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(57) Abstract

The present invention provides a method of treating a medical condition associated with increased proteolytic activity of Calpain in a mammal. The method includes administering a pharmaceutical composition containing a Calpain inhibitor compound to the mammal in an amount that is pharmacologically effective to treat the condition. The inhibitor is a compound in one of the following groups of compounds: Peptide Keto-Compounds, Substituted Heterocyclic Compounds or Halo-Ketone Peptides. Examples of conditions that can be treated using the methods of the present invention include cardiac muscle tissue damage, cataracts, smooth muscle damage and vasospasm. The present invention also provides a method of inhibiting smooth muscle cell proliferation and thereby preventing the restenosis of a blood vessel which has been undergone therapeutic angioplasty. This method includes the administration of a calpain inhibitor to the blood vessel during or after the angioplasty procedure. Further, methods of blocking the establishment of the tonically contracted state in smooth muscle and relaxing tonically contracted smooth muscle are disclosed. These methods involve the administration of a calpain inhibitor, thereby reducing or preventing smooth muscle contraction associated with vasospasm and bronchospasm.

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USE OF CALPAIN INHIBITORS IN THE INHIBITION AND TREATMENT OF MEDICAL CONDITIONS ASSOCIATED WITH INCREASED CALPAIN ACTIVITY

Background of the Invention

The present invention relates generally to medical treatments involving the inhibition of calcium-activated proteases, such as Calpain. More specifically, the present invention relates to the treatment of neurodegenerative conditions, coronary disease, circulatory pathology, cataract formation, and other medical conditions associated with calcium-activated protease activity using inhibitors of these proteases.

Neural tissues, including brain, are known to possess a large variety of proteases, including at least two calcium-stimulated proteases, termed calpain I and calpain II, which are activated by micromolar and millimolar Ca^{2+} concentrations,

respectively. Calpains are a family of calcium activated thiol proteases that are present in many tissues and use a cysteine residue in their catalytic mechanism. Calpain II is the predominant form, but calpain I is found at synapses and is thought to be the form involved in long term potentiation, synaptic plasticity and cell death.

Thiol proteases are distinguished from serine proteases, metalloproteases and other proteases by their mechanism of action and by the amino acid residue (cysteine) that participates in substrate attack. Although several thiol proteases are produced by plants, these proteases are not common in mammals, with cathepsin B (a lysosomal enzyme), other cathepsins and the calpains being among the few representatives of this family that have been described in mammals. Calpain I and calpain II are the best described of these, but several other members of the calpain family have been reported.

Other Ca^{2+}-activated thiol proteases may exist, such as those reported by Yoshihara et al., in *J. Biol. Chem.*, **265**:5809-5815 (1990). The term "Calpain" is used hereinafter to refer to any Ca^{2+}-activated thiol proteases including the Yoshihara enzyme and calpains I and II.

Although Calpains degrade a wide variety of protein substrates, cytoskeletal proteins seem to be particularly susceptible to attack. In at least some cases, the products of the proteolytic digestion of these proteins by Calpain are distinctive and persistent over time. Since cytoskeletal proteins are major components of certain types of cells, this provides a simple method of detecting Calpain activity in cells and tissues. Specifically, the accumulation of the breakdown products ("BDP's") of spectrin, a cytoskeletal protein, has been associated with the activation of Calpain. Thus, calpain activation can be measured indirectly by assaying the proteolysis of the cytoskeletal protein spectrin, which produces a large, distinctive and biologically persistent breakdown product when attacked by calpain (Siman, Baudry, and Lynch, *Proc. Natl. Acad. Sci. USA* **81**:3572-3576 (1984); incorporated herein by reference). In neural tissues, activation of Calpains, as evidenced by accumulation of these BDP's, has been observed in many neurodegenerative conditions. For example, these phenomena have been observed after denervation resulting from focal electrolytic lesions, in genetic abnormalities, after excitotoxicity, following ischemia in gerbils and rats, following administration of the toxins kainate and colchicine in rats, an in human Alzheimer's disease. Calpains have also been shown to degrade the lens proteins alpha-crystallin,

vimentin, and actin *in vitro*, and have been implicated in the degradation of cardiac muscle proteins and other tissues.

Commercially available *in vitro* inhibitors of Calpain include peptide aldehydes such as leupeptin (Ac-Leu-Leu-Arg-H) and Ac-Leu-Leu-Nle-H, as well as epoxysuccinates such as E-64. These compounds are not useful in inhibiting Calpain in Central Nervous System ("CNS") tissue *in vivo* because they are poorly membrane permeant and, accordingly, do not cross the blood brain barrier very well. Some of these compounds have also been found to have other adverse side effects. For example, leupeptin has been found to be harmful to heart cells and to adversely affect blood clotting (Toyo-Oka, et al., *Jpn. Heart J.*, **23**(5):829 (1982)). Also, many of these inhibitors are poorly specific and will inhibit a wide variety of proteases in addition to Calpain. Thus, no effective therapy has yet been developed for most neurodegenerative diseases and conditions. Millions of individuals suffer from neurodegenerative diseases and thus, there is a need for therapies effective in treating and preventing these diseases and conditions.

Cathepsin B is involved in muscular dystrophy, myocardial tissue damage, tumor metastasis, and bone resorption. In addition, a number of viral processing enzymes, which are essential for viral infection, are cysteine proteases. Inhibitors of cysteine proteases would thus have multiple therapeutic uses.

These commercially available compounds are based upon peptide structures that are believed to interact with the substrate binding site of Calpain. Active groups associated with the Calpain inhibitors then either block or attack the catalytic moiety of Calpain in order to inhibit the enzyme.

In addition, other types of compounds that are not commercially available which inhibit cysteine proteases and are thought to possess *in vitro* Calpain inhibitory activity have been reported. Examples of such compounds include the peptide diazomethanes and peptide diazomethyl ketones. See Rich, D.H., *Inhibitors of cysteine proteinases, in* Protease Inhibitors, pp153-178 (A.J. Barrett and G. Salversen, Eds., Elsevier, New York, 1986), the disclosure of which is hereby incorporated by reference. Peptide diazomethyl ketones are potentially carcinogenic and along with peptide diazomethanes are thought to be poorly membrane permeant and to have low specificity.

There is some evidence that certain particular inhibitors of Calpain have certain therapeutic utilities. For example, leupeptin can facilitate nerve repair in primates. Loxastatin (also known as EST, Ep-460 or E-64d), a derivative of E-64, is believed to have utility in the treatment of muscular dystrophy. E-64d, while not having significant protease inhibitory activity itself, is believed to be converted to more potent forms, such as to E-64c, inside a mammalian body.

Evidence from electrophysiological studies suggests that one of the earliest factors in the chain of reactions leading to cell death is an increase in intracellular-free calcium as a consequence of Ca^{2+} channel opening and/or energy depletion. Intracellular calcium is likely to produce a large number of consequences, including the activation of a large number of enzymes, including proteases, such as Calpain, lipases and kinases. An increase in intracellular calcium is also thought to induce changes in gene expression.

Ischemia, head trauma and stroke have all been associated with the release of glutamate in amounts large enough to lead to excitotoxicity, the toxicity resulting from the actions of certain amino acids on neurons of the CNS. The excess glutamate and other factors, such as free radical damage of membranes or energy depletion, cause an increase in intracellular Ca^{2+}. It is known that an excess of intracellular Ca^{2+} leads to several effects believed to be associated with neuronal cell damage, including destruction of cell structures through activation of phospholipase and Calpain, as well as free radical production resulting from activation of phospholipase and xanthine oxidase. Many other factors have been associated with neurotoxicity. For example, reductions in action potentials and changes in a wide variety of chemical markers are known to be associated with neurons exposed to ischemic conditions.

The excitotoxic death of nerve cells following ischemia is the result of a cascade of events which begins with energy depletion, followed by release of glutamate, stimulation of glutamate receptors, and an elevation of intracellular calcium. See, e.g., Meldrum, "Excitotoxicity in Ischemia: An Overview," in Cerebrovascular Diseases, Ginsberg et al. (eds.), Raven Press, New York, pp. 47-60 (1989). Since many researchers believe that excitotoxicity plays a large role in the pathology of stroke and ischemia, much recent research has focused upon developing drugs which reduce excitotoxicity by acting at specific stages of the excitotoxic cascade.

Elevations in intracellular calcium have been proposed to play a central role in the induction of excitotoxic cell death. See, e.g. Meldrum et al., *Trends Pharmacol. Sci.*, 11:379-387 (1990). Many attempts to prevent excitotoxicity have focused upon blocking the NMDA subtype of glutamate receptor, which functions as a calcium channel. Although glutamate toxicity is calcium dependent, it is clear that calcium influx through the NMDA receptor is not the sole culprit in excitotoxicity. The correlation between NMDA antagonist mediated reduction in glutamate-induced intracellular Ca^{2+} and cell rescue is poor. Further, agents acting at non-NMDA type calcium channels are effective inhibitors of glutamate toxicity and excitotoxicity appears to involve not only calcium influx through both NMDA and non-NMDA calcium channels but also the release of Ca^{2+} from intracellular stores. Thus, the mechanism by which Ca^{2+} becomes elevated is still unknown.

It is clear that elevated Ca^{2+} is a prime intracellular mediator of excitotoxicity. Elevations of intracellular calcium modulate many effects, including the activation of the calcium-dependent thiol proteases calpain I and II. Calpain has been shown to be activated during excitotoxicity, and calpain activation can be detected early following ischemia.

Calpain action results in the irreversible cleavage of cellular proteins and alterations in their function, and this degradative function fits in well with a possible role in cell death. Further, leupeptin, a calpain inhibitor, has been shown to reduce ischemic damage in gerbils and to reduce hypoxic damage in rat hippocampal slices.

Much of what is known about excitotoxicity derives from studies of neurons *in vitro*. Primary cultures of cerebral cortex, hippocampal and cerebellar neurons are killed by exposure to glutamate or glutamate analogs. Recently, glutamate has been reported to kill pheochromocytoma PC12 cells in a calcium-dependent manner.

Increases in intracellular calcium and subsequent calpain activity have also been linked to other pathological conditions. It has been found, for example, that in experimental cataracts induced in mice, increased calcium levels have been recorded just before the onset of cataract formation. The size of infarcted heart tissue in ischemic myocardium can also be reduced by the administration of calpain inhibitors (Toda, et al., *Jpn. Heart J.*, 30:375-86 (1989); Toyo-Oka, *Drug Res.*, 36(1):671-75 (1986)).

Notwithstanding the foregoing understanding of certain aspects of neurotoxicity,

no effective therapy has yet been developed for most neurodegenerative diseases and conditions of the CNS. Millions of individuals suffer from these diseases and conditions. Thus, there is a need for therapies effective in treating and preventing these diseases and conditions.

In addition to being involved in cytotoxicity, proteases such as calpain have also been linked to the regulation of cellular growth. However, the mechanisms of such regulation have not been well studied. Some protease inhibitors inhibit cellular proliferation, for example, while others enhance it. Because calpains are ubiquitously distributed in mammalian cells but apparently do not contribute to normal protein catabolism or general protein turnover, they appear to serve a regulatory role in such cells. However, the mechanisms of such regulation have not been well studied. While some calpain inhibitors have been shown to inhibit cellular proliferation and thus cell cycling, the specific point in the reproductive cycle at which such inhibition occurs is not yet known.

An understanding of the regulation of cell cycling is relevant to the development of treatments for cancer, because cancer cells grow without regulation of such cell cycling. Chemotherapy treatments for cancer sometime take the form of administering chemicals which will kill cells that are passing through the cell cycle and actively dividing while sparing those cells which are not dividing. In one such form of chemotherapy, drugs which interfere with the replication of the DNA of cells during the "S" (synthesis) phase of the cell cycle are administered to a patient. This treatment, however, will only be effective in killing cells in the S phase. Thus, a drug must be present in a patient's body for long enough so that all of the cancer cells in the patient progress through the S phase. Since chemotherapeutic agents kill non-cancerous cells which are dividing as well as cancerous cells, the timing and duration of chemotherapeutic drug administration is critical to successful therapy.

There exists, therefore, a need for compounds which are capable of manipulating the cell cycle, resulting in a shortened duration of chemotherapy and greater efficacy of the chemotherapeutic agent.

The processes of angiogenesis and vascular repair both depend upon smooth muscle cell proliferation, since smooth muscle cells play an essential part in the functioning of blood vessels as well as other organs. Smooth muscle cells are

stimulated to proliferate following vascular injury by a number of different factors, including PDGF (platelet derived growth factor). This is normally a desirable process which is necessary for healing. However, following therapeutic angioplasty for the opening of obstructed arteries, the proliferation of the smooth muscle cells can result in restenosis, the blockage of the previously opened artery. Austin G.E., et al., *J. Am. Coll. Cardiology*, **6**:369-377 (1985). This is a significant problem in the clinical use of angioplasty, and a need therefore exists for a drug which can inhibit the proliferation of the smooth muscle cells.

Additionally, proteases such as calpain have also been linked to the regulation of smooth muscle contraction. However, the mechanism by which contractility and the maintenance of the tonically contracted state is regulated in smooth muscle is not well understood. Many agents which act to decrease contractility of smooth muscle have little or no efficacy at inhibiting the establishment of the tonic state or reversing the tonic contractile state once established.

The tonic contraction of smooth muscle is a normal process. In some cases, however, such tonic contraction can lead to serious pathological conditions. For example, contraction of the bronchial smooth muscle leads to shortness of breath and other symptoms of asthma. Contraction of the coronary arteries can lead to angina, partial coronary hypoxia and subsequent loss of coronary function. Contraction of the smooth muscle in cerebral arteries can lead to cerebral vasospasm and hypoxia of the brain tissue, a serious condition that can leave patients mentally disabled and permanently brain damaged.

Summary of the Invention

One aspect of the present invention is a method of synchronizing the reproductive cycle of actively dividing cells. In this method, an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of cells from G_1 phase into S phase is administered to the cells. The Calpain Inhibitor can be one of the Peptide Keto-Compounds, the Halo-Ketone Peptides, or the Substituted Heterocyclic Compounds. In one embodiment, the cells to be treated in this method are located *in vivo* in a mammal, so that the administering step of the method comprises administering a Calpain Inhibitor to cells in a mammal. Alternatively, the administering step can comprise administering a Calpain Inhibitor to cells *in vitro*. In

one preferred embodiment, the administering step of this method comprises administering a Peptide Keto-Compound. Calpain Inhibitors can be administered in this method either intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to cells.

In another aspect, the present invention comprises a method of blocking the progression of the cell cycle from G_1 phase to S phase in actively dividing cells in a mammal. In this method, a mammal is administered an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cell cycles of actively dividing cells in the mammal from G_1 phase into S phase. The Calpain Inhibitor can be one of the Peptide Keto-Compounds, the Halo-Ketone Peptides, or the Substituted Heterocyclic Compounds. In one preferred embodiment, the Calpain Inhibitor is a Peptide Keto-Compound. Calpain Inhibitors can be administered according to this method either intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to living cells. In one embodiment, the Calpain Inhibitor is administered by direct application, where such direct application can comprise either applying a gel to an area of living cells, driving microspheres loaded with the Calpain Inhibitor into tissue comprising the living cells, or injecting a solution containing the Calpain Inhibitor directly into tissue comprising such living cells.

In yet another aspect, the present invention comprises a method of enhancing the efficacy of chemotherapy in the treatment of cancer in a human patient. This method comprises administering to the cancerous cells of the patient an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cell cycles of such cancerous cells from G_1 phase to S phase, and thereafter administering to the cells a chemotherapeutic agent. The Calpain Inhibitor in this method is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds. In one preferred embodiment, the Calpain Inhibitor is a Peptide Keto-Compound. The Calpain Inhibitor in this method can be administered intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to the cancerous cells. The chemotherapeutic agent can be administered beginning 24-48 hours after the administration of the Calpain Inhibitor, at which time the cell cycles of the patient's cancerous cells which were treatable with the Calpain Inhibitor will be synchronized.

A further aspect of the present invention includes a method of determining the effectiveness of a chemotherapeutic agent, comprising growing cancerous cells *in vitro*, administering to such cancerous cells an amount of a Calpain Inhibitor which is effective to block the progression of the cells from G_1 phase into S phase, administering to the cells the chemotherapeutic agent in an amount sufficient to kill the cells, and thereafter determining the amount of cell death that occurs. The amount of cell death that occurs in this method is indicative of the effectiveness the chemotherapeutic agent tested.

Another aspect of the present invention is a method of increasing the efficiency of cell transformation and thus increasing the efficiency of integration of foreign DNA into living cells. This method comprises administering to a population of cells comprising actively dividing cells an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cell cycles of the cells from G_1 phase into S phase, discontinuing the administration of the Calpain Inhibitor, and thereafter introducing foreign DNA into the population of cells. The Calpain Inhibitor in this method is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds. In one embodiment, the Calpain Inhibitor is a Peptide Keto-Compound. The administration of the Calpain Inhibitor in this method can continue for the length of one cell cycle in the population of living cells. The target of the Calpain Inhibitor can be a population of cells located in a mammal, which can be administered a Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to the population of cells in the mammal. In another embodiment, the Calpain Inhibitor is administered instead to a population of cells *in vitro*.

The present invention provides methods of treating a variety of medical conditions associated with calcium-activated protease activity in a mammal by administering the Calpain inhibitors of the present invention to that mammal. These Calpain inhibitors are Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds. Particularly preferred compounds for this use include the Peptide Ketoamides, such as Z-Leu-Abu-CONH-Et, Z-Leu-Phe-CONH-Et and Z-Leu-Phe-CONH$(CH_2)_2C_6H_5$. Administration of the inhibitors can be through any of a variety of routes. These routes include all of the following types of

administration: intravenous, intraperitoneal, intramuscular, oral, topical treatment such as through ointments (including ophthalmic ointments), eye drops, contact lenses, catheter, directly onto tissues such as blood vessels or cardiac tissue during surgery, or injection into the pericardial space.

Specific medical conditions which can be treated with these Calpain Inhibitors include cardiac muscle tissue damage. After a mammal with cardiac muscle tissue damage has been identified, that mammal can be treated with a Calpain Inhibitor. Mammals at risk for developing cardiac muscle tissue damage can also be treated with the present Calpain Inhibitors. Administering these Inhibitors to such mammals protects them from the cardiac tissue damage experienced by mammals which are not so protected.

In another embodiment of the present invention, cataracts are treated by the administration of a Calpain Inhibitor. If a mammal has already developed cataracts, the development of the cataracts can be slowed or arrested through the administration of a Calpain Inhibitor. On the other hand, if a mammal has been identified as being at risk for developing cataracts in the future, the development of cataracts in such a mammal can be prevented or slowed through the administration of a Calpain Inhibitor.

A variety of other tissues and conditions can also be treated with the novel Calpain Inhibitors of the present invention. Skeletal and smooth muscle damage, for example, can be treated by identifying a mammal with such tissue damage and administering a Calpain Inhibitor to that mammal. Vasospasm, a condition of a particular kind of smooth muscle, the vascular tissue, can also be reversed in a mammal identified as having this condition by the administration of Calpain Inhibitors. Erythrocytes damaged by the proteolytic activity of Calpain in hypertensive mammals can also be treated with the Calpain Inhibitors of the present invention.

In one aspect, the present invention provides methods of halting or inhibiting the proliferation of smooth muscle cells both in vivo and in vitro by administering a Calpain Inhibitor. These Calpain Inhibitors are Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds. Particularly preferred compounds for this use include the Peptide Ketoamides, such as Z-Leu-Phe-CONH-Et. Administration of the inhibitors can be through any of a variety of routes. These routes include all of the following types of administration: intravenous, intramuscular,

intraperitoneal, topical, oral, or by direct application. Preferred Peptide Keto-Compounds useful in the present invention include $(Ph)_2CHCO$-Leu-Phe-CONH-CH_2-2-Py; Z-Leu-Nva-CONH-CH_2-2-Py; Z-Leu-Phe-CONH-$CH_2CH(OH)Ph$; $(Ph)_2CHCO$-Leu-Abu-CONH-$CH_2CH(OH)Ph$; Z-Leu-Phe-$CONH_2$; Z-Leu-Abu-CONH-$CH_2CH(OH)Ph$; and Z-Leu-Phe-CONHEt.

Direct application of the Calpain Inhibitors can be through various means. Such means include using a gel or ointment containing the inhibitor to coat the surface of the balloon of a balloon catheter or onto another surgical instrument that is inserted into the blood vessel during angioplasty. Alternatively, the gel may be applied directly to an area of vascular tissue which has been treated by angioplasty during the surgical procedure. Another route of administration comprises driving microspheres which have been loaded with a Calpain Inhibitor directly into the mammal's blood vessel. This can be accomplished by applying the microspheres to the surface of the balloon or other surgical instrument used during the angioplasty procedure. The microspheres are driven into the arterial wall, where they lodge and release the Calpain Inhibitor over time.

Specific medical conditions which can be treated with these Calpain Inhibitors include the treatment of a mammal to prevent restenosis of a blood vessel following angioplasty. After a mammal which has undergone angioplasty has been identified, that mammal can be treated with a Calpain Inhibitor. Mammals at risk for developing restenosis can also be treated with the present Calpain Inhibitors. Administering these Inhibitors to such mammals protects them from the smooth muscle cell proliferation experienced by mammals which are not so protected.

In another aspect, the present invention provides a method of inhibiting tonic smooth muscle contraction in a mammal susceptible to inappropriate contraction in a smooth muscle thereof. The method includes administering to the smooth muscle an amount of a Calpain Inhibitor that is pharmacologically effective to suppress the contraction thereof. The Calpain Inhibitor is one of the Peptide Keto-Compounds, Halo-Ketone Peptides or Substituted Heterocylic Compounds. Preferably, the inhibitor is administered intravenously, intramuscularly, intraperitoneally, topically, orally, by injection into cerebrospinal fluid, by inhalation, or by direct application to the smooth muscle, such as by applying directly to an area of smooth muscle. Direct application

can also be by driving microspheres loaded with the Calpain Inhibitor into the smooth muscle. Relaxation of the smooth muscle is preferably induced.

In an additional aspect, the present invention provides a method of treating coronary vasospasm in a mammal. In this aspect, the method includes administering to the mammal an amount of a Calpain Inhibitor which is pharmacologically effective to stop vasospasm of coronary tissue in the mammal. The Calpain Inhibitor is one of the Peptide Keto-Compounds, Halo-Ketone Peptides or Substituted Heterocylic Compounds. In a preferred embodiment, the coronary tissue is surgically exposed and a solution of Calpain Inhibitor is applied directly to the tissue. Preferably, the coronary tissue comprises a coronary artery. In a preferred embodiment of this aspect, the mammal is suffering from angina and the method comprises a treatment for the angina.

In still another aspect of the invention, there is provided a method of treating bronchial vasospasm in a mammal. This method includes administering to the mammal an amount of a Calpain Inhibitor which is pharmacologically effective to stop vasospasm of bronchial tissue in the mammal. The Calpain Inhibitor is one of the Peptide Keto-Compounds, Halo-Ketone Peptides or Substituted Heterocylic Compounds. The bronchial tissue can be surgically exposed and a solution of Calpain Inhibitor applied directly to the tissue. In a preferred embodiment of the method, the mammal is suffering from asthma and the method comprises a treatment for the asthma.

Yet another aspect of the invention relates to a method of treating cerebral vasospasm in a mammal. This method includes administering to the mammal an amount of a Calpain Inhibitor which is pharmacologically effective to stop vasospasm of cerebral tissue in the mammal. The Calpain Inhibitor is one of the Peptide Keto-Compounds, Halo-Ketone Peptides and Substituted Heterocylic Compounds. The cerebral tissue can be surgically exposed and a solution of Calpain Inhibitor applied directly to the tissue. In one embodiment of this aspect of the invention, the Calpain Inhibitor can be injected into the mammal's cerebrospinal fluid.

One aspect of the present invention provides a method of medical treatment for a medical condition in a mammal. In this method, a pharmaceutical composition containing a morpholine Peptide Keto-Compound is administered to the mammal. The composition is administered in an amount that is pharmacologically effective to treat

the condition. The condition is one which is associated with increased proteolytic activity of Calpain. The morpholine Peptide Keto-Compound can be either a C-terminal or N-terminal morpholine Peptide Keto-Compound, such as cardiac muscle tissue damage, cataracts, skeletal muscle damage, vasospasm or restenosis following cardiac angioplasty.

Another aspect of the present invention also provides a method of medical treatment for a medical condition in a mammal. In this method, a pharmaceutical composition containing a Peptide Ketoamide, Subclass C is administered to the mammal. This composition is administered in an amount that is pharmacologically effective to treat the condition. The condition that can be treated with this method is also one associated with increased proteolytic activity of Calpain, such as cardiac muscle tissue damage, cataracts, skeletal muscle damage, vasospasm or restenosis following cardiac angioplasty.

One of skill in the art will recognize that the present Calpain Inhibitors can be used to counteract the harmful effects associated with calpain activity which arise in a number of medical conditions and diseases. Therefore, the treatment of such conditions with the present Calpain Inhibitors is within the scope of the present invention.

These and other features and advantages of the present invention will become apparent from the detailed description which follows, considered together with the attached drawings and claims.

Brief Description of the Figures

Figure 1 shows the percentage of inhibition of glutamate-induced cell death through the addition of glutamate and various Calpain Inhibitors relative to control where no glutamate was added.

Figure 2 shows that Calpain inhibitor reduces cell death following glutamate exposure. PC12 cells were exposed to 7.5mM glutamate with the indicated concentration of inhibitor, as described in the text, for 24 hours. Cell viability was assayed using the MTT assay. Values are expressed as % of naive control ± sem.

Figure 3 shows the dependence of the ability of Calpain inhibitors to reduce cell death on glutamate concentration. PC12 cells were incubated with the indicated concentration of glutamate and no inhibitor (circles), 20uM Z-Leu-Nva-CONH$(CH_2)_3$

morpholine (triangles), or 30uM Z-Leu-Phe-$CONHCH_2CH_3$ (squares) for 24 hours and cell viability was assayed by MTT. Values expressed as % of naive control ± sem.

Figure 4. Delayed addition of calpain inhibitor. Glutamate (7.5mM) was added at 0 time and Z-Leu-Phe-$CONHCH_2CH_3$ (squares) or Z-Leu-Nva-$CONH(CH_2)_3$ morpholine (triangles) added at the indicated times to final concentrations of 100uM each. Cell viability was measured 24 hours after the addition of glutamate by the MTT assay. Values expressed as % of naive control ± sem.

Figure 5 graphically depicts the effects of Z-Leu-Phe-CONH-Et and Z-Leu-Abu-CONH-Et on the size of infarction produced upon MCA occlusion in male rats.

Figure 6 shows the effects of Z-Leu-Abu-CO_2Et, a Peptide Keto-Compound, and CI1 (Ac-Leu-Leu-Nle-H) relative to control slices on survival of hippocampal slices exposed to 10 minutes exposure of anoxic atmosphere where both of these compounds were added at their optimal inhibitory concentration at both 1 hour and 2 hour incubation times.

Figure 7 shows the evoked potential amplitude for control, CI1 treated and Z-Leu-Abu-CO_2Et treated hippocampal slices over a time course during which the slices are exposed to anoxic atmosphere.

Figure 8 shows the percent recovery of EPSP from severe hypoxia over the course of one hour incubation for Z-Leu-Phe-CONH-Et and Z-Leu-Phe-CO_2Et.

Figure 9 shows a comparison of the effect of the presence of CI1 or Z-Leu-Phe-CO_2Et on survival of hippocampal slices expressed as the duration of anoxia (in minutes) before fiber volley disappearance.

Figure 10 shows the effects of CI1 compared with control on the behavioral and convulsive effects of kainic acid.

Figure 11 shows the amount of spectrin BDP's in rat brains exposed to kainate for control and CI1 treated rats.

Figure 12 graphically depicts the effect of several different Calpain Inhibitors on contraction of isolated arteries induced by endothelin (ET-1). Drug A is Z-Leu-Abu-CONHEt, Drug B is Z-Leu-Phe-CONHEt, Drug C is 1,10-Phenanthroline and Drug D is TLCK (Tosyl-Lysine-chloromethylketone).

Figure 13 graphically depicts the effect of several other Calpain Inhibitors on contraction of isolated arteries induced by endothelin (ET-1). Drug E is Z-Leu-Phe,

Drug F is Z-Leu-Phe-CONHEt (the same as drug B), Drug G is Z-Leu-Phe-$CONH(CH_2)_2Ph$, Drug H is Ac-Leu-Leu-Nle-H (Calpain Inhibitor I), Drug I is Gly-Gly-Gly and Drug J is $(Ph)_2CHCO$-Leu-Abu-CONH-$CH_2CH(OH)Ph$.

Figure 14 shows the effect of Calpain Inhibitors on contraction of isolated arteries induced by phorbol dibutyrate (PDB). Drugs E through J are as in Figure 15.

Figure 15 graphically depicts the effect of Calpain Inhibitors on smooth muscle resting tension. Drugs E through J are as in Figure 13.

Figure 16 shows the dose-dependent inhibition of oxyhemoglobin-induced constriction by a Calpain Inhibitor, Z-Leu-Phe-$CONH(CH_2)_3$, of the present invention.

Figure 17 shows an example of the time course of artery constriction in an artery constricted by subarachnoid hemorrhage (SAH) and treated with a Calpain Inhibitor, Z-Leu-Phe-$CONH(CH_2)_3$, of the present invention.

Figure 18 shows the summary of data from three animals in which a Calpain Inhibitor, Z-Leu-Phe-$CONH(CH_2)_3$, of the present invention reversed constrictions caused by SAH.

Figure 19 graphically depicts the effects of Z-Leu-Phe-CONHEt and Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$ on the proliferation of cultured bovine smooth muscle cells.

Figure 20 shows the continued viability of smooth muscle cells after treatment with a Calpain Inhibitor, despite a complete inhibition of cell proliferation.

Figure 21 graphically depicts the blocking of the progression into S phase of bovine aortic smooth muscle cells (BASMC) after treatment with the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$. In this graph, "Drug C" is Ph_2-CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$ ("Drug C" elsewhere may be a different compound).

Figure 22 graphically depicts the synchronous progression into S phase of HeLa and AT-2 cells after the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$ was washed out of the medium in which such cells were maintained. In this graph, "Drug C" is also Ph_2-CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$ (though "Drug C" elsewhere may be a different compound).

Detailed Description of the Invention

A. INTRODUCTION

We have discovered that Calpain activation is an event central to many cases of brain atrophy and degeneration and that inhibition of Calpain alone is sufficient to inhibit or prevent cell deterioration and loss. Thus, we have further discovered that inhibition of Calpain provides protection from neurotoxicity associated with many neurodegenerative conditions and diseases.

In accordance with the foregoing discoveries, we believe that the elevation of intracellular calcium associated with neuropathological conditions in neuronal cells activates Calpain and sets in motion the digestion of neuronal cells from within. We believe there may be other mechanisms of activation of Calpains associated with these conditions. Accordingly, one aspect of the present invention is directed to inhibition and treatment of the neurodegeneration and other diseases associated with this digestion through the inhibition of Calpain activity. Thus, part of this aspect of the present invention is to prevent the neurodegeneration and other pathology caused by this digestion through the *in vivo* administration of Calpain inhibitors. By way of example, and not of limitation, diseases and conditions which can be treated using this aspect of the present invention include neurodegeneration following excitotoxicity, HIV-induced neuropathy, ischemia, denervation following ischemia or injury, subarachnoid hemorrhage, stroke, multiple infarction dementia, Alzheimer's Disease (AD), Parkinson's Disease, Huntington's Disease, surgery-related brain damage and other neuropathological conditions.

As stated above, spectrin BDP's have been found to be associated with Calpain activation *in vivo*. We have observed that in each instance of neurodegeneration in which BDP's characteristic of Calpain activation are detected, Calpain activation is localized to the brain areas most vulnerable to the particular pathogenic manipulation. In addition, as judged by histological methods, Calpain activation precedes overt evidence of neurodegeneration. Accordingly, Calpain activation is spatially and temporally linked to impending or ongoing cell death in the brain. Thus, we believe that Calpain activation is an important mechanism of cell damage and death in many pathological conditions, including neuropathological conditions. Moreover, there is evidence that the activation of Calpains is an early event in the death of cells including

neural cells. This is in contrast to other known proteases which are activated at later stages of cell death. Thus, we believe that, advantageously, inhibition of Calpain activity provides intervention at an early stage of cell death, prior to significant deterioration of cellular machinery.

Another aspect of the involvement of Calpains in neurodegeneration is the involvement of these proteins in regenerating systems. It is known that developing or regenerating axons are somehow inhibited from further development in a stabilization process called the "stop pathway." This stabilization can occur when axons have reached their targets; however, in some systems stabilization can also occur at inappropriate places. One researcher has developed evidence that this stop pathway operates at least in part by the activation of intracellular Calpain and that inhibition of Calpain can interfere with stabilization (Luizzi, 1990). We believe that Calpain inhibitors, when used in accordance with the present invention, can advantageously aid regeneration and recovery of neural tissue after injury, in addition to inhibiting neurodegeneration.

Another aspect of the present invention is our discovery that at least three classes of compounds, the substituted isocoumarins, the peptide keto-compounds and the Halo-Ketone Peptides have Calpain inhibitory activity. We have further discovered, as will be described hereinbelow, that these three classes of compounds exhibit additional properties that render them especially useful as therapeutically effective compounds in the treatment of neurodegenerative conditions and diseases.

Calpain has also been implicated in the pathogenesis of a number of other medical conditions. The inhibition of Calpain is capable of slowing the progress of these diseases and of preventing certain conditions altogether.

The formation of cataracts, for example, has been linked to Calpain activity in mammalian lenses. In *in vivo* models of cataract formation, increased Calpain activity has been documented just before the onset of detectable cataract formation. Calpain activity has also been observed to decrease after a cataract has formed in a lens, leading to the inference that calpain activity is involved in the formation of cataracts. Moreover, we have shown that there are increased levels of spectrin breakdown products found in *in vitro* models of cataract formation. The presence of such spectrin breakdown products is known to be reflective of increased Calpain activity. Thus, we

believe that by administering the Calpain Inhibitors of the present invention, the formation of cataracts can be prevented or slowed.

Calpain activity has also been implicated in producing myocardial infarctions. Calpain activity is regulated by intracellular calcium concentrations, and increased intracellular calcium in myocardial tissues has been observed when the myocardium is cut off from its supply of oxygen due to ischemia. Cell damage and ultimately cell death results from such ischemia. The increased proteolytic activity of Calpain due to increased levels of intracellular calcium during ischemia is therefore a contributor to or direct cause of cell death during cardiac ischemia. Cardiac tissue damage can thus be prevented or minimized with the present Calpain Inhibitors.

Calpain is also believed to be an important regulator of cell growth. Several Calpain Inhibitors have been found, for example, to inhibit smooth muscle cell proliferation. Such proliferation is in fact necessary to repair injured smooth muscle tissue. Following therapeutic angioplasty, however, smooth muscle cell proliferation may result in restenosis of the opened blood vessel. Calpain Inhibitors may thus be used to prevent the smooth muscle cell proliferation which results in the restenosis of blood vessels.

Other disease conditions can be treated with Calpain Inhibitors as well. Calpain has been shown to degrade the constituents of skeletal and smooth muscle cells, and has been implicated in causing vasospasm. Increased Calpain activity has also been shown in the blood cells of hypertensive patients, and has been shown to be five times as active in degrading proteins in such cells as in the cells of non-hypertensive patients. Calpain Inhibitors therefore can reduce or eliminate the harmful effects of Calpain activity in these tissues.

We have also found that Calpain Inhibitors inhibit tonic smooth muscle contraction. These compounds are useful in the treatment of animals or humans for the purpose of preventing or reducing the smooth muscle contraction associated with vasospasm and bronchospasm.

The present invention includes the use of a variety of Calpain Inhibitors and methods for using these inhibitors to treat disease conditions. Specifically, Substituted Heterocyclic Compounds, Peptide Keto-Compounds, and Halo-Ketone Peptides have been found to be effective in treating the foregoing conditions as well as other diseases.

Unless otherwise stated, the Calpain Inhibitors of the present invention refers to the novel Substituted Heterocyclic Compounds, Peptide Keto-Compounds, and Halo-Ketone Peptides described herein.

Several Calpain Inhibitors have also been found to play a role in the regulation of the reproductive cycle of the cell. These compounds can be used in the treatment of cancer in animals or humans along with other chemotherapeutic agents in order to enhance the effectiveness of such agents. By synchronizing the growth of rapidly dividing cells, these compounds can increase the effectiveness of chemotherapeutics that act at a specific stage in the cell cycle, such as at DNA replication.

By synchronizing the cell cycles of cells, Calpain Inhibitors are also useful in increasing the efficiency of cell transformation. Transformation results from the incorporation of foreign DNA into a cell. Such incorporation is increased when cells are synthesizing DNA. Thus, by synchronizing cells to the DNA synthetic portion of the cell cycle, the cells will be more efficiently transformed by foreign DNA introduced into the cells.

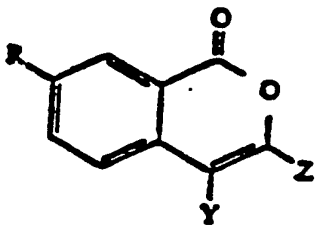
B. SUBSTITUTED HETEROCYCLIC COMPOUNDS

One particular class of compounds exhibiting Calpain inhibitory activity, when used in accordance with the present invention, are the substituted heterocyclic compounds. These compounds include the substituted isocoumarins. The substituted heterocyclic compounds are known to be excellent inhibitors of serine proteases. As discussed hereinbelow, we have now discovered that these compounds are also inhibitors of calpain I and calpain II, and also of other Calpains. Additionally, as also discussed below, we have found that, unlike most known inhibitors of Calpains, these substituted heterocyclic compounds are not effective as inhibitors of papain or cathepsin B. Thus, we believe that the substituted heterocyclic compounds provide a relatively specific means of inhibiting Calpains while not affecting other thiol proteases.

One particular class of substituted heterocyclic compounds with Calpain inhibitory activity are the isocoumarins having cationic substituents. These substituted heterocyclic compounds are referred to herein as the "Class I Substituted Isocoumarins." The Class I Substituted Isocoumarins are known to be excellent inhibitors of several serine proteases, including bovine thrombin, human thrombin,

human factor Xa, human factor XIa, human factor XIIa, bovine trypsin, human plasma plasmin, human tissue plasminogen activator, human lung tryptase, rat skin tryptase, human leukocyte elastase, porcine pancreatic elastase, bovine chymotrypsin and human leukocyte cathepsin G. The Class I Substituted Isocoumarins inhibit the serine proteases by reaction with the active site serine to form an acyl enzyme, which in some cases may further react with another active site nucleophile to form an additional covalent bond. We have discovered that the Class I Substituted Isocoumarins also react with Calpain. We believe that the mechanism of action of Calpain inhibition is similar to that of the inhibition of serine proteases since the reaction mechanism of Calpains is similar to that of the serine proteases.

The Class I Substituted Isocoumarins having Calpain inhibitory activity have the following structural formula:

(I) 

or a pharmaceutically acceptable salt, wherein

Z is selected from the group consisting of C_{1-6} alkoxy with an amino group attached to the alkoxy group, C_{1-6} alkoxy with an isothiureido group attached to the alkoxy group, C_{1-6} alkoxy with a guanidino group attached to the alkoxy group, C_{1-6} alkoxy with an amidino group attached to the alkoxy group, C_{1-6} alkyl with an amino group attached to the alkyl group, C_{1-6} alkyl with an isothiureido group attached to the alkyl group, C_{1-6} alkyl with an guanidino group attached to the alkyl group, C_{1-6} alkyl with an amidino group attached to the alkyl group,

R is selected from the group consisting of O=C=N-, S=C=N-, AA-NH-, AA-AA-NH-, AA-O, AA-AA-O-, M-NH-, M-AA-NH, M-AA-AA-NH-, M-O-, M-AA-O-, M-AA-AA-O-,

wherein AA represents alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine or sarcosine,

wherein M represents NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X-NH-CS, X-NH-SO_2, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-,

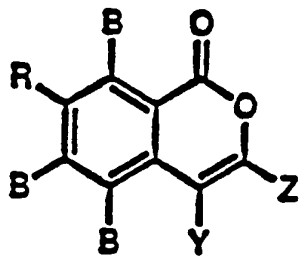
wherein X represents C_{1-6} alkyl, C_{1-6} fluoroalkyl, C_{1-6} alkyl substituted with K, C_{1-6} fluoroalkyl substituted with K, phenyl, phenyl substituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, naphthyl, naphthyl substituted with J, naphthyl disubstituted with J, naphthyl trisubstituted with J, C_{1-6} alkyl with an attached phenyl group, C_{1-6} alkyl with two attached phenyl groups, C_{1-6} alkyl with an attached phenyl group substituted with J, or C_{1-6} alkyl with two attached phenyl groups substituted with J,

wherein J represents halogen, COOH, OH, CN, NO_2, C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkylamine, C_{1-6} dialkylamine, or C_{1-6} alkyl-O-CO-,

wherein K represents halogen, COOH, OH, CN, NO_2, NH_2, C_{1-6} alkylamine, C_{1-6} dialkylamine, or C_{1-6} alkyl-O-CO-,

Y is selected from the group consisting of H, halogen, trifluoromethyl, methyl, OH and methoxy.

The compounds of Formula (I) can also contain one or more substituents at position B as shown in the following structure:



wherein electronegative substituents such as NO_2, CN, CI, COOR, and COOH will increase the reactivity of the isocoumarin, and electropositive substituents such as

NH_2, OH, alkoxy, thioalkyl, alkyl, alkylamino, and dialkylamino will increase its stability. Neutral substituents could also increase the stability of acyl enzyme and improve the effectiveness of the inhibitors.

The following compounds are representative of the Class I Substituted Isocoumarins of the present invention:

4-chloro-3-(3-isothiureidopropoxy)isocoumarin (CiTPrOIC)

7-(benzylcarbamoylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin ($PhCH_2NHCONH$-CiTPrOIC)

7-(phenylcarbamoylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (PhNHCONH-CiTPrOIC)

7-(acetylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (CH_3CONH-CiTPrOIC)

7-(3-phenylpropionylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin ($PhCH_2CH_2CONH$-CiTPrOIC)

7-(phenylacetylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin ($PhCH_2CONH$-CiTPrOIC)

7-(L-phenylalanylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (L-Phe-NH-CiTPrOIC)

7-(N-t-butyloxycarbonyl-L-phenylalanylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (Boc-L-Phe-NH-CiTPrOIC)

7-(D-phenylalanylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (D-Phe-NH-CiTPrOIC)

7-(N-t-butyloxycarbonyl-D-phenylalanylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (Boc-D-Phe-NH-CiTPrOIC)

7-(benzylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin ($PhCH_2NHCONH$-CiTEtOIC)

7-(phenylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (PhNHCONH-CiTEtOIC)

7-(isopropylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin ($(CH3)_2CHNHCONH$-CiTEtOIC)

7-(phenylacetylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin ($PhCH_2CONH$-CiTEtOIC)

7-(L-phenylalanylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (L-Phe-NH-CiTEtOIC)

7-(N-t-butyloxycarbonyl-L-phenylalanylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (Boc-L-Phe-NH-CiTEtOIC)

7-(D-phenylalanylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (D-Phe-NH-CiTEtOIC)

7-(N-t-butyloxycarbonyl-D-phenylalanylamino)-4-chloro-3-(2--isothiureidoethoxy)isocoumarin (Boc-D-Phe-NH-CiTEtOIC)

7-(N-t-butyloxycarbonyl-L-alanyl-L-alanylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (Boc-Ala-Ala-NH-CiTEtOIC)

7-(L-alanyl-L-alanylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (Ala-Ala-NH-CiTEtOIC)

7-(1-naphthylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (NaphthylNH-CiTEtOIC)

7-((S)-α-methylbenzylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (S-$C_6H_5(CH_3)$CHNHCONH-CiTEtOIC)

7-((R)-α-methylbenzylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (R-$C_6H_5(CH_3)$CHNHCONH-CiTEtOIC)

7-dansylamino-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (DansylNH-CiTEtOIC)

7-phenylthiocarbamoylamino-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (PhNHCSNH-CiTEtOIC)

7-(m-carboxyphenylthiocarbamoyl)amino-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (m-COOH-PhNHCSNH-CiTEtOIC)

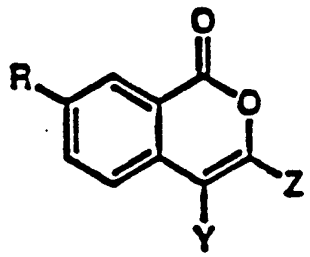
7-(p-carboxyphenylthiocarbamoyl)amino-4-chloro-3-(2-isothiureidoethoxy)isocoumarin (p-COOH-PhNHCSNH-CiTEtOIC)

7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (ACITIC)

Isocoumarins with basic substituents are also known to be effective inhibitors of serine proteases. See Powers et al, U.S. Patent No. 4,845,242, the disclosure of which is hereby incorporated by reference. This class of compounds, referred to herein as the "Class II Substituted Isocoumarins," along with the other substituted heterocyclic compounds, is believed to be effective in the use of the present invention.

The Class II Substituted Isocoumarins have the following structural formula:

(II)



or a pharmaceutically acceptable salt, wherein:

R is selected from the group consisting of -N-H-C(=NH)-NH_2, -C(=NH)NH_2, C_{1-6} alkyl with an attached amino, and C_{1-6} alkyl with an attached isothiureido of the formula -S-C(+NH_2+)NH_2,

Z is selected from the group consisting of H, halogen, C_{1-6} alkyl, C_{1-6} alkyl with an attached phenyl, C_{1-6} fluorinated alkyl, C_{1-6} alkyl with an attached hydroxyl, C_{1-6} alkyl with an attached C_{1-6} alkoxy, C_{1-6} alkoxy, C_{1-6} fluorinated alkoxy, C_{1-6} alkoxy with an attached phenyl, benzyloxy, 4-fluorobenzyloxy, -$OCH_2C_6H_4$R' (2-substituent), -$OCH_2C_6H_4$R' (3-substituent), -$OCH_2C_6H_4$R' (4-substituent), -$OCH_2C_6H_3R_2$' (2,3-substituents), -$OCH_2C_6H_3R_2$' (2,4-substituents), -$OCH_2C_6H_3R_2$' (2,5-substituents), -$OCH_2C_6H_3R_2$' (2,6-substituents), -$OCH_2C_6H_3R_2$' (3,4-substituents), and $OCH_2C_6H_3R_2$' (3,5-substituents).

R' is selected from the group consisting of H, halogen, trifluoromethyl, NO_2, cyano, methyl, methoxy, acetyl, carboxyl, OH, and amino.

Y is selected from the group consisting of H, halogen, trifluoromethyl, methyl, OH, and methoxy.

Alternately, the Class II Substituted Isocoumarins are represented by structure (II) where,

Z is selected from the group consisting of C_{1-6} alkoxy with an attached isothiureido, C_{1-6} alkoxy with an attached guanidino, C_{1-6} alkoxy with an attached amidino, C_{1-6} alkyl with an attached amino, C_{1-6} alkyl with an attached isothiureido, C_{1-6} alkyl with an attached guanidino, C_{1-6} alkyl with an attached amidino,

R is selected from the group consisting of H, OH, NH_2, NO_2 halogen, C_{1-6} alkoxy, C_{1-6} fluorinated alkoxy, C_{1-6} alkyl, C_{1-6} alkyl with an attached amino, M-AA-NH-, M-AA-O-,

wherein AA represents alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, beta-alanine, norleucine, norvaline, alpha-aminobutyric and epsilon-aminocaponic acid, citrulline, hydroxyproline, ornithine and sarcosine,

wherein M represents H, lower alkanoyl having 1 to 6 carbons, carboxyalkanoyl, hydroxyalkanoyl, amin-alkanoyl, benzene sulfonyl, tosyl, benzoyl, and lower alkyl sulfonyl having 1 to 6 carbons,

Y is selected from the group consisting of H, halogen, trifluoromethyl, methyl, OH and methoxy.

As a further alternative, the Class II Substituted Isocoumarins are represented by structure (II) where

R is selected from the group consisting of -N-H-C(=NH)-NH_2, -C(=NH)NH_2, C_{1-6} alkyl with an attached amino, C_{1-6} alkyl with an attached isothiureido,

Z is selected from the group consisting of C_{1-6} alkoxy with an attached amino, C_{1-6} alkoxy with an attached isothiureido, C_{1-6} alkoxy with an attached guanidino, C_{1-6} alkoxy with an attached amidino, C_{1-6} alkyl with an attached amino, C_{1-6} alkyl with an attached guanidino, C_{1-6} alkyl with an attached amidino,

Y is selected from the group consisting of H, halogen, trifluoromethyl, methyl, OH and methoxy.

The following compounds are representative of the Class II Substituted Isocoumarins:

3-(3-aminopropoxy)isocoumarin,

3-(3-aminopropoxy)-4-chloroisocoumarin,

3-(2-isothiureidoethoxy)-4-chloroisocoumarin,

3-(3-isothiureidopropoxy)-4-chloroisocoumarin,

7-amino-3-(3-isothiureidopropoxy)-4-chloroisocoumarin,

7-guanidino-3-methoxyisocoumarin,

7-guanidino-3-methoxy-4-chloroisocoumarin,

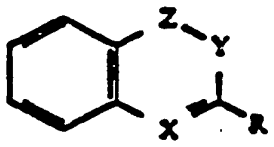
7-guanidino-3-ethoxyisocoumarin,

7-guanidino-3-ethoxy-4-chloroisocoumarin,

7-guanidino-3-(2-phenylethoxy)isocoumarin,

7-guanidino-3-(2-phenylethoxy)-4-chloroisocoumarin.

Still another class of susbstituted heterocyclic compounds useful in the present invention is referred to herein as the "Class III Heterocyclic Compounds" and have the following structural formula:

(III) 

wherein

Z is selected from the group consisting of CO, SO, SO_2, CCl and CF,

Y is selected from the group consisting of O, S and NH,

X is selected from the group consisting of N and CH, and

R is selected from the group consisting of C_{1-6} alkyl (such as methyl, ethyl and propyl), C_{1-4} alkyl containing a phenyl (such as benzyl), and C_{1-6} fluoroalkyl (such as trifluoromethyl, pentafluoroethyl, and heptafluoropropyl).

The Z group must be electrophilic since it interacts with the active site serine OH group of the serine protease. The R group must be uncharged and hydrophobic. One or more of the carbons in the R group could be replaced by O, S, NH and other such atomic groups as long as the R group maintains its hydrophobic character.

The following compounds are representative of the Class III Heterocyclic Compounds:

2-trifluoromethyl-4H-3,1-benzoxazine-4-one,

2-pentafluoroethyl-4H-3,1-benzoxazine-4-one,

2-heptafluoropropyl-4H-3,1-benzoxazine-4-one,

2-methyl-4H-3,1-benzoaxazine-4-one,
2-propyl-4H-3,1-benzoaxazine-4-one,
2-benzyl-4H-3,1-benxoaxazine-4-one,
2-heptafluoropropyl-4-quinazolinone,
2-propyl-4-quinazolinone,
2-benzyl-4-quinazolinone,
2-($C_6H_5CCl_2$)-4-chloroquinazoline, and
2-propyl-4-chloroquinazoline.

The Class III Heterocyclic Compounds are disclosed in Powers et al., U.S. Patent No. 4,847,202, the disclosure of which is hereby incorporated by reference.

Other substituted heterocyclic compounds have been prepared earlier for other purposes, such as 3-chloroisocoumarin, Davies and Poole, *J. Chem. Soc.*, pp. 1616-1629 (1928); 3-chloro and 3,4-dichloroisocoumarin, Milevskaya, et al., *Zhur. Org. Khim.*, **9**:2145-2149 (1973); 3-methyl and 4-carboxy-3-methylisocoumarin, Tirodkar and Usgaonkar, *Ind. J. Chem.*, **7**:1114-1116 (1969); 7-nitro and 7-aminoisocoumarin, Choksey and Usgaonkar, *Ind. J. Chem.*, **14B**:596-598 (1976). The disclosures of all of the preceding articles are hereby incorporated by reference. These other substituted isocoumarins are also believed to exhibit Calpain inhibitory activity when used in accordance with the present invention.

Still other substituted isocoumarins which have been prepared recently for inhibition of serine proteases are 3-chloroisocoumarin, Harper, et al., *J. A. Chem. Soc.*, **105**:6518-6520 (1983); 3,4-dichloroisocoumarin, Harper, et al., *Biochemistry*, **24**:1831-1841 (1985); 3-alkoxy-7-amino-4-chloroisocoumarin, Harper and Powers, *J. Am. Chem. Soc.*, **106**:7618-7619 (1984), Harper and Powers, *Biochemistry*, **24**:7200-7213 (1983); additional substituted isocoumarins with basic groups (aminoalkoxy, guanidino or isothiureidoalkoxy), Kam, et al., *Biochemistry*, **27**:2547-2557 (1988); 7-substituted 3-alkoxy-4-chloroisocoumarins, Powers, et al., *J. Cell Biochem.*, **39**:33-46 (1989) and Powers, et al., *Biochemistry*, **29**:3108-3118 (1990). The disclosures of all of the preceding articles are hereby incorporated by reference. We believe that the foregoing compounds, which exhibit serine protease inhibitory activity, also exhibit Calpain inhibitory activity when used in accordance with the present invention. All of the foregoing isocoumarin compounds, including the Class I and II Substituted

Isocoumarins, the Class III Substituted Heterocyclic Compounds and the other substituted heterocyclic compounds useful in the practice of the present invention shall be referred to collectively hereinafter as the "Substituted Heterocyclic Compounds." The term "Substituted Heterocyclic Compound" shall be used to refer to any particular species of these compounds.

The preparation of the various Substituted Heterocyclic Compounds is illustrated by Examples SHC1-SHC9.

EXAMPLE SHC1

Preparation of 7-(phenylcarbamoylamino)-4-chloroisocoumarin was synthesized as previously described in Powers, et al., *Biochemistry,* **29**:3108-3118 (1990). This compound (0.32 g, 1 mmole) was mixed with phenyl isocyanate (0.12g, 1 mmole) in 5 ml of THF and the reaction mixture was stirred at r.t. overnight. The product 7-(phenylcarbamoylamino)-4-chloro-3-(2-bromoethoxy)isocoumarin precipitated out, yield 40%, m.p. 215-217°C, mass spectrum m/e = 437.9 (M+)> Anal. Calc. for $C_{18}H_{14}N_2O_4ClBr$: C, 49.40; H, 3.22; N, 6.40; Cl, 8.10. Found: C,49.48; H, 3.25; N,6.34; Cl, 8.12. The phenylcarbamoylamino compound (0.1 g, 0.23 mmole) was heated with 0.02 g of thiourea (0.26 mmole) in 10 ml of THF at 70°C overnight. The final product precipitated out, yield 0.04 g, 36%, m.p. 161-163°C (dec.), mass spectrum (FAB+) m/e = 433 (M-Br). Anal. Calc. for $C_{19}H_{18}N_4O_4ClBrS$:0.25 THF: C, 45.12; H, 3.86; N, 10.53; Cl, 6.67. Found: C, 44.83; H, 3.92; N, 10.12; Cl, 6.41.

7-(Ethylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin, 7-(t-butylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin, 7-(benzylthiocarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin, 7-(ethylthiocarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy)isocoumarin, 7-(4-fluorobenzyl) thiocarbamoylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, and 7-(2,5-dimethylbenzyl) thiocarbamoylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin can be prepared by the same procedure.

EXAMPLE SHC2

Preparation of 7-(acetylamino)-4-chloro-3-(3-isothiureidopropoxy) isocoumarin:

7-Amino-3(3-bromopropoxy)-4-chloroisocoumarin was synthesized as previously described (Kam, et al., *supra*). This compound (0.33 g, 1 mmole) was heated with 0.15 g of acetic anhydride (1.5 mmole) in 20 ml of dry THF. After a few minutes, a yellow

solid precipitated out. After 3 hrs, the solution was concentrated to 5 ml, and the solid was filtered to give 0.37 g of 7-(acetylamino)-4-chloro-3-(3-bromopropoxy) isocoumarin, m.p. 170-172°C; mass spectrum: m/e = 375 (M+). The acetylated isocoumarin (0.15 g, 0.4 mmole) was treated with thiourea (0.036 g, 0.47 mmole) to give 0.9 g of the final product, (yield 50%), m.p. 180-181°C, mass spectrum m/e = 370 (M+-Br). Anal. Calc. for $C_{15}H_{17}N_3O_4ClBrS$: C, 39.97; H, 3.80; N, 9.32; Cl 7.87. Found: C, 39.86; H 3.83; N, 9.29; Cl, 7.85.

7-trifluoroacetylamino-4-chloro-3-(3-isothiureidopropoxy) isocoumarin, 7-heptafluorobutyroylamino-4-chloro-3-(3-isothiureidopropoxy) isocoumarin, 7-succinylamino-4-chloro-3-(3-isothiureidopropoxy) isocoumarin, and 7-(o-phthalyl)amino-4-chloro-3-(3-isothiureidopropoxy) isocoumarin can be prepared by the same procedure.

EXAMPLE SHC3

Preparation of 7-(benzylcarbamoylamino)-4-chloro-3-(3-isothiureidopropoxy) isocoumarin:

7-(benzylcarbamoylamino)-4-chloro-3(3-bromopropoxy) isocoumarin was prepared from the reaction of benzyl isocyanate with 7-amino-4-chloro-3-(3-bromopropoxy) isocoumarin as described above, m.p. 188-189°C, mass spectrum: m/e = 359 (M+ -benzyl). The final product was obtained from the reaction of 7-(benzylcarbamoylamino)-4-chloro-3-(3-bromopropoxy) isocoumarin with thiourea as described above (yield 74%), m.p. 165-166°C; mass spectrum (FAB+) m/e = 461 (M+-Br). Anal. Calc. for $C_{21}H_{22}N_4O_4ClBrS$:0.75 THF: C, 48.36; H, 4.70; N, 9.40; Cl, 6.56. Found: C, 48.13; H, 4.87; N, 9.65; Cl, 6.15.

EXAMPLE SHC4

Preparation of 7-(phenylacetylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin:

7-Amino-4-chloro-3-(2-bromoethoxy) isocoumarin (0.15 g, 0.47 mmole) was first mixed with phenylacetyl chloride (0.09 g, 0.55 mmole) in 10 ml of THF, triethylamine (0.05 g, 0.47 mmole) was then added and the reaction mixture was stirred at r.t. overnight. After $Et_3N{\cdot}HCl$ salt was removed by filtration, the product 7-(phenylacetylamino)-4-chloro-3-(2-bromoethoxy) isocoumarin was crystallized from THF and Pet. ether (yield, 73%), m.p. 165-169°C; mass spectrum; m/e = 436.7 (M+). The phenylacetyamino derivative (0.1 g) was heated with thiourea (0.02 g) to give the

product 0.05 g (yield, 40%), m.p. 115-120°C; mass spectrum (FAB+) m/e = 432 (M+ -Br). Anal. Calc. for $C_{20}H_{19}N_3O_4ClBrS \cdot 0.5\ H_2O$: C 45.99; H, 3.83; N, 8.05; Cl, 6.80. Found: C, 46.09; H, 4.17; N, 8.02; Cl, 6.79.

EXAMPLE SHC5

Preparation of 7-(R-α-methylbenzylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin:

7-(R-α-methylbenzylcarbamoylamino)-4-chloro-3-(2-bromoethoxy) isocoumarin was synthesized in the same manner as described above, m.p. 183-185°C; mass spectrum m/e = 464 (M+). This compound (0.1 g) reacted with thiourea (0.02 g) under the same condition described above to form the final product 7-(R-α-methylbenzylcarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin (0.078 g), m.p. 143-150°C; mass spectrum (FAB+) m/e = 461 (M+ -Br). Anal. Calc. for $C_{21}H_{22}N_4O_4ClBrS \cdot 0.5H_2O$: C, 45.75; H, 4.35; N, 10.17; Cl, 6.44. Found: C, 44.95; H, 4.31; N, 10.02; Cl, 6.36.

EXAMPLE SHC6

Preparation of 7-(D-phenylalanylamino)-4-chloro-3(2-isothiureidoethoxy) isocoumarin:

Boc-D-Phe (0.33 g, 1.2 mmole) reacted with 1,3-dicyclohexylcarbodiimide (0.13 g, 0.6 mmole) in 10 ml THF at 0°C for 1 hour to form the symmetric anhydride, and then 7-amino-4-chloro-3(2-bromoethoxy) isocoumarin (0.2g, 0.6 mmole) was added. The reaction was stirred at r.t. overnight and the precipitate 7-(Boc-D-Phe-amino)-4-chloro-3-(2-bromoethoxy) isocoumarin was formed (0.29 g, 71%). TLC one spot, m.p. 180-182°C; mass spectrum m/3 = 566(M^+). Anal. Calc. for $C25H_{26}N_2O_6ClBr$: C, 53.07; H, 4.63; N, 4.95; Cl 6.27. Found: C, 53.25: H, 4.66; N, 4.87; Cl, 6.24. Boc-D-Phe compound (0.2 g, 0.35 mmole) was reacted with thiourea (0.027 g, 0.35 mmole) in the same manner to give 7-(Boc-D-phenylalanylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin (0.14 g), yield 62%, mass spectrum (FAB^+) m/e = 561 (M^+ -Br). This compound (0.1 g) was dissolved in 3 ml of THF at 0°C and then the solvent was evaporated to dryness. The final product precipitated out after addition of ether, one spot on TLC (CH_3CN:$H_2$0:Ac0H = 8:1:1); mass spectrum (FAB^+) m/e = 462 (M^+ - Br -CF_3C00).

7-Boc-alanylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, 7-benzoylamino-Ala-4-chloro-3(2-isothiureidoethoxy) isocoumarin, 7-benzoylamino-Phe-4-chloro-3-(2-isothiureidoethoxy) isocoumarin and 7-Boc-valylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin can be prepared by the same procedure.

EXAMPLE SHC7

Preparation of 7-(Boc-alanylalanylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin:

7-(Boc-alanylalanylamino)-4-chloro-3-(2-bromoethoxy) isocoumarin was synthesized in the same manner, m.p. 147-151°C; mass spectrum m/e = 561 (M^+). Anal. Calc: C, 47.12: H, 4.85. Found: C, 47.18; H, 4.87. This compound (0.2 g) was reacted with thiourea (0.03 g) by the same procedure to form 7-(Boc-alanylalanylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin (0.04 g), mass spectrum m/e = 556 (M^+ -Br).

7-(Alanylalanylamino)-4-chloro-3(2-isothiureidoethoxy) isocoumarin was prepared by deblocking of Boc-Ala-Ala-NH-CiTEtOIC with trifluoroacetic acid, mass spectrum (FAB^+) m/e = 456 (M^+ -Br -CF_3COO).

EXAMPLE SHC8

Preparation of 7-(phenylthiocarbamoylamino)-4-chloro-3-(2-isothiureidoethoxy) isocoumarin:

7-(Phenylthiocarbamoylamino)-4-chloro-3-(2-bromoethoxy) isocoumarin was prepared from the reaction of phenyl isothiocyanate with 7-amino-4-chloro-3-(2-bromoethoxy) isocoumarin, yield 59%, m.p. 157-158°C; mass spectrum m/e = 361 (M^+ -$PhNH_+1$). Anal. Calc.: C, 48.36; H, 3.39. Found: C, 48.26; H, 3.40. The bromoethoxy compound was then reacted with thiourea by the same procedure to give the final product, yield 32%; mass spectrum (FAB^+) m/e 449 (M^+ -Br).

EXAMPLE SHC9

Preparation of 7-(m-carboxyphenylthiocarbamoylamino)-4-chloro-3-(2-bromoethoxy) isocoumarin was prepared from the reaction of m-carboxyphenyl isothiocyanate with 7-amino-4-chloro-3-(2-bromoethoxy) isocoumarin, yield 64%, m.p. 157-158°C; mass spectrum m/e 361 (M^+ -(COOH)$PhNH_+$-Br).

7-(3-Fluorobenzoyl)amino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, 7-(3-nitrobenzoyl) amino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, 7-

diphenylacetylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, 7-diphenylpropionylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, 7-(p-toluenesulfonyl) amino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, and 7-(α-toluenesulfonyl) amino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin can be prepared from the reaction of corresponding 7-substituted-4-chloro-3-(2-bromoethoxy) isocoumarin with thiourea as described above. 7-substituted-4-chloro-3-(2-bromoethoxy) isocoumarin can be synthesized by reacting 7-amino-4-chloro-3-(2-bromoethoxy) isocoumarin with appropriate acid chloride or sulfonyl chloride in the presence of Et_3N.

7-Ethoxycarbonylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, 7-benzyloxycarbonylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin, and 7-phenoxycarbonylamino-4-chloro-3-(2-isothiureidoethoxy) isocoumarin can be prepared from the reaction of 7-substituted-4-chloro-3-(2-bromoethoxy) isocoumarin with thiourea. 7-Ethoxycarbonylamino-4-chloro-3-(2-bromoethoxy) isocoumarin, 7-benzyloxycarbonylamino-4-chloro-3-(2-bromoethoxy) isocoumarin and 7-phenoxycarbonylamino-4-chloro-3-(2-bromoethoxy) isocoumarin can be synthesized by reacting 7-amino-4-chloro-3-(2-bromoethoxy) isocoumarin with the corresponding chloroformate.

C. PEPTIDE KETO-COMPOUNDS

Peptide α-ketoesters, peptide α-ketoacids, and peptide α-ketoamides are transition state analog inhibitors for serine proteases and cysteine proteases. While these subclasses of compounds are chemically distinguishable, for simplicity, all of these compounds will be referred to collectively herein as the "Peptide Keto-Compounds".

The interactions of peptides with serine and cysteine proteases are designated herein using the nomenclature of Schechter and Berger, *Biochem. Biophys. Res. Commun.*, **27**:157-162 (1967), incorporated herein by reference. The individual amino acid residues of a substrate or inhibitor are designated P1, P2, etc. and the corresponding subsites of the enzyme are designated S1, S2, etc. The scissile bond of the substrate is P1-P1'. The primary recognition site of serine proteases is S1. The most important recognition subsites of cysteine proteases are S1 and S2. There are additional recognition sites at the prime subsites such as S1' and S2'.

Amino acid residues and blocking groups are designated using standard abbreviations using nomenclature rules presented in *J. Biol. Chem.*, **260**:14-42 (1985), incorporated herein by reference. An amino acid residue (AA) in a peptide or inhibitor structure refers to the part structure -NH-CHR_1-CO-, where R_1 is the side chain of the amino acid AA. A peptide α-ketoester residue would be designated -AA-CO-OR which represents the part structure -NH-CHR_1-CO-CO-OR. Thus, the ethyl ketoester derived from benzoyl alanine would be designated Bz-Ala-CO-OEt which represents C_6H_5CO-NH-CHMe-CO-CO-OEt. Likewise, peptide ketoacid residues would be designated -AA-CO-OH. Further, peptide ketoamide residues are designated -AA-CO-NH-R. Thus, the ethyl keto amide derived from Z-Leu-Phe-OH would be designated Z-Leu-Phe-CO-NH-Et which represents $C_6H_5CH_2OCO$-NH-$CH(CH_2CHMe_2)$-CO-NH-$CH(CH_2Ph)$-CO-CO-NH-Et.

Peptide α-ketoesters containing amino acid residues with hydrophobic side chain at the P1 site have also been found to be excellent inhibitors of several cysteine proteases including papain, cathepsin B and calpain. Calpains can be inhibited by peptide inhibitors having several different active groups. Structure-activity relationships with the commercially available *in vitro* inhibitors of Calpain, such as peptide aldehydes, have revealed that Calpains strongly prefer Leu or Val in the P2 position. These enzymes are inhibited by inhibitors having a wide variety of amino acids in the P1 position, but are generally more effectively inhibited by inhibitors having amino acids with nonpolar or hydrophobic side chains in the P1 position. Thus, we have discovered that another particular class of compounds exhibiting Calpain inhibitory activity, when used in accordance with the present invention, are the Peptide Keto-Compounds. These are compounds of the general structure:

$$M\text{-}(aa)_n\text{-}\overset{\overset{O}{\|}}{C}\text{-}Q\text{-}R$$

or a pharmaceutically acceptable salt, wherein:

M represents NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X-NH-CS-, X-NH-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-, H, acetyl, carbobenzoxy, succinyl, methyloxysuccinyl, butyloxycarbonyl;

X is selected from the group consisting of C_{1-6} alkyl, C_{1-6} fluoroalkyl, C_{1-6} alkyl substituted with J, C_{1-6} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-6} alkyl with an attached phenyl group, C_{1-6} alkyl with two attached phenyl groups, C_{1-6} alkyl with an attached phenyl group substituted with K, and C_{1-6} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-6} alkoxy, C_{1-6} alkylamine, C_{1-6} dialkylamine, C_{1-6} alkyl-O-CO-, C_{1-6} alkyl-O-CO-NH, and C_{1-6} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-6} alkyl, C1-6 perfluoroalkyl, C_{1-6} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-6} alkylamino, C_{2-12} dialkylamino, C_{1-6} acyl, and C_{1-6} alkoxy-CO-, and C_{1-6} alkyl-S-;

aa represents a blocked or unblocked amino acid of the L or D configuration, preferably selected from the group consisting of: alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine (nle), norvaline (nva), alpha-aminobutyric acid (abu), epsilon-aminocaproic acid, citrulline, hydroxyproline, homoarginine, ornithine or sarcosine;

n is a number from 1 to 20;

Q is O or NH,

R represents H, C_{1-6} alkyl, C_{1-6} fluoroalkyl, C_{1-6} chloroalkyl, benzyl, C_{1-6} alkyl substituted with phenyl, C_{1-6} alkyl with an attached phenyl group substituted with K.

Thus, the Peptide Keto-Compounds can be divided into the Peptide Ketoesters, Peptide Ketoacids and Peptide Ketoamides. Each of the compounds can also be classified based on the number of amino acids contained within the compound, such as an amino acid peptide, dipeptide, tripeptide, tetrapeptide, pentapeptide and so on.

We have found certain subclasses of Peptide α-Ketoester compounds to be particularly useful as Calpain Inhibitors when used in accordance with the present invention. These subclasses are referred to herein as the Dipeptide α-Ketoesters (Subclass A), the Dipeptide α-Ketoesters (Subclass B), the Tripeptide α-Ketoesters (Subclass A), the Tripeptide α-Ketoesters (Subclass B), the Tetrapeptide α-Ketoesters and the Amino Acid Peptide α-Ketoesters. All of these subclasses are considered to be to be within the class of Peptide Keto-Compounds.

The Dipeptide α-Ketoesters (Subclass A) are compounds of the formula:

$$M_1\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}O\text{-}R_1$$

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of $C_{1\text{-}10}$ alkyl, $C_{1\text{-}10}$ fluoroalkyl, $C_{1\text{-}10}$ alkyl substituted with J, $C_{1\text{-}10}$ fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, $C_{1\text{-}10}$ alkyl with an attached phenyl group, $C_{1\text{-}10}$ alkyl with two attached phenyl groups, $C_{1\text{-}10}$ alkyl with an attached phenyl group substituted with K, and $C_{1\text{-}10}$ alkyl with two attached phenyl groups substituted with K, $C_{1\text{-}10}$ alkyl with an attached phenoxy group, and $C_{1\text{-}10}$ alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, $C_{1\text{-}10}$ alkoxy, $C_{1\text{-}10}$ alkylamine, $C_{2\text{-}12}$ dialkylamine, $C_{1\text{-}10}$ alkyl-O-CO-, $C_{1\text{-}10}$ alkyl-O-CO-NH-, and $C_{1\text{-}10}$ alkyl-S-;

K is selected from the group consisting of halogen, $C_{1\text{-}10}$ alkyl, $C_{1\text{-}10}$ perfluoroalkyl, $C_{1\text{-}10}$ alkoxy, NO_2, CN, OH, CO_2H, amino, $C_{1\text{-}10}$ alkylamino, $C_{2\text{-}12}$ dialkylamino, C_1-C_{10} acyl, and $C_{1\text{-}10}$ alkoxy-CO-, and $C_{1\text{-}10}$ alkyl-S-;

AA_1 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, serine, threonine, cysteine, tyrosine, asparagine, glutamine,

aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

AA_2 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_1 is selected from the group consisting of H, $C_{1\text{-}20}$ alkyl, $C_{1\text{-}20}$ alkyl with a phenyl group attached to the $C_{1\text{-}20}$ alkyl, and $C_{1\text{-}20}$ alkyl with an attached phenyl group substituted with K.

The Dipeptide α-Ketoesters (Subclass B) are compounds of the structure:

M_1-AA-NH-CHR_2-CO-CO-O-R

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_2 represents C_{1-8} branched and unbranched alkyl, C_{1-8} branched and unbranched cyclized alkyl, or C_{1-8} branched and unbranched fluoroalkyl;

R is selected from the group consisting of H, C_{1-20} alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, and C_{1-20} alkyl with an attached phenyl group substituted with K.

The Tripeptide α-Ketoesters (Subclass A) are compounds of the structure:

M_3-AA-AA-AA-CO-O-R

or a pharmaceutically acceptable salt, wherein

M_3 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, T-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

T is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{2-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R is selected from the group consisting of H, C_{2-20} alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, and C_{1-20} alkyl with an attached phenyl group substituted with K.

The Tripeptide α-Ketoesters (Subclass B) are compounds of the structure:

M_3-AA-AA-NH-CHR_2-CO-CO-O-R

or a pharmaceutically acceptable salt, wherein

M_3 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, T-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

T is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{2-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{1-10} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_2 represents C_{1-8} branched and unbranched alkyl, C_{1-8} branched and unbranched cyclized alkyl, or C_{1-8} branched and unbranched fluoroalkyl;

R is selected from the group consisting of H, C_{1-20} alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, and C_{1-20} alkyl with an attached phenyl group substituted with K.

The Tetrapeptide α-Ketoesters are compounds of the structure:

M_3-AA_4-AA-AA-AA-CO-O-R

or a pharmaceutically acceptable salt, wherein

M_3 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, T-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

T is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{2-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine,

glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;;

AA_4 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of leucine, isoleucine, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R is selected from the group consisting of H, $C_{1\text{-}20}$ alkyl, $C_{1\text{-}20}$ alkyl with a phenyl group attached to the $C_{1\text{-}20}$ alkyl, and $C_{1\text{-}20}$ alkyl with an attached phenyl group substituted with K.

The Amino Acid Peptide α-Ketoesters are compounds of the structure:

M_1-AA-CO-O-R

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, Y-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

Y is selected from the group consisting of C_{6-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH,

NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R is selected from the group consisting of H, C_{1-20} alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, and C_{1-20} alkyl with an attached phenyl group substituted with K.

A few amino acid and peptide ketoesters and ketoacids have been previously reported. Cornforth and Cornforth in *J. Chem. Soc.*, 93-96 (1953), incorporated herein by reference, report the synthesis of the ketoacids PhCH2CO-Gly-CO-OH and Ac-Gly-CO-OH upon hydrolysis of heterocyclic molecules. Charles et al. in *J. Chem. Soc. Perkin* I:1139-1146 (1980), incorporated herein by reference, use ketoesters for the synthesis of bicyclic heterocycles. They report the synthesis of n-Bu-CO-Ala-CO-OEt, Pr-CO-Ala-CO-OEt, cyclopentyl-CO-Ala-CO-OEt, Pr-CO-Phg-CO-OEt, and Bz-Ala-CO-OEt. Hori et al. in *Peptides: Structure and Function-Proceedings of the Ninth American Peptide Symposium* (Deber, Hruby, and Kopple, Eds., Pierce Chemical Co.), pp 819-822 (1985), incorporated herein by reference, report Bz-Ala-CO-OEt, Bz-Ala-CO-OH, Z-Ala-Ala-Abu-CO-OEt, Z-Ala-Ala-Abu-CO-OBzl, and Z-Ala-Ala-Ala-Ala-CO-OEt (Abu = 2-aminobutanoic acid or a-aminobutyric acid) and report that these compounds inhibit elastase. Trainer in *Trends Pharm. Sci.* 8:303-307 (1987), incorporated herein by reference, comments on one of these compounds. Burkhart, J. *et al.* in *Tetrahedron Lett.* 29:3433-3436 (1988), incorporated herein by reference, report the synthesis of Z-Val-Phe-CO-OMe and Bz-Phe-CO-OMe.

Angelastro *et al.* in *J. Med. Chem.* 33:13-16 (1990), incorporated herein by reference, report some a-ketoesters which are inhibitors of calpain and chymotrypsin. Hu and Abeles in *Arch. Biochem. Biophys.* 281:271-274 (1990), incorporated herein by reference, report some peptidyl a-ketoamides and a-ketoacids which are inhibitors of cathepsin B and papain. Peet *et al.* in *J. Med. Chem.* 33:394-407 (1990), incorporated herein by reference, report some peptidyl a-ketoesters which are inhibitors of porcine pancreatic elastase, human neutrophil elastase, and rat & human neutrophil cathepsin G.

The following Peptide Ketoester compounds are representative of the Peptide Keto-Compounds found to be useful as Calpain inhibitors within the context of the present invention:

Bz-DL-Ala-COOEt

Bz-DL-Ala-COOBzl

Bz-DL-Ala-COOnBu

Bz-DL-Phe-COOEt

Bz-DL-Ala-COOCH2-C_6H_4-CF_3 (para)

Bz-DL-Arg-COOEt

Bz-DL-Lys-COOEt

Z-Ala-DL-Ala-COOEt

Z-Ala-DL-Ala-COOBzl

Z-Ala-DL-Ala-COOnBu

MeO-Suc-Ala-DL-Ala-COOMe

Z-Leu-Nva-COOEt

Z-Leu-Nle-COOEt

Z-Leu-Phe-COOEt

Z-Leu-Abu-COOEt

Z-Leu-Met-COOEt

Z-Phe-DL-Phe-COOEt

H-Gly-DL-Lys-COOEt

H-Ala-DL-Lys-COOEt

H-Pro-DL-Lys-COOEt

H-Phe-DL-Lys-COOEt

Z-Ala-Ala-DL-Ala-COOEt

Z-Ala-Pro-DL-Ala-COOEt

Z-Ala-Ala-DL-Abu-COOEt

Z-Ala-Ala-DL-Abu-COOBzl

Z-Ala-Ala-DL-Abu-COOCH2-C_6H_4-CF_3 (para)

MeO-Suc-Val-Pro-DL-Phe-COOMe

H-Leu-Ala-DL-Lys-COOEt

Z-Ala-Ala-Ala-DL-Ala-COOEt

MeO-Suc-Ala-Ala-Pro-DL-Abu-COOMe.

Z-Leu-Phe-COOEt

PhCO-Abu-COOEt

$(CH_3)_2CH(CH_2)_2CO$-Abu-COOEt

$CH_3CH_2CH)_2CHCO$-Abu-COOEt

$Ph(CH_2)_6CO$-Abu-COOEt

Z-Leu-4-Cl-Phe-COOEt

Z-Leu-Leu-Abu-COOEt

Z-Leu-Leu-Phe-COOEt

2-$NapSO_2$-Leu-Abu-COOEt

2-$NapSO_2$-Leu-Leu-Abu-COOEt

Z-Leu-NLeu-CO_2Et

Z-Leu-Phe-CO_2Bu

Z-Leu-Abu-CO_2Bu

Z-Leu-Phe-CO_2Bzl

Z-Leu-Abu-CO_2Bzl.

We have found certain subclasses of Peptide Ketoacid Compounds to be particularly useful when used in accordance with the present invention. These are subclasses are the Dipeptide α-Ketoacids (Subclass A), the Dipeptide α-Ketoacids (Subclass B), the Tripeptide α-Ketoacids, the Tetrapeptide α-Ketoacids and the Amino Acid peptide α-Ketoacids. All of these are considered to be within the class of Peptide Keto-Compounds.

The Dipeptide α-Ketoacids (Subclass A) are compounds of the structure:

M_1-AA-NH-CHR_2-CO-CO-OH

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl

trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_2 represents C_{1-8} branched and unbranched alkyl, C_{1-8} branched and unbranched cyclized alkyl, or C_{1-8} branched and unbranched fluoroalkyl.

The Dipeptide α-Ketoacids (Subclass B) are compounds of the structure:

M_1-AA_2-AA_1-CO-OH

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA_1 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homcarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

AA_2 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine,

glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine.

The Tripeptide α-Ketoacids are compounds of the structure:

M_1-AA-AA-AA-CO-OH

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of

alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine.

The Tetrapeptide α-Ketoacids are compounds of the structure:

M_1-AA-AA-AA-AA-CO-OH

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, Y_1-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

Y_1 is selected from the group consisting of C_{2-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two

attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine.

The Amino Acid Peptide α-Ketoacids are compounds of the structure:

M_1-AA-CO-OH

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, Y_2-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two

attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

Y_2 is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, and C_{1-10} alkyl with two attached phenyl groups substituted with K;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, alpha-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine.

The following Peptide Ketoacid compounds are representative of the Peptide Keto-Compounds found to be useful as Calpain inhibitors within the context of the present invention:

Bz-DL-Lys-COOH

Bz-DL-Ala-COOH

Z-Leu-Phe-COOH

Z-Leu-Abu-COOH.

The peptide α-ketoamides are transition state analogue inhibitors for cysteine proteases, such as Calpain. We have found that Peptide α-ketoamides containing amino acid residues with hydrophobic side chains at the P_1 site are excellent inhibitors of several cysteine proteases including calpain I and calpain II.

We have found six subclasses of the peptide ketoamides to be particularly effective in inhibiting Calpain. These subclasses are referred to herein as Dipeptide α-Ketoamides (Subclass A), Dipeptide α-Ketoamides (Subclass B), Dipeptide α-Ketoamides (Subclass C, Types 1 through 6), Tripeptide α-Ketoamides, Tetrapeptide α-Ketoamides and Amino Acid α-Ketoamides. All of these subclasses are considered herein to be within the class of Peptide Keto-Compounds.

The Dipeptide α-Ketoamides (Subclass A) have the following structural formula:

$$M_1\text{-AA-NH-CHR}_2\text{-CO-CO-NR}_3\text{R}_4$$

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of $C_{1\text{-}10}$ alkyl, $C_{1\text{-}10}$ fluoroalkyl, $C_{1\text{-}10}$ alkyl substituted with J, $C_{1\text{-}10}$ fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, $C_{1\text{-}10}$ alkyl with an attached phenyl group, $C_{1\text{-}10}$ alkyl with two attached phenyl groups, $C_{1\text{-}10}$ alkyl with an attached phenyl group substituted with K, $C_{1\text{-}10}$ alkyl with two attached phenyl groups substituted with K, $C_{1\text{-}10}$ alkyl with an

attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, α-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_2 is selected from the group consisting of C_{1-8} branched and unbranched alkyl, C_{1-8} branched and unbranched cyclized alkyl, and C_{1-8} branched and unbranched fluoroalkyl;

R_3 and R_4 are selected independently from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [-$N(CH_2CH_2)O$] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached

through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, -$CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), and -NH-CH_2CH_2-(3-indolyl).

The Dipeptide α-Ketoamides (Subclass B) have the following structural formula:

$$M_1\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NR_3R_4$$

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA_1 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, α-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-

azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

AA_2 is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, α-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_3 and R_4 are selected independently from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [-N(CH_2CH_2)O] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, -$CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), and -NH-CH_2CH_2-(3-indolyl).

The Dipeptide α-Ketoamides (Subclass C, Type 1) have the following structural formula:

$$M_1CO\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NH\text{-}CH_2CH(OH)\text{-}R_1$$

or a pharmaceutically acceptable salt, wherein

M_1 is selected from the group consisting of C_{1-4} alkyl monosubstituted with phenyl, C_{1-4} alkyl disubstituted with phenyl, C_{1-4} alkyl monosubstituted with 1-naphthyl, C_{1-4} alkyl monosubstituted with 2-naphthyl, C_{1-4} alkoxy monosubstituted with phenyl, C_{1-4} alkoxy disubstituted with phenyl, $ArCH_2O$-, ArO-, $ArCH_2NH$-, and ArNH-;

wherein Ar is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, 1-naphthyl, 1-naphthyl monosubstituted with J, 2-naphthyl, and 2-naphthyl monosubstituted with J;

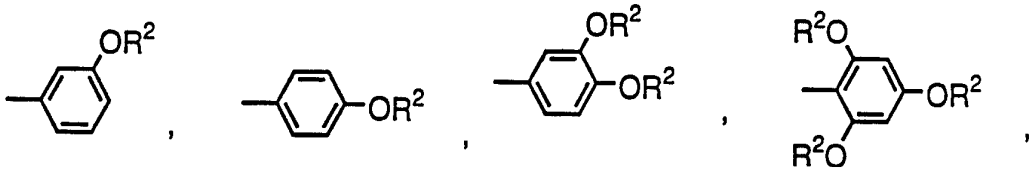
J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $-N(CH_2CH_2)_2O$;

AA_2 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

AA_1 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, arginine, lysine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH,

NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

R_1 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, pentafluorophenyl,



1-naphthyl, 1-naphthyl monosubstituted with J, 1-naphthyl disubstituted with J, 2-naphthyl, 2-naphthyl monosubstituted with J, 2-naphthyl disubstituted with J, 2-pyridyl, 2-quinolinyl, and 1-isoquinolinyl;

R_2 represents C_{1-4} alkyl substituted with phenyl, phenyl and phenyl substituted with J.

Dipeptide α-Ketoamides (Subclass C, Type 2) have the following structural formula:

$$M_1CO\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NH\text{-}(CH_2)_n\text{-}R_3$$

or a pharmaceutically acceptable salt, wherein

M_1 is selected from the group consisting of C_{1-4} alkyl monosubstituted with phenyl, C_{1-4} alkyl disubstituted with phenyl, C_{1-4} alkyl monosubstituted with 1-naphthyl, C_{1-4} alkyl monosubstituted with 2-naphthyl, C_{1-4} alkoxy monosubstituted with phenyl, C_{1-4} alkoxy disubstituted with phenyl, $ArCH_2O$-, ArO-, $ArCH_2NH$-, and ArNH-;

wherein Ar is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, 1-naphthyl, 1-naphthyl monosubstituted with J, 2-naphthyl, and 2-naphthyl monosubstituted with J;

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $-N(CH_2CH_2)_2O$;

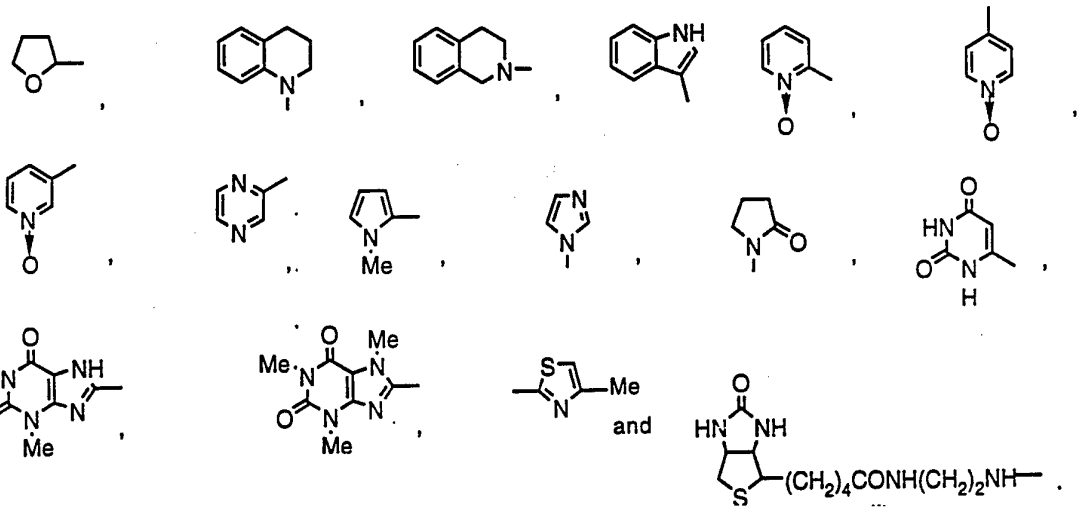
AA_2 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid,

O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

AA_1 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, arginine, lysine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

n = 1-3;

R_3 is selected from the group consisting of 2-furyl, 2-furyl monosubstituted with J, 2-pyridyl, 2-pyridyl monosubstituted with J, 3-pyridyl, 3-pyridyl monosubstituted with J, 4-pyridyl, 4-pyridyl monosubstituted with J, 2-quinolinyl, 2-quinolinyl monosubstituted with J, 1-isoquinolinyl, 1-isoquinolinyl monosubstituted with J,



Dipeptide α-Ketoamides (Subclass C, Type 3) have the following structural formula:

$$M_3\text{-}(CH_2)_q\text{-}CO\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NH\text{-}CH_2CH(OH)\text{-}R_1$$

or a pharmaceutically acceptable salt, wherein

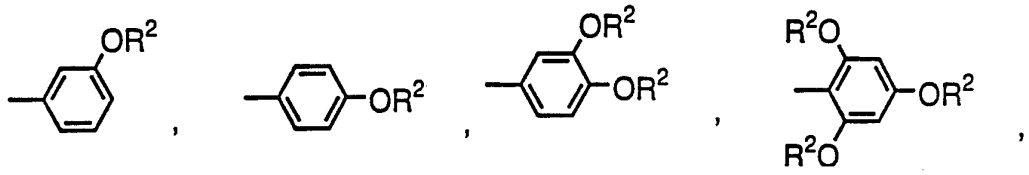
M_3 is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrazinyl, 2-quinolinyl, 1-tetrahydroquinolinyl, 1-isoquinolinyl, 2-tetrahydroisoquinolinyl, and $-N(CH_2CH_2)_2O$;

q = 0-2;

AA_2 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

AA_1 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, arginine, lysine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

R_1 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, pentafluorophenyl,



1-naphthyl, 1-naphthyl monosubstituted with J, 1-naphthyl disubstituted with J, 2-naphthyl 2-naphthyl monosubstituted with J, 2-naphthyl disubstituted with J, 2-pyridyl, 2-quinolinyl, and 1-isoquinolinyl;

R_2 represents C_{1-4} alkyl substituted with phenyl, phenyl and phenyl substituted with J.

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $N(CH_2CH_2)_2O$;

Dipeptide α-Ketoamides (Subclass C, Type 4) have the following structural formula:

$$M_3\text{-}(CH_2)_q\text{-}CO\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NH\text{-}(CH_2)_n\text{-}R_3$$

or a pharmaceutically acceptable salt, wherein

M_3 is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrazinyl, 2-quinolinyl, 1-tetrahydroquinolinyl, 1-isoquinolinyl, 2-tetrahydroisoquinolinyl, and $-N(CH_2CH_2)_2O$;

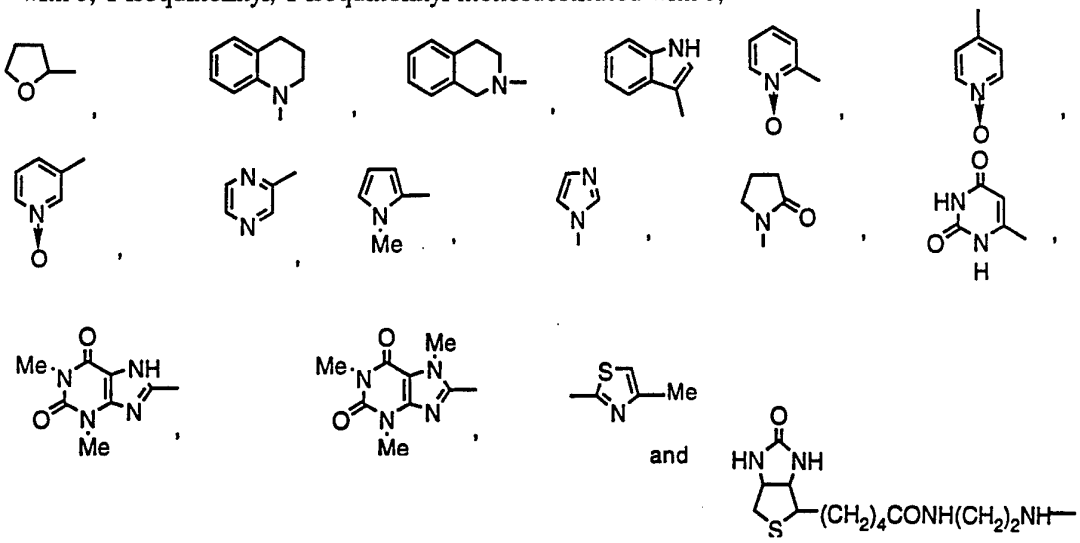
q = 0-2;

AA_2 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

AA_1 is an amino acid with the L configuration, D configuration, or DL configuration at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, arginine, lysine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

n = 1-3;

R_3 is selected from the group consisting of 2-furyl, 2-furyl monosubstituted with J, 2-pyridyl, 2-pyridyl monosubstituted with J, 3-pyridyl, 3-pyridyl monosubstituted with J, 4-pyridyl, 4-pyridyl monosubstituted with J, 2-quinolinyl, 2-quinolinyl monosubstituted with J, 1-isoquinolinyl, 1-isoquinolinyl monosubstituted with J,



and

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $N(CH_2CH_2)_2O$;

Dipeptide α-Ketoamides (Subclass C, Type 5) have the following structural formula:

$$M_4\text{-}(CH_2)_q\text{-}O\text{-}CO\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NH\text{-}CH_2CH(OH)\text{-}R_1$$

or a pharmaceutically acceptable salt, wherein

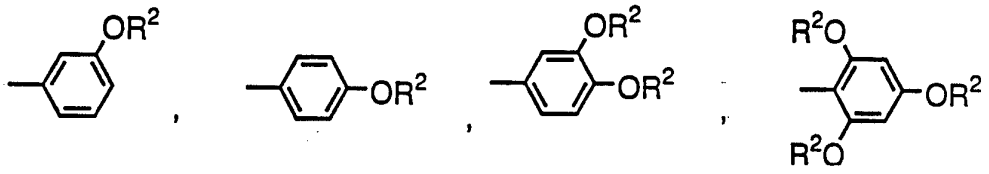
M_4 is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyridyl, 2-pyrazinyl, 2-quinolinyl, 2-tetrahydroquinolinyl, 1-isoquinolinyl, and 1-tetrahydroisoquinolinyl;

q = 0-2;

AA_2 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

AA_1 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, arginine, lysine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

R_1 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, pentafluorophenyl,



1-naphthyl, 1-naphthyl monosubstituted with J, 1-naphthyl disubstituted with J, 2-naphthyl 2-naphthyl monosubstituted with J, 2-naphthyl disubstituted with J, 2-pyridyl, 2-quinolinyl, and 1-isoquinolinyl;

R_2 represents C_{1-4} alkyl substituted with phenyl, phenyl and phenyl substituted with J.

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $N(CH_2CH_2)_2O$;

Dipeptide α-Ketoamides (Subclass C, Type 6) have the following structural formula:

$$M_4\text{-}(CH_2)_q\text{-}O\text{-}CO\text{-}AA_2\text{-}AA_1\text{-}CO\text{-}NH\text{-}(CH_2)_n\text{-}R_3$$

or a pharmaceutically acceptable salt, wherein

M_4 is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyridyl, 2-pyrazinyl, 2-quinolinyl, 2-tetrahydroquinolinyl, 1-isoquinolinyl, and 1-tetrahydroisoquinolinyl;

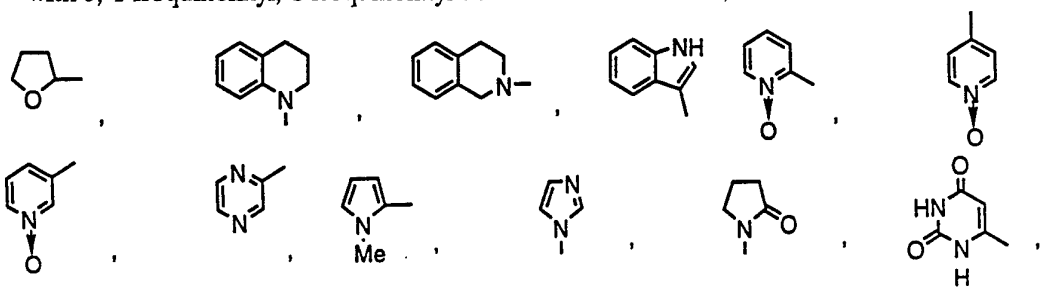
q = 0-2;

AA_2 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

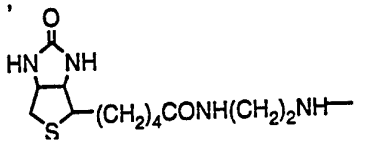
AA_1 is an amino acid with the L configuration, D configuration, or DL configuration at the a-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, histidine, methionine, methionine sulfoxide, phenylalanine, arginine, lysine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, phenylglycine, norleucine, norvaline, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

n = 1-3;

R_3 is selected from the group consisting of 2-furyl, 2-furyl monosubstituted with J, 2-pyridyl, 2-pyridyl monosubstituted with J, 3-pyridyl, 3-pyridyl monosubstituted with J, 4-pyridyl, 4-pyridyl monosubstituted with J, 2-quinolinyl, 2-quinolinyl monosubstituted with J, 1-isoquinolinyl, 1-isoquinolinyl monosubstituted with J,



and



J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $N(CH_2CH_2)_2O$.

The Tripeptide α-Ketoamides have the following structural formula:

M_1-AA-AA-AA-CO-NR_3R_4

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, α-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-

cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_3 and R_4 are selected independently from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [-N(CH_2CH_2)O] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, -$CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), and -NH-CH_2CH_2-(3-indolyl).

The Tetrapeptide α-Ketoamides have the following structural formula:

$$M_1\text{-AA-AA-AA-AA-CO-}NR_3R_4$$

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, α-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-aminoheptanoic acid, NH_2-$CH(CH_2$-1-napthyl)-COOH, NH_2-$CH(CH_2$-2-napthyl)-COOH, NH_2-$CH(CH_2$-cyclohexyl)-COOH, NH_2-$CH(CH_2$-cyclopentyl)-COOH, NH_2-$CH(CH_2$-cyclobutyl)-COOH, NH_2-$CH(CH_2$-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_3 and R_4 are selected independently from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [$-N(CH_2CH_2)O$] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, $-CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), and -NH-CH_2CH_2-(3-indolyl).

The Amino Acid α-Ketoamides have the following structural formula:

$$M_1\text{-AA-CO-}NR_3R_4$$

or a pharmaceutically acceptable salt, wherein

M_1 represents H, NH_2-CO-, NH_2-CS-, NH_2-SO_2-, X-NH-CO-, X_2N-CO-, X-NH-CS-, X_2N-CS-, X-NH-SO_2-, X_2N-SO_2-, X-CO-, X-CS-, X-SO_2-, X-O-CO-, or X-O-CS-;

X is selected from the group consisting of C_{1-10} alkyl, C_{1-10} fluoroalkyl, C_{1-10} alkyl substituted with J, C_{1-10} fluoroalkyl substituted with J, 1-admantyl, 9-fluorenyl, phenyl, phenyl substituted with K, phenyl disubstituted with K, phenyl trisubstituted with K, naphthyl, naphthyl substituted with K, naphthyl disubstituted with K, naphthyl trisubstituted with K, C_{1-10} alkyl with an attached phenyl group, C_{1-10} alkyl with two attached phenyl groups, C_{1-10} alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with two attached phenyl groups substituted with K, C_{1-10} alkyl with an attached phenoxy group, and C_{1-10} alkyl with an attached phenoxy group substituted with K on the phenoxy group;

J is selected from the group consisting of halogen, COOH, OH, CN, NO_2, NH_2, C_{1-10} alkoxy, C_{1-10} alkylamine, C_{2-12} dialkylamine, C_{1-10} alkyl-O-CO-, C_{1-10} alkyl-O-CO-NH-, and C_{1-10} alkyl-S-;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_1-C_{10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

AA is a side chain blocked or unblocked amino acid with the L configuration, D configuration, or no chirality at the α-carbon selected from the group consisting of alanine, valine, leucine, isoleucine, proline, methionine, methionine sulfoxide, phenylalanine, tryptophan, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, phenylglycine, beta-alanine, norleucine, norvaline, α-aminobutyric acid, epsilon-aminocaproic acid, citrulline, hydroxyproline, ornithine, homoarginine, sarcosine, indoline 2-carboxylic acid, 2-azetidinecarboxylic acid, pipecolinic acid (2-piperidine carboxylic acid), O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, α-aminoheptanoic acid, NH_2-CH(CH_2-1-napthyl)-COOH, NH_2-CH(CH_2-2-napthyl)-COOH, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, trifluoroleucine, and hexafluoroleucine;

R_3 and R_4 are selected independently from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [$-N(CH_2CH_2)O$] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, $-CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, $-NH-CH_2CH_2$-(4-hydroxyphenyl), and $-NH-CH_2CH_2$-(3-indolyl).

The Applicants are aware of only a single peptide ketoamide reported in the literature. This compound is Z-Phe-$NHCH_2$CO-CO-NH-Et (Z-Phe-Gly-CO-NH-Et). The compound is reported by Hu and Abeles (*supra*) to be an inhibitor of papain (K_i = 1.5 mM) and cathepsin B (K_i = 4 mM).

The following Peptide Ketoamide compounds are representative of the Peptide Keto-Compounds found to be useful as Calpain inhibitors within the context of the present invention:

Z-Leu-Phe-CONH-Et
Z-Leu-Phe-CONH-nPr
Z-Leu-Phe-CONH-nBu
Z-Leu-Phe-CONH-iBu
Z-Leu-Phe-CONH-Bzl
Z-Leu-Phe-CONH-$(CH_2)_2$Ph
Z-Leu-Abu-CONH-Et
Z-Leu-Abu-CONH-nPr
Z-Leu-Abu-CONH-nBu
Z-Leu-Abu-CONH-iBu
Z-Leu-Abu-CONH-Bzl
Z-Leu-Abu-CONH-$(CH_2)_2$Ph
Z-Leu-Abu-CONH-$(CH_2)_3$-$N(CH_2CH_2)_2O$

Z-Leu-Abu-CONH-$(CH_2)_7CH_3$

Z-Leu-Abu-CONH-$(CH_2)_2OH$

Z-Leu-Abu-CONH-$(CH_2)_2O(CH_2)_2OH$

Z-Leu-Abu-CONH-$(CH_2)_{17}CH_3$

Z-Leu-Abu-CONH-CH_2-C_6H_3[3,5-$(OCH_3)_2$]

Z-Leu-Abu-CONH-CH_2-C_4H_4N

Z-Leu-Abu-CONH-$(CH_2)_5OH$

Z-Leu-Abu-CONH-$CH_2CH(OCH_3)_2$

Z-Leu-Abu-CONH-$CH_2CH(OC_2H_5)_2$

Z-Leu-Abu-CONH-CH_2-C_6H_8[1,3,3-$(CH_3)_3$-5-OH]

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(4-OH)

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(2-OCH_3)

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(3-OCH_3)

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(4-OCH_3)

Z-Leu-Abu-CONH-$CH_2CH(OH)Ph$

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(4-OCH_3)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_2$[2,4,6-$(OCH_3)_3$]

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$[4-$N(CH_3)_2$]

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6F_5$

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(3-CF_3)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(3-OPh)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(4-OPh)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(4-OCH_2Ph)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$-3-OC_6H_4(3-CF_3)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$-3-OC_6H_3(3,4-Cl_2)

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_3$[3,4-$(OCH_2Ph)_2$]

Z-Leu-Abu-CONH-$CH_2CH(OH)$-1-$C_{10}H_7$

Z-Leu-Abu-CONH-$CH_2CH(OH)$-2-$C_{10}H_7$

Z-Leu-Phe-CONH-$CH_2CH(OH)Ph$

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$[4-$N(CH_3)_2$]

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6F_5$

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(3-CF_3)

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(3-OPh)

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(4-OPh)

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(4-OCH_2Ph)

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$-3-OC_6H_4(3-CF_3)

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$-3-OC_6H_3(3,4-Cl_2)

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_3$(3,4-$(OCH_2Ph)_2$)

Z-Leu-Abu-CONH-CH_2-2-furyl

Z-Leu-Abu-CONH-CH_2-2-tetrahydrofuryl

Z-Leu-Abu-CONH-CH_2-2-pyridyl

Z-Leu-Abu-CONH-CH_2-3-pyridyl

Z-Leu-Abu-CONH-CH_2-4-pyridyl

Z-Leu-Abu-CONH-$(CH_2)_2$-2-pyridyl

Z-Leu-Abu-CONH-CH_2-2-pyridyl(3-$COOCH_3$)

Z-Leu-Abu-CONH-CH_2-2-pyridyl(5-$COOCH_3$)

Z-Leu-Abu-CONH-$(CH_2)_2$-2-(N-methylpyrrolyl)

Z-Leu-Abu-CONH-$(CH_2)_3$-1-imidazolyl

Z-Leu-Abu-CONH-$(CH_2)_2$-4-morpholinyl

Z-Leu-Abu-CONH-$(CH_2)_3$-4-morpholinyl

Z-Leu-Abu-CONH-$(CH_2)_3$-1-pyrrolidinyl-2-one

Z-Leu-Abu-CONH-$CH_2)_2$-3-indolyl

Z-Leu-Abu-CONH-CH_2-2-quinolinyl

Z-Leu-Abu-CONH-CH_2-1-isoquinoline

Z-Leu-Abu-CONH-$(CH_2)_3$-1-tetrahydroquinolinyl

Z-Leu-Abu-CONH-$(CH_2)_3$-2-tetrahydroisoquinolinyl

Z-Leu-Abu-CONH-CH_2-8-caffeinyl

Z-Leu-Abu-CONH-CH_2-2-(4-methyl-2-thiazolyl)

Z-Leu-Abu-CONH-CONH-$(CH_2)_2$NH-biotinyl

Z-Leu-Abu-CONH-CH_2-3-pyridyl-N-oxide

Z-Leu-Abu-CONH-CH_2-6-uracil

Z-Leu-Phe-CONH-CH_2-2-pyridyl

Z-Leu-Phe-CONH-$(CH_2)_3$-4-morpholinyl

Z-Leu-Phe-CONH-CH_2-2-quinolinyl

Z-Leu-Phe-CONH-CH_2-1-isoquinolinyl

Z-Leu-Phe-CONH-$(CH_2)_3$-1-tetrahydroquinolinyl

Z-Leu-Phe-CONH-$(CH_2)_3$-2-tetrahydroisoquinolinyl

Z-Leu-Phe-CONH-$(CH_2)_2$-NH-biotinyl

Z-Leu-Nva-CONH-$CH_2CH(OH)Ph$

Z-Leu-Nva-CONH-CH_2-2-pyridyl

Z-Leu-Nva-CONH-$(CH_2)_3$-4-morpholinyl

$CH_3OCO(CH_2)_2CO$-Leu-Abu-CONHEt

2-furyl-CO-Leu-Abu-CONHEt

2-tetrahydrofuryl-CO-Leu-Abu-CONHEt

3-pyridyl-CO-Leu-Abu-CONHEt

2-pyrazyl-CO-Leu-Abu-CONHEt

2-quinolinyl-CO-Leu-Abu-CONHEt

1-isoquinolinyl-CO-Leu-Abu-CONHEt

4-morpholinyl-CO-Leu-Abu-CONHEt

$Ph(CH_2)_2CO$-Leu-Abu-CONHEt

1-$C_{10}H_7CH_2CO$-Leu-Abu-CONHEt

Ph_2CHCO-Leu-Abu-CONHEt

Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$

Ph_2CHCO-Leu-Abu-CONH-CH_2-2-pyridyl

Ph_2CHCO-Leu-Abu-CONH-$(CH_2)_3$-4-morpholinyl

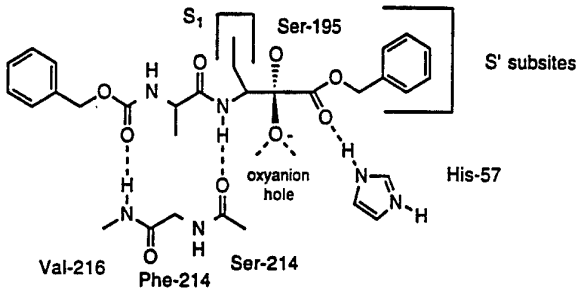
Ph_2CHCO-Leu-Phe-CONH-$CH_2CH(OH)Ph$

Ph_2CHCO-Leu-Phe-CONH-CH_2-2-pyridyl

Ph_2CHCO-Leu-Phe-CONH-$(CH_2)_3$-4-morpholinyl

We studied the inhibition mechanism of the Peptide Keto-Compounds in both serine and thiol proteases. A crystal structure of one α-ketoester bound into the active site of the serine protease, porcine pancreatic elastase, has been completed. The active site Ser-195 oxygen of the enzyme adds to the carbonyl group of the ketoester to form a tetrahedral intermediate which is stabilized by interactions with the oxyanion hole. This structure resembles the tetrahedral intermediate involved in peptide bond hydrolysis and proves that α-ketoesters are transition-state analogs. His-57 is hydrogen bonded to the carbonyl group of the ester functional group, the peptide backbone on a

section of the PPE polypeptide backbone hydrogen bonds to the inhibitor to form a ß-sheet, and the benzyl ester is directed toward the S' subsites. The side chain of the P1 amino acid residue is located in the S1 pocket of the enzyme. Interactions with ketoamides would be similar except that there is the possibility of forming an additional hydrogen bond with the NH group of the ketoamide functional group. If R is a longer substituent, then it would make favorable interactions with the S' subsites of the enzyme.



In the case of ketoacids, there would be no R group to interact with the S' subsites. Therefore, these inhibitors would be expected to be slightly less potent than the ketoesters and ketoamides. However, unexpectedly, certain ketoacid compounds have been found to have surprisingly high activity when used in the context of the present invention. In particular, Z-Leu-Phe-COOH and Z-Leu-Abu-COOH have been found to be extremely potent inhibitors of Calpains.

The active site of cysteine proteases shares several features in common with serine proteases including an active site histidine residue. In place of the Ser-195, cysteine proteases have an active site cysteine residue which would add to the ketonic carbonyl group of the peptide ketoacids, ketoesters, or ketoamides to form an adduct very similar to the structure described above except with a cysteine residue replacing the serine-195 residue. Additional interactions would occur between the extended substrate binding site of the cysteine protease and the inhibitor that would increase the binding affinity and specificity of the inhibitors.

The Peptide Keto-Compounds bind to the proteases inhibited thereby using many of the interactions that are found in complexes of a particular individual enzyme with its substrates. In order to design an inhibitor for a particular cysteine protease, it is necessary to: 1) find the amino acid sequences of good peptide substrates for that enzyme, and 2) place those or similar amino acid sequences into a Peptide Keto-

Compound. This design strategy will also work when other classes of peptide inhibitors are used in place of the peptide substrate to gain information on the appropriate sequence to place in the Peptide Keto-Compound inhibitor. Thus, we are able to predict the structure of new inhibitors for other proteases based on knowledge of their substrate specificities. Once a good inhibitor structure for a particular enzyme is found, it is then possible to change other characteristics such as solubility or hydrophobicity by adding substituents to the M or R groups.

Additional interactions with the enzyme can be obtained by tailoring the R group of the inhibitor to imitate the amino acid residues which are preferred by an individual protease at the S1' and S2' subsites. For example, ketoamides with R = alkyl substituted with phenyl would interact effectively with serine and cysteine proteases which prefer Phe, Tyr, Trp residues at P1' and/or P2'. Likewise, the M1 group can be tailored to interact with the S subsites of the enzyme. This design strategy will also work when other classes of peptide inhibitors are used in place of the peptide substrate to gain information on the appropriate sequence to place in the ketoamide inhibitor. Thus, we are able to predict the structure of new inhibitors for other serine and cysteine proteases based on knowledge of their substrate specificities. Once a good inhibitor structure for a particular enzyme is found, it is then possible to change other characteristics such as solubility or hydrophobicity by adding substituents to the M1 or R groups.

In the case of Calpain, a cysteine protease, a known inhibitor sequence is the peptide aldehyde, Ac-Leu-Leu-Nle-H (also known as Calpain Inhibitor 1 and hereinafter designated as "CI1"). This inhibitor, in addition to a related peptide aldehyde inhibitor Ac-Leu-Leu-Nme-H (also known as Calpain Inhibitor II) are commercially available from Calbiochem of La Jolla, California. We have discovered that peptide α-ketoesters with aromatic amino acid residues in P1 are good inhibitors of the thiol proteases, cathepsin B, papain and Calpain. Additionally, we have discovered that peptide α-ketoester and peptide α-ketoamides with either aromatic amino acid residues or small hydrophobic alkyl amino acid residues at P1 are good inhibitors of Calpain.

Our discovery of Peptide Keto-Compounds effective as Calpain Inhibitors was made through assay of the Peptide Keto-Compounds as reversible inhibitors. Various

concentrations of inhibitors in dimethylsulfoxide (DMSO) were added to the assay mixture, which contained buffer and substrate. The reaction was started by the addition of the enzyme and the hydrolysis rates were followed spectrophotometrically or fluorimetrically. 88 mM KH_2PO_4, 12 mM Na_2HPO_4, 1.33 mM EDTA, 2.7 mM cysteine, pH 6.0 was used as a buffer for cathepsin B; and 20 mM Hepes, 10 mM $CaCl_2$, 10 mM ß-mercaptoethanol, pH 7.2 buffer was utilized for calpain I and calpain II.

All peptide thioester hydrolysis rates were measured with assay mixtures containing 4,4'-dithiodipyridine (e324 = 19800 $M^{-1}cm^{-1}$; Grasetti & Murray, *Arch. Biochem. Biophys.*, **119**:41-49 (1967)). Papain was assayed with Bz-Arg-AMC or Bz-Arg-NA (Kanaoka et al., *Chem. Pharm. Bull.*, **25**:3126-3128 (1977)), and the AMC (7-amino-4-methylcoumarin) release was followed fluorimetrically (excitation at 380 nm, and emission at 460 nm). Cathepsin B was assayed with Z-Arg-Arg-AFC (Barrett and Kirschke, *Methods Enzymol.*, **80**:535-561 (1981)), and the AFC (7-amino-4-trifluoromethylcoumarin) release was followed fluorimetrically (excitation at 400 nm, and emission at 505 nm). Calpain I from human erythrocytes and calpain II from rabbit were assayed using Suc-Leu-Tyr-AMC (Sasaki et al., *J. Biol. Chem.* **259**:12489-12494 (1984), hereby incorporated by reference), and the AMC (7-amino-4-methylcoumarin) release was followed fluorimetrically (excitation at 380 nm, and emission at 460 nm). Enzymatic hydrolysis rates were measured at various substrate and inhibitor concentrations, and K_i values were determined by Dixon plot.

Table PKC1 shows the inhibition constants (K_i) for papain, cathepsin B, calpain I, and calpain II.

The inhibition constants for papain shown in Table PKC1 were measured in 0.05 M Tris-HCl, pH 7.5 buffer, containing 2mM EDTA, 5mM cysteine (freshly prepared), 1% DMSO, at 25°C, using N^{α}-Benzoyl-Arg-AMC as a substrate, except that those values of inhibition constants for papain marked with an "e" in Table PKC1 were measured in 50 mM Tris-HCl, pH 7.5 buffer, containing 20 mM EDTA, 5 mM cysteine, 9% DMSO, at 25°C, using N^{α}-Benzoyl-Arg-NA as a substrate.

TABLE PKC1

Inhibition of Cysteine Proteases by
Peptide Ketoesters and Ketoacids

Compounds	K_i(μM)			
	P[a]	CB[b]	CI[c]	CII[d]
Z-Leu-Abu-COOEt			0.04	0.4
Z-Leu-Phe-COOEt			0.23	0.4
Z-Leu-Nle-COOEt			0.12	0.18
Z-Leu-Nva-COOEt		30		1.2
Bz-DL-Phe-COOEt	500[e]	64		
Z-Phe-DL-Phe-COOEt	1.8	0.1		
Z-Phe-DL-Ala-COOEt	3.6	3.2		
Z-Ala-Ala-DL-Ala-COOEt	1.5	2.2	200	
Z-Ala-Ala-DL-Abu-COOEt	0.9	10	50	200
Z-Ala-Ala-DL-Abu-COOBzl	30	60		
Z-Ala-Ala-DL-Nva-COOEt	30	0.1		
Z-Ala-Pro-DL-Ala-COOEt	26	66		
MeO-Suc-Val-Pro-DL-Phe-COOMe	1.1	0.1		
	2.9[e]			
Z-Ala-Ala-Ala-DL-Ala-COOEt	2.1	10.0		
MeO-Suc-Ala-Ala-Pro-Abu-COOMe	0.7	6.0	100	

[a]P = Papain [c]CI = Calpain I

[b]CB = Cathepsin B [d]CII = Calpain II

It can be seen from the data in Table PKC1 that the dipeptide ketoesters with Abu, Phe, or Nle in the P1 site and Leu in the P2 site are potent inhibitors of calpain I and calpain II. Tripeptides with Abu or Ala in the P1 site and Ala in the P2 site are also seen to be inhibitors of Calpain, albeit somewhat weaker inhibitors than the dipeptides. Thus, in accordance with the foregoing description of the design of Peptide Keto-Compound inhibitors, we believe that Peptide Keto-Compounds based on these and similar structures will exhibit Calpain inhibitory activity.

Assay of Inhibitory Potency of Peptide a-ketoamides. HEPES, heparin, and A23187 were obtained from Calbiochem. Suc-Leu-Tyr-AMC and chromogenic substrates were obtained from Sigma. Calpain I was purified from human erythrocytes according to the method of Kitahara (Kitahara, *et al., J. Biochem.* **95**:1759-1766 (1984)) omitting the Blue-Sepharose step. Calpain II from rabbit muscle and cathepsin B were purchased from Sigma. Papain was purchased from Calbiochem.

Peptide α-ketoamides were assayed as reversible enzyme inhibitors. Various concentrations of inhibitors in Me_2SO were added to the assay mixture which contained buffer and substrate. The reaction was started by the addition of the enzyme and the hydrolysis rates were followed spectrophotometrically or fluorimetrically.

Calpain I from human erythrocytes and calpain II from rabbit were assayed using Suc-Leu-Tyr-AMC (Sasaki *et al., J. Biol. Chem.* **259**:12489-12494 (1984); incorporated herein by reference), and the AMC (7-amino-4-methylcoumarin) release was followed fluorimetrically (excitation at 380 nm, and emmision at 460 nm). Calpains were assayed in 25 mM Tris pH = 8.0, 10 mM CaCl2. Fluorescence was followed using a Gilson FL-1A fluorometer or a Perkin-Elmer 203 Fluorescence spectrometer. Cathepsin B was assayed in 20 mM sodium acetate pH = 5.2, 0.5 mM dithiothreitol using Bz-Phe-Val-Arg-*p*-nitroanilide as substrate. Alternately, cathepsin B was assayed with Z-Arg-Arg-AFC (Barrett and Kirschke, *Methods Enzymol.* **80**:535-561 (1981); incorporated herein by reference), and the AFC (7-amino-4-trifluoromethylcoumarin) release was followed fluorimetrically (excitation at 400 nm and emmision at 505 nm). Papain was assayed in 100 mM KPO_4, 1 mM EDTA, 2.5 mM cysteine pH = 6.0 using Bz-Arg-AMC or Bz-Arg-NA (Kanaoka *et al.*, *Chem. Pharm. Bull.* **25**:3126-3128 (1977); incorporated herein by reference) as a substrate. The AMC (7-amino-4-methylcoumarin) release was followed fluorimetrically (excitation at 380 nm,

and emmision at 460 nm). Enzymatic hydrolysis rates were measured at various substrate and inhibitor concentrations, and K_i values were determined by either Lineweaver-Burk plots or Dixon plots.

A 0.1 M Hepes, 0.5 M NaCl, pH 7.5 buffer was utilized for human leukocyte elastase (HLE), porcine pancreatic elastase (PPE), chymotrypsin and cathepsin G. A 0.1 Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer was utilized for trypsin, plasmin, and coagulation enzymes. A 50 mM Tris.HCl, 2 mM EDTA, 5 mM cysteine, pH 7.5 was used as a buffer for papain. A 88 mM KH_2PO_4, 12 mM Na_2HPO_4, 1.33 mM EDTA, 2.7 mM cysteine, pH 6.0 solution was used as a buffer for cathepsin B. A 20 mM Hepes, 10 mM $CaCl_2$, 10 mM mercatoethanol, pH 7.2 buffer was utilized for calpain I and calpain II.

HLE and PPE were assayed with MeO-Suc-Ala-Ala-Pro-Val-NA and Suc-Ala-Ala-Ala-NA, respectively (Nakajima *et al.*, *J. Biol. Chem.* **254**:4027-4032 (1979); incorporated herein by reference). Human leukocyte cathepsin G and chymotrypsin Aa were assayed with Suc-Val-Pro-Phe-NA (Tanaka *et al.*, *Biochemistry* **24**:2040-2047 (1985); incorporated herein by reference). The hydrolysis of peptide 4-nitroanilides was measured at 410 nm (e410 = 8800 $M^{-1}cm^{-1}$; Erlanger *et al.*, *Arch. Biochem. Biophys.* **95**:271-278 (1961); incorporated herein by reference). Trypsin, thrombin, human plasma kallikrein, porcine pancreatic kallikrein, human factor XIa, and human plasmin were assayed with Z-Arg-SBzl or Z-Gly-Arg-SBu-i (McRae *et al.*, *Biochemistry* **20**:7196-7206 (1981); incorporated herein by reference). All peptide thioester hydrolysis rates were measured with assay mixtures containing 4,4'-dithiodipyridine (e324 = 19800 $M^{-1}cm^{-1}$; Grasetti & Murray, *Arch. Biochem. Biophys.* **119**:41-49 (1967); incorporated herein by reference).

Structure-Activity Relationships. Table PKC2 shows the inhibition constants (K_i) for calpain I, calpain II and cathepsin B. Changing the R group on the amide significantly improves the inhibitory potency toward calpains. Dipeptide α-ketoamides with Abu, Phe, and Nva in the P1 site and Leu in the P2 site are potent inhibitors of these cysteine proteases. The presence of a hydrogen bond donor in the S1' subsite of the cysteine proteases which may be interacting with the N-H on the ketoamide functional group is indicated since disubstituted amides were much less effective inhibitors. Derivatives of Z-Leu-AA-CONHR where the R group contained a hydroxy

or alkoxy group, such as $(CH_2)_5OH$ and $CH_2CH(OC_2H_5)_2$, are very good inhibitors of the calpains. The prescence of an aromatic group in P1' position of the peptide ketoamide inhibitor resulted in improved inhibitory potency for calpains which indicates the prescence of hydrophobic residues in the S' subsites of both calpains. The derivatives Z-Leu-AA-CONH$(CH_2)_n$R where R was phenyl, phenyl substituted with hydroxy or alkoxy groups and naphthyl, are also very good inhibitors of calpains and cathepsin B. Derivatives of Z-Leu-Abu-CONH$(CH_2)_n$R where the R group contained a heterocylic group which has both a hydrophobic moiety with an electronnegative atom, are among the best inhibitors for calpains and cathepsin B. For example Z-Leu-Nva-CONHCH$_2$-2-pyridyl is the best inhibitor of calpain I. Z-Leu-Abu-CONHCH$_2$-2-pyridyl is the best inhibitor of calpain II respectively in this series, but its isomers, Z-Leu-Abu-CONH-CH$_2$-3-pyridyl and Z-Leu-Abu-CONH-CH$_2$-4-pyridyl, are substantially poorer inhibitors.

TABLE PKC2. Inhibition of Cysteine Proteases by Peptide a-Ketoamides with the Structures Z-Leu-AA-CONHR.

R	K_i (μM)	
Cal I	Cal II	Cat B
AA = a-aminobutyric acid		
$(CH_2)_2OH$	0.8	0.078
4.5		
$(CH_2)_5OH$	0.5	0.051
0.28		
$(CH_2)_2O(CH_2)_2OH$	0.65	0.16
2.0		
$CH_2CH(OCH_3)_2$	0.5	
$CH_2CH(OC_2H_5)_2$	0.2	
CH_2-C_6H_8(1,3,3-$(CH_3)_3$-5-OH)	0.42	0.069
0.89		
$(CH_2)_2C_6H_4$(4-OH)	0.38	0.06
$(CH_2)_2C_6H_4$(2-OCH_3)	0.13	0.16
0.63		

	$(CH_2)_2C_6H_4(3\text{-}OCH_3)$	0.11	0.086
	0.31		
	$(CH_2)_2C_6H_4(4\text{-}OCH_3)$	0.12	0.046
	0.44		
5	$CH_2C_6H_3(3,5\text{-}(OCH_3)_2)$	2.3	0.022
	1.8		
	CH_2-2-furyl	0.80	0.033
	6.0		
	CH_2-2-tetrahydrofuryl	0.33	0.066
10	4.5		
	CH_2-2-pyridyl	0.64	0.017
	3.0		
	CH_2-3-pyridyl		0.12
	1.2		
15	CH_2-4-pyridyl	1.1	0.11
	6.4		
	$(CH_2)_2$-2-pyridyl	0.41	0.47
	0.20		
	CH_2-2-pyridyl(3-$COOCH_3$)	ca.110	
20	CH_2-2-pyridyl(5-$COOCH_3$)	ca.28	
	$(CH_2)_2$-2-(N-methylpyrrole)	0.16	0.076
	1.2		
	$(CH_2)_3$-1-imidazolyl	0.29	0.068
	9.9		
25	$(CH_2)_2$-4-morpholinyl	1.0	0.16
	2.5		
	$(CH_2)_3$-4-morpholinyl	0.14	0.041
	6.9		
	$(CH_2)_3$-1-pyrrolidine-2-one	1.2	0.27
30	2.0		
	$(CH_2)_2$-3-indolyl	0.3	0.05
	CH_2-2-quinolinyl	0.13	

	CH_2-1-isoquinolinyl	0.25	
	0.3		
	$(CH_2)_3$-1-tetrahydroquinolinyl	0.37	
	$(CH_2)_3$-2-tetrahydroisoquinolinyl	0.31	
5	8		
	CH_2-8-caffeine	32.0	
	CH_2-2-(4-methylthiazole)	34.0	
	$(CH_2)_2$NH-biotinyl	0.65	
	CH_2-3-pyridyl-N-oxide	9.5	
10	CH_2-6-uracil	9.0	
	AA = phenylalanine		
	CH_2-2-pyridyl		0.65
	0.27		
	$(CH_2)_3$-4-morpholinyl	0.22	
15	CH_2-2-quinolinyl	0.11	0.023
	0.34		
	CH_2-1-isoquinolinyl	2.4	
	9.6		
	$(CH_2)_3$-1-tetrahydroquinolinyl	0.38	
20	$(CH_2)_3$-2-tetrahydroisoquinolinyl	0.22	
	$(CH_2)_2$NH-biotinyl	0.22	
	AA = Norvaline		
	CH_2-2-pyridyl	0.019	0.12
	$(CH_2)_3$-4-morpholinyl	0.25	0.10
25	4.2		

Table PKC3 shows the inhibition constants (K_i) of Z-Leu-AA-CONH-CH_2CH(OH)R. The hydrophobic moiety substituted with CH_2CH-X (X = electronegative atoms such as O, N) resulted in good inhibitor structures.
30 Z-Leu-Abu-CONH-CH_2CH(OH)C_6F_5 is the best inhibitor for calpain I, and Z-Leu-Abu- CONH-CH_2CH(OH)Ph is the best inhibitor for calpain II respectively in this series.

TABLE PKC3. Inhibition of Cysteine Proteases by Peptide a-Ketoamides with the Structures Z-Leu-AA-CONH-$CH_2CH(OH)$-R.

R	K_i	
	Cal I	Cal IICat B
AA = a-aminobutyric acid		
Ph	1.1	0.0150.37
$C_6H_4(4\text{-}OCH_3)$	0.24	
$C_6H_2(2,4,6\text{-}(OCH_3)_3)$	0.38	
$C_6H_4(4\text{-}N(CH_3)_2)$	0.33	
C_6F_5 0.05		
$C_6H_4(3\text{-}CF_3)$	0.35	
$C_6H_4(3\text{-}OPh)$	0.90	
$C_6H_4(4\text{-}OPh)$	0.10	
$C_6H_4(4\text{-}OCH_2Ph)$	0.08	
$C_6H_4\text{-}3\text{-}OC_6H_4(3\text{-}CF_3)$	0.07	
$C_6H_4\text{-}3\text{-}OC_6H_3(3,4\text{-}Cl_2)$	0.27	
$C_6H_3(3,4\text{-}(OCH_2Ph)_2)$	0.23	
$1\text{-}C_{10}H_7$	0.12	
$2\text{-}C_{10}H_7$	0.35	
AA = phenylalanine		
Ph	1.3	0.052.1
$C_6H_4(4\text{-}N(CH_3)_2)$	0.62	
C_6F_5	0.70	
$C_6H_4(3\text{-}CF_3)$	0.46	
$C_6H_4(3\text{-}OPh)$	0.60	
$C_6H_4(4\text{-}OPh)$	0.20	
$C_6H_4(4\text{-}OCH_2Ph)$	0.20	
$C_6H_4\text{-}3\text{-}OC_6H_4(3\text{-}CF_3)$	0.18	
$C_6H_4\text{-}3\text{-}OC_6H_3(3,4\text{-}Cl_2)$	0.59	
$C_6H_3(3,4\text{-}(OCH_2Ph)_2)$		
AA = Norvaline		
Ph	7.8	11

In general, replacement of the Z group ($PhCH_2OCO$-) by related aromatic groups also resulted in good inhibitor structures (Table PKC4).

Table PKC4. Inhibition of Cysteine Proteases by Peptide α-ketoamides with the Structures M_1CO-Leu-AA-CONH-R.

M_1	R	KI (μM)	
		Cal I	Cal II
Cat B			
AA = a-aminobutyric acid			
Z	Et	0.5	0.232.4
$CH_3OCO(CH_2)_2$	Et	3.8	
2-furyl Et	0.85		
2-tetrahydrofuryl	Et	18.5	
3-pyridyl	Et	1.30	
2-pyrazinyl	Et	0.30	
2-quinolinyl	Et	0.5	
1-isoquinolinyl	Et	0.35	
4-morpholinyl	Et	7.9	
$Ph(CH_2)_2$	Et		
1-$C_{10}H_7CH_2$	Et		
Ph_2CH	Et	5.0	
Ph_2CH	$CH_2CH(OH)Ph$	0.75	0.20
Ph_2CH	CH_2-2-pyridyl	0.5	0.092.8
Ph_2CH	$(CH_2)_3$-4-morpholinyl	0.8	0.112.3
AA = phenylalanine			
Ph_2CH	$CH_2CH(OH)Ph$	10	0.73
Ph_2CH	CH_2-2-pyridyl	1.1	0.362.2
Ph_2CH	$(CH_2)_3$-4-morpholinyl	0.76	0.0743.8

Preparation of peptide α-ketoesters. The peptide α-ketoesters are prepared by a two step Dakin-West procedure. This procedure can be utilized with either amino acid derivatives, dipeptide derivatives, tripeptide derivatives, or tetrapeptide derivatives as shown in the following scheme:

$$M\text{-}(AA)_n\text{-}OH \dashrightarrow \text{Enol Ester} \dashrightarrow M\text{-}(AA)_n\text{-}C(=O)\text{-}R.$$

The precursor peptide ($(AA)_n$) can be prepared using standard peptide chemistry procedures, including those that are well described in publications such as The Peptides, Analysis, Synthesis, Biology, **1-9** (1979-1987), published by Academic Press ("The Peptides") and *Synthese von Peptiden* in Houben-Weyl Methoden der Organischen Chemie, **15**, Parts 1 and 2, (1974) published by Georg Thieme Verlag ("Houben-Weyl"); both references hereby incorporated herein by reference.

The M group can be introduced using a number of different reaction schemes. For example, it could be introduced directly on an amino acid as shown in the following scheme:

$$H\text{-}(AA)_n\text{-}OH \dashrightarrow M\text{-}(AA)_n\text{-}OH.$$

Alternatively, the M group can be introduced by reaction with an amino acid ester, followed by removal of the ester group to give the same product, as shown in the following scheme:

$$H\text{-}(AA)_n\text{-}OR' \dashrightarrow M\text{-}(AA)_n\text{-}OR' \dashrightarrow M\text{-}(AA)_n\text{-}OH.$$

These and other techniques for introduction of the M group are well documented in the The Peptides, Houben-Weyl, and many other textbooks on organic synthesis. For example reaction with cyanate or p-nitrophenyl cyanate would introduce a carbamyl group (M = NH_2CO-). Reaction with p-nitrophenyl thiocarbamate would introduce a thio carbamyl group (M = NH_2CS-). Reaction with $NH_2S_4O_2Cl$ would introduce the NH_2SO_2- group. Reaction with a substituted alkyl or aryl isocyanate would introduce the X-NH-CO- group where X is a substituted alkyl or aryl group. Reaction with a substituted alkyl or aryl isothiocyanate would introduce the X-NH-CS- group where X is a substituted alkyl or aryl group. Reaction with X-SO_2-Cl would introduce the X-SO_2- group. Reaction with a substituted alkyl or aryl acid chloride would introduce an acyl group (M = Y-CO-). For example, reaction with MeO-CO-CH_2CH_2-CO-Cl would give the Y-CO- group when Y is a C_2 alkyl

substituted with a C1 alkyl-OCO- group. Reaction with a substituted alkyl or aryl thioacid chloride would introduce a thioacyl group (M = Y-CS-). Reaction with an a substituted alkyl or aryl sulfonyl chloride would introduce an X-SO_2- group. For example reaction with dansyl chloride would give the X-SO_2- derivative where X was a napthyl group monosubstituted with a dimethylamino group. Reaction with a substituted alkyl or aryl chloroformate would introduce a X-O-CO- group. Reaction with a substituted alkyl or aryl chlorothioformate would introduce a X-O-CS-. There are many alternate reaction schemes which could be used to introduce all of the above M groups to give either M-AA-OH or M-AA-OR'.

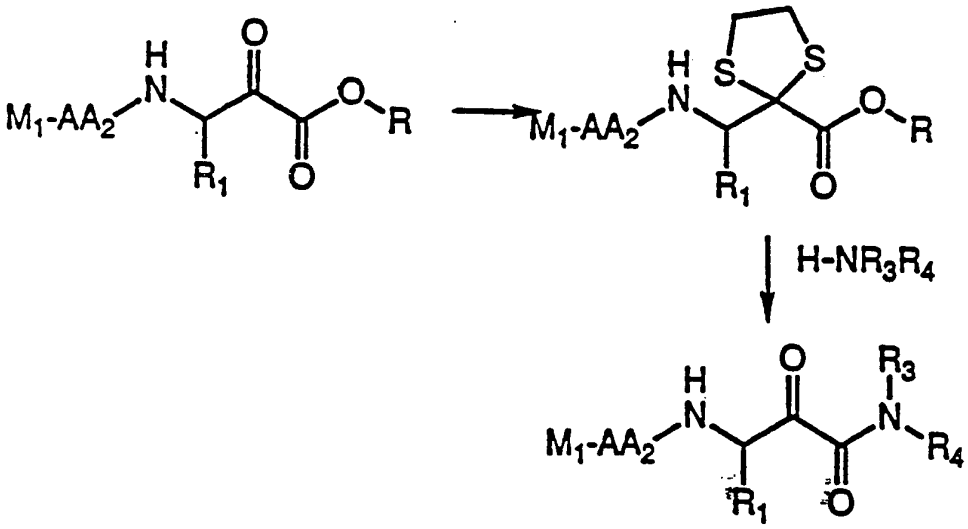
The M-AA-OH derivatives could then be used directly in the Dakin-West reaction or could be converted into the dipeptides, tripeptides, and tetrapeptides M-AA-AA-OH, M-AA-AA-AA-OH, or M-AA-AA-AA-AA-OH which could be be used in the Dakin-West reaction. The substituted peptides M-AA-AA-OH, M-AA-AA-AA-OH, or M-AA-AA-AA-AA-OH could also be prepared directly from H-AA-AA-OH, H-AA-AA-AA-OH, or H-AA-AA-AA-AA-OH using the reactions described above for introduction of the M group. Alternately, the M group could be introduced by reaction with carboxyl blocked peptides M-AA-AA-OR', M-AA-AA-AA-OR', or M-AA-AA-AA-AA-OR', followed by the removal of the blocking group R'.

The R group in the ketoester structures is introduced during the Dakin-West reaction by reaction with an oxalyl chloride Cl-CO-CO-O-R. For example, reaction of M-AA-AA-OH with ethyl oxaiyl chloride Cl-CO-CO-O-Et gives the keto ester M-AA-AA-CO-O-Et. Reaction of M-AA-AA-AA-AA-OH with Cl-CO-CO-O-Bzl would give the ketoester M-AA-AA-AA-AA-CO-O-Bzl. Clearly a wide variety of R groups can be introduced into the ketoester structure by reaction with various alkyl or arylalkyl oxalyl chlorides (Cl-CO-CO-O-R).

The oxalyl chlorides are easily prepared by reaction of an alkyl or arylalkyl alcohol with oxalyl chloride Cl-CO-CO-Cl. For example, Bzl-O-CO-CO-Cl and n-Bu-O-CO-CO-Cl are prepared by reaction of benzyl alcohol and butanol, respectively, with oxalyl chloride in yields of 50% and 80% (Warren and Malee, *J. Chromat.*, **64**:219-222 (1972); incorporated herein by reference).

Ketoacids M-AA-CO-OH, M-AA-AA-CO-OH, M-AA-AA-AA-CO-OH, M-AA-AA-AA-AA-CO-OH, are generally prepared from the corresponding ketoesters M-AA-CO-OR, M-AA-AA-CO-OR, M-AA-AA-AA-CO-OR, M-AA-AA-AA-AA-CO-OR by alkaline hydrolysis. In some cases, it may be necessary to use other methods such as hydrogenolysis of a benzyl group (R = Bzl) or acid cleavage (R = t-butyl) to obtain the ketoacid. The alternate methods would be used when the M group was labile to alkaline hydrolysis.

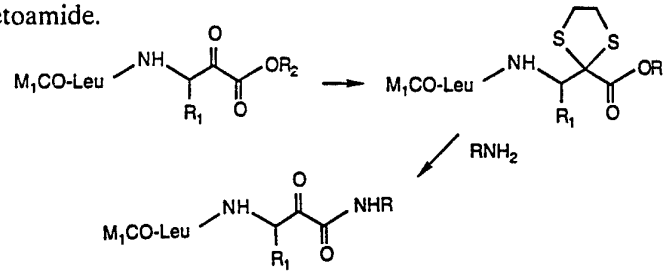
The various peptide ketoamide subclasses, including M-AA-NH-CHR_2-CO-CO-NR_3R_4 (Dipeptide Ketoamides, Subclass A), M-AA-AA-CO-NR_3R_4 (Dipeptide Ketoamides, Subclass B), M_1CO-AA_2-AA_1-CO-NH-CH_2CH(OH)-R_1 and five others presented above (Dipeptide α-Ketoamides, Subclass C, Types 1 through 6), M-AA-AA-AA-CO-NR_3R_4 (Tripeptide Ketoamides), M-AA-AA-AA-AA-CO-NR_3R_4 (Tetrapeptide Ketoamides) and M_1-AA-CO-NR_3R_4 (Amino Acid Ketoamides), were prepared indirectly from the corresponding ketoesters. The ketone carbonyl group was first protected as shown in the following scheme and then the ketoamide was prepared by reaction with an amine H-NR_3R_4. The illustrated procedure should also work with other protecting groups.



In addition to the scheme outlined above, a ketoacid could be used as a precursor to produce a corresponding ketoamide. Blocking the ketone carbonyl group of the ketoacid and then coupling with an amine H-NR_3R_4 using standard peptide

coupling reagents would yield an intermediate which could then be deblocked to form the ketoamide.

Ketoamides M_1CO-AA-AA-CONHR were prepared indirectly from the ketoesters. The ketone carbonyl group is first protected as shown in the following scheme and then the ketoamide is prepared by reaction with an amine RNH_2. The product is easily isolated from the reaction mixture when using this procedure. This procedure will also work with other ketone protecting groups. In addition, the corresponding ketoacid can be used as a precursor to the a-ketoamide via coupling with an amine RNH_2 using standard peptide coupling reagents would result in formation of the peptide a-ketoamide.



General Synthetic Methods for Peptide Keto-Compounds

The techniques for synthesis of a wide variety of amines are described in many publications. For example, Evans *et al.* in *J.Org.Chem.* **39**:914 (1974) reported the syntheses of phenylethanol derivatives with alkylamino, alkoxyamino and phenyloxyamino groups. Katrizky *et al.* in *J. Chem. Soc.*:2404-2408 (1956), Fife *et al.* in *Heterocycles* **22**(1):93-96 (1984), and *Heterocycles* **22**(5):1121-1124 (1984), and Isoda *et al.* in *Chemical and Pharmaceutical Bulletin* **28**:1408-1414 (1980) reported the syntheses of pyridine derivatives with alkylamino and COOR groups. Nagata *et al.* in *Yakugaku Zasshi* **83**:679-682 (1963) reported the syntheses of quinoline derivatives with alkylamino groups. Zimmer *et al.* in *Tetrahedron Letters* **24**:2805-2807 (1968) reported the syntheses of isoquinoline derivatives with alkylamino groups. Aroyan *et al.* in *Izv. Akad. Nauk Arm. SSR, Khim. Nauki* **18**(1):76-82 (1965) reported the syntheses of tetrahydroquinoline derivatives with alkylamino groups. Yonan, (U.S. 3,245,997 (Cl. 260-288), April 12, 1966. 2 pp) reported the syntheses of tetrahydroisoquinoline derivatives with alkylamino groups. Rybar *et al.* in *Chem. Commun.* **35**:1415-1433 (1970), Golovchinskaya *et al.* in *J. General Chem.* **22**:599-603 (1952), and Nantka-Namirski *et al.* in *Acta. Polon. Pharm.* **1**:5-12 (1974) reported the syntheses of caffeine derivatives with alkylamino groups. Goldberg *et al.* in

J.Chem.Soc.:1372 (1947) reported the syntheses of methylthiazole derivatives with alkylamino groups. Mizuno *et al.* in *J.Org.Chem.* **39**:1250 (1974) reported the syntheses of pyridine-N-oxide derivatives with alkylamino groups. Wade in *J.Heterocyclic Chem.* **23**:981 (1986) reported the syntheses of uracil derivatives with alkylamino groups. All of the above citations are incorporated herein by reference.

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Melting points were taken with a Büchi capillary apparatus and are uncorrected. ^{1}H NMR spectra were determined on a Varian Gemini 300. Chemical shifts are expressed in ppm (δ) relative to internal tetramethylsilane. Flash column chromatography was performed with Universal Scientific Inc. silica gel 0-63. Electron-impact mass spectra (MS) of novel compounds were determined with a Varian MAT 112S spectrometer. The purity of all compounds was checked by thin- layer chromatography on Baker Si250F silica gel plates using the following solvent system: A, $CHCl_3$:MeOH = 20:1 v/v; B, $CHCl_3$:MeOH = 100:1 v/v; C, AcOEt; D, $CHCl_3$:MeOH = 10:1 v/v; E, n-BuOH:AcOH;py:H_2O = 4:1:1:2 v/v; F, $CHCl_3$:MeOH = 5:1 v/v; G, AcOEt:MeOH = 10:1 v/v; H, $(i\text{-}Pr)_2O$; I, $CHCl_3$:MeOH:AcOH = 80:10:5 v/v; J, $CHCl_3$:MeOH:AcOH = 95:5:3 v/v; K, AcOEt:AcOH = 200:1 v/v; L, $CHCl_3$; M, $CHCl_3$:MeOH = 50:1 v/v.

Amino acid methyl ester hydrochlorides were prepared according to M. Brenner et al., *Helv. Chem. Acta* **33**:568 (1950); **36**:1109 (1953) in a scale over 10 mmol or according to Rachele, *J. Org. Chem.* **28**:2898 (1963) in a scale of 0.1-1.0 mmol.

	Yield (%)	mp (°C)	m.p. (literature)
DL-Nva-OCH_3·HCl,	100	113-116	116-117
L-Ile-OCH_3·HCl,	98	90-91	98-100
L-Phe-OCH_3·HCl,	98	159-161	158-160
DL-Abu-OCH_3·HCl,	100	148-150	150-151
L-Leu-OCH_3·HCl	100	145.5-146.5	147
DL-Nle-OCH_3·HCl	93	120-121	122-123
4-Cl-Phe-OCH_3·HCl	98	184-185 (decomp.)	185-186

N-Acylamino acids was synthesized via Schotten-Baumann reaction as in Bergmann and Zervas, *Chem. Ber.*, **65**:1192 (1932) in the case when the acyl group was phenylsulphonyl, 2- naphthylsulphonyl or benzoyl.

	Yield (%)	mp (°C)	TLC (R_f, eluent)
2-NapSO_2-L-Leu-OH	49	115-116	0.58I
2-NapSO_2-DL-Abu-OH	51	150-151	0.50I
2-NapSO_2-L-Phe-OH	57	148-148.5	0.48K
PheSO_2-DL-Abu-OH	44	142-143	0.51K
PhCO-DL-Abu-OH	64	141-142	0.64K

N-Acylamino acids with 4-methylpentanoic, 2-(1- propyl)pentanoic and 7-phenylheptanoic group was synthesized in a two step synthesis. The N-acylamino acid methyl ester was obtained first and then was hydrolysed to the free N-acylamino acid.

N-Acylamino Acid Methyl Esters (General Procedure). To a chilled (10 °C) slurry of the appropriate amino acid methyl ester hydrochloride (20 mmol) in 100 ml benzene was added slowly (temp. 10-15 °C) 40 mmol triethylamine or N- methylmorpholine and then the reaction mixture was stirred for 30 minutes at this temperature. Then 18 mmol of appropriate acid chloride (temp. 10-15 °C) was added slowly to the reaction mixture and the reaction mixture was stirred overnight at room temperature. The precipiatated hydrochloride was filtered, washed on a funnel with 2 x 20 ml benzene, and the collected filtrate was washed successively with 2 x 50 ml 1 M HCl, 2 x 50 ml 5% $NaHCO_3$, 1 x 100 ml H_2O, 2 x 50 ml satd. NaCl and dried over $MgSO_4$. After evaporation of the solvent in vacuo (rotavaporator), the residue was checked for purity (TLC) and used for the next step (hydrolysis).

	Yield (%)	mp (°C)
$(CH_3)_2CH(CH_2)_2CO$-DL-Abu-OCH_3	80	oil
$(CH_3CH_2CH_2)_2CHCO$-DL-Abu-OCH_3	96	117-118
$Ph(CH_2)_6CO$-DL-Abu-OCH_3	72	oil

Hydrolysis (General Procedure). To a solution of 10 mmole of the appropriate N-acylamino acid methyl ester in 100 ml of methanol was added in one portion 11.25 ml of 1 M NaOH (11.25 mmol) and the reaction mixture was stirred three hours at room temperature. Then the reaction mixture was cooled to 0 °C (ice- salt bath) and acidified to pH = 2 with 1 M HCl aq. To this reaction mixture was added 100 ml ethyl acetate, transferred to a separatory funnel and organic layer separated. The water layer was saturated with solid NaCl or $(NH_4)_2SO_4$ and reextracted with 2 x 50 ml AcOEt. The collected organic layer was washed with 2 x 50 ml H_2O, decolorized with carbon, and dried

over $MgSO_4$. After evaporation of the solvent in vacuo (rotavaporator), the residue was checked for purity (TLC) and in the case of contamination was crystallized from an appropriate solvent.

	Yield (%)	mp (°C)
$(CH_3)_2CH(CH_2)_2CO$-DL-Abu-OH	92	110.5-112
$(CH_3CH_2CH_2)_2CHCO$-DL-Abu-OH	99	126-127 (n-octane)
$Ph(CH_2)_6CO$-DL-Abu-OH	89	110-112 (n-octane)

N-Acyldipeptide methyl esters were synthesized via the HOBt-DCC method in a DMF solution as in König and Geiger, *Chem. Ber.*, **103**:788 (1970).

	Yield (%)	mp (°C)	TLC (R_f, eluent)
Z-Leu-DL-NVa-OCH_3	80	112-113	0.37 B
Z-Leu-L-Phe-OCH_3	83	86-87	0.85 A
			0.39 B
Z-Leu-L-Ile-OCH_3	97	oil	0.79 A
			0.43 B
Z-Leu-DL-Abu-OCH_3	99	86-88	0.33 B
			0.26 H
Z-Leu-L-Leu-OCH_3	80	91-92	0.79 G
Z-Leu-DL-NLeu-OCH_3	97	111-111.5	
Z-Leu-4-Cl-Phe-OCH_3	65	112-132	0.77 J
		(liquid crystal?)	0.68 K
2-NapSO_2-Leu-DL-Abu-OCH_3	99	oil	0.59 A
2-NapSO_2-Leu-L-Leu-OCH_3	90	97-98.5	0.63 A

N-Acyldipeptides were obtained by hydrolysis of the appropriate methyl esters via a general hydrolysis procedure. In the case of N-sulphonyldipeptide methyl esters, 1 equivalent of the methyl ester was hydrolyzed with 2.25 equivalent of 1 molar NaOH because of form a sulfonamide sodium salt.

	Yield (%)	mp (°C)	TLC (R_f, eluent)
Z-Leu-DL-NVa-OH	100	117-118.5	0.11 A
Z-Leu-L-Phe-OH	92	105-106.5	0.28 C
			0.55 G
Z-Leu-L-ILe-OH	79	77-79	0.22 A
			0.52 C
Z-Leu-DL-Abu-OH	99	glass	0.61 G
Z-Leu-L-Leu-OH	97	glass	0.56 I
Z-Leu-DL-NLeu-OH	98	95-96	
Z-Leu-4-Cl-Phe-OH	87	104-114 (liquid crystal?)	0.48 K
2-$NapSO_2$-Leu-DL-Abu-OH			
	97.4	180-195 (decomp)	0.58 I
2-$NapSO_2$-Leu-L-Leu-OH			
	94.0	68-70	0.52 I

N-Acytripeptide methyl esters were synthesized via HOBt- DCC method in DMF solution as in König and Geiger, *supra*.

	Yield (%)	mp (°C)	TLC (R_f, eluent)
Z-Leu-Leu-Abu-OCH_3	87	140-141.5	0.50 A
Z-Leu-Leu-Phe-OCH_3	76	158-159	0.83 J
2-$NapSO_2$-Leu-Leu-Abu-OCH_3			
	97	>200	0.52 A

N-Acyltripeptide were obtained through hydrolysis of the appropriate methyl esters via general hydrolysis procedure. In the case of N-sulphonyltripeptide methyl ester, 1 equivalent of methyl ester was hydrolyzed with 2.25 equivalent of 1 molar NaOH to form the sulfonamide sodium salt.

	Yield	mp (°C)	TLC (R_f, eluent)
Z-Leu-Leu-Abu-OH	97	glass	0.69 I
Z-Leu-Leu-Phe-OH	98	glass	0.44 K
2-$NapSO_2$-Leu-Leu-Abu-OH			
	85	193-195 (decomp.)	0.53 I
			0.32 J

We have also discovered a process for the synthesis of a-ketoamides with the structures

M-CO-AA_2-AA_1-CO-NH-R and

M-CO-AA3-AA_2-AA_1-CO-NH-R,

wherein

M is selected from the group consisting of C_{1-4} alkyl monosubstituted with phenyl, C_{1-4} alkyl disubstituted with phenyl, C_{1-4} alkyl monosubstituted with 1-naphthyl, C_{1-4} alkyl monosubstituted with 2-naphthyl, C_{1-4} alkoxy monosubstituted with phenyl, C_{1-4} alkoxy disubstituted with phenyl, Ar_1CH_2O-, Ar_1O-, Ar_1CH_2NH-, Ar_1NH- and $Heterocycle_1(CH_2)_q$-;

Ar_1 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, 1-naphthyl, 1-naphthyl monosubstituted with J, 2-naphthyl, and 2-naphthyl monosubstituted with J;

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and -$N(CH_2CH_2)_2O$;

$Heterocycle_1$ is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyrazinyl, 3-pyridyl, 4-pyridyl, 2-quinolinyl, 1-tetrahydroquinolinyl, 1-isoquinolinyl, 2-tetrahydroisoquinolinyl, and -$N(CH_2CH_2)_2O$;

q = 0-2;

AA_1, AA_2 and AA_3 are side chain blocked or unblocked a-amino acids with the L configuration, D configuration, or DL configuration at the a-carbon selected independently from the group consisting of alanine, valine, leucine, isoleucine, histidine, proline, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, arginine, lysine, tryptophan, glycine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-$CH(CH_2CHEt_2)$-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH,

NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

R is selected from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [-N(CH_2CH_2)O] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, -$CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), -NH-CH_2CH_2-(3-indolyl), $CH_2CH(OH)$-Ar_2 and $(CH_2)_n$-$Heterocycle_2$;

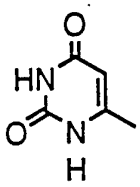
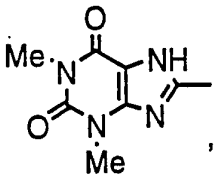
K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_{1-10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

Ar_2 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, pentafluorophenyl, $C_6H_4(3\text{-}OR_2)$, $C_6H_4(4\text{-}OR_2)$, $C_6H_3(3,4\text{-}(OR_2)_2$, $C_6H_2(2,4,6\text{-}(OR_2)_3$, 1-naphthyl, 1-naphthyl monosubstituted with J, 1-naphthyl disubstituted with J, 2-naphthyl, 2-naphthyl monosubstituted with J, 2-naphthyl disubstituted with J, 2-pyridyl, 2-quinolinyl, and 1-isoquinolinyl;

R_2 represents C_{1-4} alkyl substituted with phenyl, phenyl and phenyl substituted with J.

$Heterocycle_2$ is selected from the group consisting of 2-furyl, 2-furyl monosubstituted with J, 2-tetrahydrofuryl, 2-pyridyl, 2-pyridyl monosubstituted with J, 3-pyridyl, 3-pyridyl monosubstituted with J, 4-pyridyl, 4-pyridyl monosubstituted with J, 2-pyrazinyl, 2-quinolinyl, 2-quinolinyl monosubstituted with J, 1-isoquinolinyl, 1-isoquinolinyl monosubstituted with J, 1-tetrahydroquinolinyl, 2-tetrahydroisoquinolinyl,

3-indolyl, 2-pyridyl-N-oxide, 3-pyridyl-N-oxide, 4-pyridyl-N-oxide, 2-(N-methyl-2-pyrrolyl), 1-imidazolyl, 1-pyrrolidinyl-2-one, 2-(5-methyl-3-thiazolyl), $(CH_2)_2$-NH-biotin;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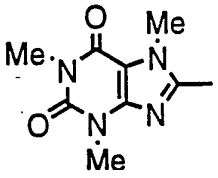


comprising the steps:

(a) Protecting the α-ketone carbonyl of a peptidyl α-ketoester with the structures

M-CO-AA_2-AA_1-$COOR_6$ and

M-CO-AA_3-AA_2-AA_1-$COOR_6$,

wherein

R_6 is selected from the group consisting of C_{1-6} alkyls and C_{1-6} alkyls monosubstituted with phenyl,

by treatment with a blocking reagent in the presence of a Lewis acid in an organic solvent at 0-100 °C for 1-48 hours, wherein

the preferred blocking reagent is 1,2-ethanedithiol;

the preferred Lewis acids are selected from the group consisting of $BF3.Et_2O$, 4-toluene sulfonic acid, $AlCl_3$ and $ZnCl_2$;

the preferred organic solvents are selected from the group consisting of CH_2Cl_2, $CHCl_3$, Et_2O and THF;

(b) Treating the product with a primary amine RNH_2 in an organic solvent at 0-100 °C for 1-72 hours, wherein

the preferred organic solvents are selected from the group consisting of EtOH, THF, CH_2Cl_2 and DMF;

(c) Removing the blocking group from the α-carbonyl to give the desired peptidyl α-ketoamide.

We have also discovered another process for the synthesis of peptidyl α-ketoamides with the structures

M-CO-AA_2-AA_1-CO-NH-R and

M-CO-AA_3-AA_2-AA_1-CO-NH-R,

wherein

M is selected from the group consisting of C_{1-4} alkyl monosubstituted with phenyl, C_{1-4} alkyl disubstituted with phenyl, C_{1-4} alkyl monosubstituted with 1-naphthyl, C_{1-4} alkyl monosubstituted with 2-naphthyl, C_{1-4} alkoxy monosubstituted with phenyl, C_{1-4} alkoxy disubstituted with phenyl, Ar_1CH_2O-, Ar_1O-, Ar_1CH_2NH-, Ar_1NH- and $Heterocycle_1(CH_2)_q$-;

Ar_1 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, 1-naphthyl, 1-naphthyl monosubstituted with J, 2-naphthyl, and 2-naphthyl monosubstituted with J;

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $-N(CH_2CH_2)_2O$;

$Heterocycle_1$ is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrazinyl, 2-quinolinyl, 1-tetrahydroquinolinyl, 1-isoquinolinyl, 2-tetrahydroisoquinolinyl, and $-N(CH_2CH_2)_2O$;

q = 0-2;

AA_1, AA_2 and AA_3 are side chain blocked or unblocked a-amino acids with the L configuration, D configuration, or DL configuration at the a-carbon selected independently from the group consisting of alanine, valine, leucine, isoleucine, histidine, proline, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, arginine, lysine, tryptophan, glycine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

R is selected from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group

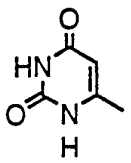
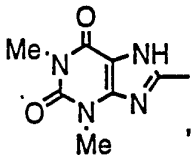
trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [-N(CH_2CH_2)O] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, -$CH_2CH_2OCH_2CH_2OH$, C_{1-10} with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), -NH-CH_2CH_2-(3-indolyl), $CH_2CH(OH)$-Ar_2 and $(CH_2)_n$-$Heterocycle_2$;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_{1-10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

Ar_2 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, pentafluorophenyl, $C_6H_4(3\text{-}OR_2)$, $C_6H_4(4\text{-}OR_2)$, $C_6H_3(3,4\text{-}(OR_2)_2$, $C_6H_2(2,4,6\text{-}(OR_2)_3$, 1-naphthyl, 1-naphthyl monosubstituted with J, 1-naphthyl disubstituted with J, 2-naphthyl, 2-naphthyl monosubstituted with J, 2-naphthyl disubstituted with J, 2-pyridyl, 2-quinolinyl, and 1-isoquinolinyl;

R_2 represents C_{1-4} alkyl substituted with phenyl, phenyl and phenyl substituted with J.

Heterocycle$_2$ is selected from the group consisting of 2-furyl, 2-furyl monosubstituted with J, 2-tetrahydrofuryl, 2-pyridyl, 2-pyridyl monosubstituted with J, 3-pyridyl, 3-pyridyl monosubstituted with J, 4-pyridyl, 4-pyridyl monosubstituted with J, 2-pyrazinyl, 2-quinolinyl, 2-quinolinyl monosubstituted with J, 1-isoquinolinyl, 1-isoquinolinyl monosubstituted with J, 1-tetrahydroquinolinyl, 2-tetrahydroisoquinolinyl, 3-indolyl, 2-pyridyl-N-oxide, 3-pyridyl-N-oxide, 4-pyridyl-N-oxide, 2-(N-methyl-2-pyrrolyl), 1-imidazolyl, 1-pyrrolidinyl-2-one, 2-(5-methyl-3-thiazolyl), (CH2)$_2$-NH-biotin;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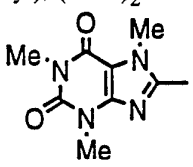


comprised of the steps:

(a) Hydrolyzing a peptidyl α-ketoester with the structures M-CO-AA_2-AA_1-$COOR_6$ and

M-CO-AA_3-AA_2-AA_1-$COOR_6$,

wherein

R_6 is selected from the group consisting of C_{1-6} alkyls and C_{1-6} alkyls monosubstituted with phenyl;

by treating the peptidyl α-ketoester with a hydrolysis reagent in an appropriate solvent at 0-100 °C for 1-24 hours to give the corresponding peptidyl α-ketoacid, wherein

the preferred hydrolysis reagents are selected from the group consisting of NaOH, KOH, EtONa and EtOK;

the preferred solvent are selected from the group consisting of water, MeOH, EtOH, THF and DMF;

(b) Coupling the product peptidyl α-ketoacid with a primary amine RNH_2 in an organic solvent at 0-100 °C for 1-72 hours to give the desired peptidyl α-ketoamide, wherein

the preferred coupling conditions are selected from the group consisting of treatment with 1,1-carbonyldiimidazole, treatment with dicyclohexylcarbodiimide, and treatment with dicyclohexylcarbodiimide-1-hydroxybenzotriazole;

the preferred organic solvents are selected from the group consisting of CH_2Cl_2, $CHCl_3$, DMF and THF.

We have also discovered a process for the synthesis of peptidyl α-ketoamides with the structures

M-CO-AA_2-AA_1-CO-NH-R and

M-CO AA_3-AA_2-AA_1-CO-NH-R,

wherein

M is selected from the group consisting of C_{1-4} alkyl monosubstituted with phenyl, C_{1-4} alkyl disubstituted with phenyl, C_{1-4} alkyl monosubstituted with 1-naphthyl, C_{1-4} alkyl monosubstituted with 2-naphthyl, C_{1-4} alkoxy monosubstituted with phenyl, C_{1-4} alkoxy disubstituted with phenyl, Ar_1CH_2O-, Ar_1O-, Ar_1CH_2NH-, Ar_1NH- and $Heterocycle_1(CH_2)_q$-;

Ar_1 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, 1-naphthyl, 1-naphthyl monosubstituted with J, 2-naphthyl, and 2-naphthyl monosubstituted with J;

J is selected from the group consisting of halogen, OH, CN, NO_2, NH_2, COOH, CO_2Me, CO_2Et, CF_3, C_{1-4} alkoxy, C_{1-4} alkylamine, C_{2-8} dialkylamine, C_{1-4} perfluoroalkyl, and $-N(CH_2CH_2)_2O$;

$Heterocycle_1$ is selected from the group consisting of 2-furyl, 2-tetrahydrofuryl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrazinyl, 2-quinolinyl, 2-tetrahydroquinolinyl, 1-isoquinolinyl, 1-tetrahydroisoquinolinyl, and $-N(CH_2CH_2)_2O$;

q = 0-2;

AA_1, AA_2 and AA_3 are side chain blocked or unblocked a-amino acids with the L configuration, D configuration, or DL configuration at the a-carbon selected independently from the group consisting of alanine, valine, leucine, isoleucine, histidine, proline, methionine, methionine sulfoxide, phenylalanine, serine, threonine, phenylglycine, norleucine, norvaline, arginine, lysine, tryptophan, glycine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, alpha-aminobutyric acid, O-methylserine, O-ethylserine, S-methylcysteine, S-ethylcysteine, S-benzylcysteine, NH_2-CH(CH_2CHEt_2)-COOH, alpha-aminoheptanoic acid, NH_2-CH(CH_2-cyclohexyl)-COOH, NH_2-CH(CH_2-cyclopentyl)-COOH, NH_2-CH(CH_2-cyclobutyl)-COOH, NH_2-CH(CH_2-cyclopropyl)-COOH, NH_2-CH(CH_2-1-naphthyl)-COOH, NH_2-CH(CH_2-2-naphthyl)-COOH, 5,5,5-trifluoroleucine, and hexafluoroleucine;

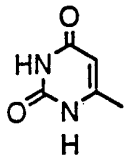
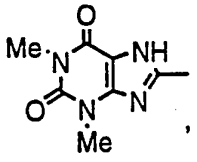
R is selected from the group consisting of H, C_{1-20} alkyl, C_{1-20} cyclized alkyl, C_{1-20} alkyl with a phenyl group attached to the C_{1-20} alkyl, C_{1-20} cyclized alkyl with an attached phenyl group, C_{1-20} alkyl with an attached phenyl group substituted with K, C_{1-20} alkyl with an attached phenyl group disubstituted with K, C_{1-20} alkyl with an attached phenyl group trisubstituted with K, C_{1-20} cyclized alkyl with an attached phenyl group substituted with K, C_{1-10} alkyl with a morpholine [$-N(CH_2CH_2)O$] ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a piperidine ring attached through nitrogen to the alkyl, C_{1-10} alkyl with a pyrrolidine ring attached through nitrogen to the alkyl, C_{1-20} alkyl with an OH group attached to the alkyl, $-CH_2CH_2OCH_2CH_2OH$, C1-10 with an attached 4-pyridyl group, C_{1-10} with an attached 3-pyridyl group, C_{1-10} with an attached 2-pyridyl group, C_{1-10} with an attached cyclohexyl group, -NH-CH_2CH_2-(4-hydroxyphenyl), -NH-CH_2CH_2-(3-indolyl), $CH_2CH(OH)$-Ar_2 and (CH_2)n-$Heterocycle_2$;

K is selected from the group consisting of halogen, C_{1-10} alkyl, C_{1-10} perfluoroalkyl, C_{1-10} alkoxy, NO_2, CN, OH, CO_2H, amino, C_{1-10} alkylamino, C_{2-12} dialkylamino, C_{1-10} acyl, and C_{1-10} alkoxy-CO-, and C_{1-10} alkyl-S-;

Ar_2 is selected from the group consisting of phenyl, phenyl monosubstituted with J, phenyl disubstituted with J, phenyl trisubstituted with J, pentafluorophenyl, $C_6H_4(3\text{-}OR_2)$, $C_6H_4(4\text{-}OR_2)$, $C_6H_3(3,4\text{-}(OR_2)_2$, $C_6H_2(2,4,6\text{-}(OR_2)_3$, 1-naphthyl, 1-naphthyl monosubstituted with J, 1-naphthyl disubstituted with J, 2-naphthyl, 2-naphthyl monosubstituted with J, 2-naphthyl disubstituted with J, 2-pyridyl, 2-quinolinyl, and 1-isoquinolinyl;

R_2 represents C_{1-4} alkyl substituted with phenyl, phenyl and phenyl substituted with J.

$Heterocycle_2$ is selected from the group consisting of 2-furyl, 2-furyl monosubstituted with J, 2-tetrahydrofuryl, 2-pyridyl, 2-pyridyl monosubstituted with J, 3-pyridyl, 3-pyridyl monosubstituted with J, 4-pyridyl, 4-pyridyl monosubstituted with J, 2-pyrazinyl, 2-quinolinyl, 2-quinolinyl monosubstituted with J, 1-isoquinolinyl, 1-isoquinolinyl monosubstituted with J, 1-tetrahydroquinolinyl, 2-tetrahydroisoquinolinyl, 3-indolyl, 2-pyridyl-N-oxide, 3-pyridyl-N-oxide, 4-pyridyl-N-oxide, 2-(N-methyl-2-pyrrolyl), 1-imidazolyl, 1-pyrrolidinyl-2-one, 2-(5-methyl-3-thiazolyl), $(CH2)_2$-NH-biotin;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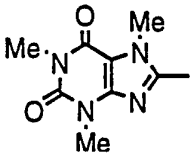


consisting of treating a peptidyl a-enolester derived from a peptidyl a-ketoester with the structures

M-CO-AA_2-AA_1-$COOR_6$ and

M-CO-AA_3-AA_2-AA_1-$COOR_6$,

wherein

R_6 is selected from the group consisting of C_{1-6} alkyls and C_{1-6} alkyls monosubstituted with phenyl;

with a primary amine RNH_2 in an organic solvent at 0-100 °C for 1-72 hours to give the desired peptidyl α-ketoamide, wherein

the preferred organic solvents are selected from the group consisting of CH_2Cl_2, EtOH, DMF and THF.

The following examples, Examples PKC1-PKC65, are given to illustrate the synthesis of Peptide Keto-Compounds:

EXAMPLE PKC1

Z-Ala-DL-Ala-COOEt. This compound was synthesized by a modified Dakin-West procedure as in Charles et al., *J. Chem. Soc. Perkin* I:1139-1146 (1980). To a stirred solution of Z-Ala-Ala-OH (880 mg, 3 mmole), 4-dimethylaminopyridine (15 mg, 0.31 mmole), and pyridine (0.8 mL, 10 mmole) in tetrahydrofuran (3 mL) was added ethyl oxalyl chloride (0.7 mL, 6 mmole) at a rate sufficient to initiate refluxing. The mixture was gently refluxed for 3.5 h. The mixture was treated with water (3 mL) and stirred vigorously at room temperature for 30 min. The mixture was extracted with ethyl acetate. The organic extracts were dried and evaporated to obtain the residue (1.45 g). The residue was chromatographed on silica gel and eluted with CH_2Cl_2 to give the enol ester product, oil (500 mg, 37%); single spot on tlc, R_f^2 = 0.67 ($CHCl_3$:MeOH = 9:1); MS, m/e = 451 ($M^+ + 1$). To a stirred suspension of the enol ester (210 mg, 0.47 mmol) in anhydrous ethanol (1 mL) at room temperature was added dropwise a solution of sodium ethoxide in ethanol until a clear yellow solution resulted. The ethanol was then removed and the residue was treated with ether. The ether solution was washed with water, dried, and evaporated to give a residue. This residue was chromatographed on a silica gel and the product was eluted with methylene chloride. The solvent was removed, and the peptide ketoester Z-Ala-DL-Ala-CO_2Et was obtained as an semi-solid (150 mg, 92 %); single spot on tlc, R_f^1 0.58 ($CHCl_3$:MeOH = 5:1); MS, m/e = 351 ($M^+ + 1$). Anal. Calcd. for $C_{17}H_{22}O_6N_2 \cdot 1/3\ H_2O$: C, 57.29; H, 6.22; N, 7.86. Found: C, 57.23; H, 6.36; N, 8.17.

EXAMPLE PKC2

Z-Ala-Ala-DL-Ala-CO_2Et. This compound was prepared from Z-Ala-Ala-Ala-OH using the same procedure as described in Example PKC1. The product was crystallized from ethyl ether in 23% yield; single spot on tlc, R_f^2 = 0.31 ($CHCl_3$:MeOH = 9:1); mp 143-144 °C; MS, m/e = 421 (M^+). Anal. Calcd. for $C_{20}H_{27}O_7N_3$: C, 56.99; H, 6.46; N, 9.97. Found: C, 56.96; H, 6.49; N, 9.92.

EXAMPLE PKC3

Z-Ala-Ala-DL-Abu-CO_2Et. This compound was prepared from Z-Ala-Ala-DL-Abu-OH in 11% yield by the procedure described in Example PKC1; single spot on tlc, R_f^2 = 0.60 ($CHCl_3$:MeOH = 9:1); mp 111-113 °C; MS, m/e = 436 ($M^+ + 1$). Anal.

Calcd. for $C_{21}H_{29}O_7N_3 \cdot 1/3\ H_2O$: C, 57.13; H, 6.75; N, 9.51. Found: C, 57.38; H, 6.82; N, 9.62.

EXAMPLE PKC4

Z-Ala-Ala-DL-Nva-CO_2Et. This compound was prepared from Z-Ala-Nva-OH in 20% yield by the procedure described in Example PKC1; single spot on tlc, R_f^1 = 0.64 ($CHCl_3$:MeOH = 5:1); MS, m/e = 450 ($M^+ + 1$). Anal. Calcd. for $C_{22}H_{31}O_7N_3 \cdot H_2O$: C, 56.51; H, 7.11; N, 8.99. Found: C, 56.42; H, 7.08; N, 9.06.

EXAMPLE PKC5

Z-Ala-Pro-DL-Ala-CO_2Et. This compound was prepared from Z-Ala-Pro-Ala-OH·dicyclohexylamine in 19% yield by the procedure described in Example PKC1; single spot on tlc, R_f^2 = 0.55 ($CHCl_3$:MeOH = 9:1); MS, m/e = 447 (M^+). Anal. Calcd. for $C_{22}H_{29}O_7N_3 \cdot 1/2\ H_2O$: C, 57.88; H, 6.62; N, 9.21. Found: C, 57.65; H, 6.68; N, 9.17.

EXAMPLE PKC6

Z-Ala-Ala-Ala-DL-Ala-CO_2Et. The compound was prepared from Z-Ala-Ala-Ala-Ala-OH in 7% yield by the procedure described in Example PKC1; single spot on tlc, R_f^2 =0.40 ($CHCl_3$:MeOH = 9:1); mp. 163-165 °C; MS, m/e = 493 ($M^+ + 1$). Anal. Calcd. for $C_{23}H_{32}O_8N_4 \cdot 1/2$ H2O: C, 55.08; H, 6.63; N, 11.17. Found: C, 54.85; H, 6.53; N, 11.14.

EXAMPLE PKC7

Bz-DL-Phe-CO_2Et. This compound was prepared from Bz-Phe-OH in 36% yield by the procedure described in Example PKC1, oil, single spot on tlc, R_f^2 = 0.61 ($CHCl_3$:MeOH = 9:1); MS, m/e = 325 (M^+). Anal. Calcd. for $C_{19}H_{19}O_4N \cdot 1/3\ H_2O$: C, 68.86; H, 5.98; N, 4.22. Found: C, 69.10; H, 6.09; N, 4.38.

EXAMPLE PKC8

MeO-Suc-Ala-DL-Ala-CO_2Me. This compound was prepared from MeO-Suc-Ala-Ala-OH in 22% yield by the same procedure as described in Example PKC1, except that sodium methoxide in methanol was used for enol ester hydrolysis, single spot on tlc, R_f^2 = 0.43 ($CHCl_3$:MeOH = 9:1); MS, m/e = 317 ($M^+ + 1$). Anal. Calcd. for $C_{13}H_{20}O_7N_4 \cdot 1/3\ H_2O$: C, 48.44; H, 6.46; N, 8.69. Found: C, 48.56; H, 6.39; N, 8.69.

EXAMPLE PKC9

MeO-Suc-Ala-Ala-Pro-DL-Abu-CO_2Me. This compound was prepared from MeO-Suc-Ala-Ala-Pro-DL-Abu-OH in 22% yield by the procedure described in Example PKC8; foam, single spot on tlc, R_f^1 = 0.66 ($CHCl_3$:MeOH = 5:1). Anal. Calcd. for $C_{22}H_{34}O_9N_4 \cdot H_2O$: C, 51.53; H, 7.02; N, 10.85. Found: C. 51.11; H, 7.03; N, 10.88.

EXAMPLE PKC10

MeO-Suc-Val-Pro-DL-Phe-CO_2Me. This compound was prepared from MeO-Suc-Val-Pro-Phe-OH in 42% yield by the same procedure as described in Example PKC8; foam, single spot on tlc, R_f^2 0.57 ($CHCl_3$:MeOH = 9:1); MS, m/e = 517 (M^+). Anal. Calcd. for $C_{26}H_{35}O_8N_3 \cdot 2/3\ H_2O$: C, 58.96; H, 6.90; N, 7.93. Found: C, 58.92; H, 6.96; N, 7.89.

EXAMPLE PKC11

Bz-DL-Ala-CO_2-n-Bu. This compound was prepared from Bz-Ala-OH in 45% yield by the procedure described in Example PKC1, except that *n*-butyl oxalylchloride was used for the Dakin-West reaction and sodium *n*-butoxide in *n*-butanol was used for enol ester hydrolysis; colorless oil, single spot on tlc, R_f^2 = 0.72 ($CHCl_3$:MeOH = 9:1); MS, m/e = 277 (M^+).

EXAMPLE PKC12

Bz-DL-Ala-CO_2Bzl. This compound was prepared from Bz-Ala-OH in 26% yield by the procedure described in Example PKC1, except that benzyl oxalyl chloride was used in place of ethyl oxayl chloride and sodium benzyloxide in benzyl alcohol was used for enol ester hydrolysis; single spot on tlc, R_f^2 =0.69 ($CHCl_3$:MeOH = 9:1); mp 95-97 °C; MS, m/e = 312 ($M^+ + 1$). Anal. Calcd. for $C_{18}H_{17}O_4N.1/2$ H2O: C, 67.48; H, 5.66; N, 4.37. Found: C, 67.78; H, 5.55; N, 4.66.

EXAMPLE PKC13

Z-Ala-DL-Ala-CO_2-n-Bu. This compound was prepared from Z-Ala-Ala-OH in 14% yield by the procedure described in Example PKC1, except that *n*-butyl oxalyl chloride was used in the Dakin-West reaction and sodium *n*-butoxide was used for enol ester hydrolysis; oil, single spot on tlc, R_f^2 = 0.45 ($CHCl_3$:MeOH = 9:1); MS, m/e = 378 (M^+). Anal. Calcd. for $C_{19}H_{26}O_6N_2 \cdot 1/3\ H_2O$: C, 59.35; H, 7.00; N, 7.29. Found: C, 59.41; H, 7.03; N, 7.10.

EXAMPLE PKC14

Z-Ala-DL-Ala-CO_2Bzl. This compound was prepared from Z-Ala-Ala-OH in 36% yield by the procedure described in Example PKC1, except that benzyl oxalyl chloride was used in the Dakin-West reaction and sodium benzyloxide in benzyl alcohol was used for enol ester hydrolysis; single spot on tlc, R_f^2 = 0.55 ($CHCl_3$:MeOH = 9:1); MS, m/e = 413 (M^++1). Anal. Calcd. for $C_{22}H_{24}O_6N_2$: C, 64.06; H, 5.87; N, 6.79. Found: C, 63.79; H, 5.95; N, 6.72.

EXAMPLE PKC15

Z-Ala-Ala-DL-Abu-CO_2Bzl. This compound was prepared from Z-Ala-Ala-Abu-OH in 31% yield by the procedure described in Example PKC1, except that benzyl oxalyl chloride was used in the Dakin-West reaction and sodium benzyloxide in benzyl alcohol was used for enol ester hydrolysis; single spot on tlc, R_f^2 = 0.40 ($CHCl_3$:MeOH = 9:1); mp 124-125 °C; MS, m/e = 498 (M^++1). Anal. Calcd. for $C_{26}H_{31}O_7N_3$·2/3 H_2O: C, 61.28; H, 6.39; N, 8.24. Found: C, 61.14; H, 6.65; N, 7.94.

EXAMPLE PKC16

Bz-DL-Ala-COOH. The hydrolysis procedure of Tsushima et al., *J. Org. Chem.*, **49**:1163-1169 (1984) was used. Bz-DL-Ala-CO_2Et (540 mg, 2.2 mmol) was added to a solution of 650 mg of sodium bicarbonate in an aqueous 50% 2-propanol solution (7.5 mL of H_2O and 2-propanol) and stirred at 40 °C under nitrogen. After adding ethyl acetate and a saline solution to the reaction mixture, the aqueous layer was separated and acidified with 2N HCl and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate and the solvent was removed under reduced pressure. The crude hydrolysis product was chromatographed on silica gel and eluted with methylene chloride and methanol to obtain an oil (150 mg, 31%); single spot on tlc, R_f^4 = 0.68 (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2). Anal. Calcd. for $C_{11}H_{11}O_4N$.3/4 H_2O: C, 56.28; H, 5.37; N, 5.97. Found: C, 56.21; H, 5.46; 5.66.

EXAMPLE PKC17

Z-Leu-DL-Nva-COOEt. This compound was prepared from Z-Leu-Nva-OH in 60 % yield by the procedure described in Example PKC1; oil, one spot on tlc, R_f = 0.49 ($CHCl_3$:MeOH = 20:1). NMR ($CDCl_3$) δ: 0.91 (t, 9H), CH_3; 1.25 (t, 3H), CH_3; 1.38 (q, 2H), OCH_2CH_3; 1.64 (m, 6H), CH_2; 1.85 (m, 1H), $CH(CH_3)_2$; 4.34 (m, 1H)

$CH_2CH(NHCOOCH_2Ph)CONH$; 5.12 (d, 3H) $NHCH(CO)CH_2$ and OCH_2Ph; 5.32 (d, 1H) NH; 6.71 (d, 1H) NH; 7.36 (s, 5H) Ph.

Z-Leu-DL-Nva-enol ester, the precursor of Z-Leu-DL-Nva-COOEt was synthesized by the same procedure as described in Example PKC1 and purified by column chromatography, oil, one spot on tlc. NMR ($CDCl_3$) δ: 0.96 (t, 9H); 1.25 (t, 3H); 1.41 (t, 2H); 1.54 (m, 4H); 1.72 (m, 3H); 2.80 (t, 2H); 4.20 (q, 2H); 4.43 (q, 2H); 5.16 (q, 2H); 5.23 (s, 1H); 7.37 (m, 5H); 11.33 (s, 1H).

EXAMPLE PKC18

Z-Leu-DL-Phe-COOEt. This compound was prepared from Z-Leu-Phe-OH in 30 % yield by the procedure described in Example PKC1; oil, one spot on tlc, R_f = 0.47 ($CHCl_3$:MeOH = 50:1). NMR ($CDCl_3$) δ: 0.88 (d, 9H), OCH_2CH_3 and $(CH_3)_2CH$; 1.35 (q, 2H), OCH_2CH_3; 1.56 (q, 2H), $(CH_3)_2CHCH_2CH$; 3.03 (m, 1H), $(CH_3)_2CH$; 4.32 (m, 2H), $NHCH(CO)CH_2$; 5.08 (s, 4H) CH_2Ph; 5.40 (m, 1H) NH; 6.61 (d, 1H) NH; 7.31 (s, 5H) Ph; 7.35.(s, 5H) Ph.

Z-Leu-DL-Phe-enol ester, the precursor of Z-Leu-DL-Phe-COOEt was synthesized by the same procedure as described in Example PKC1 and purified by column chromatography, oil, one spot on tlc. NMR ($CDCl_3$) δ: 0.86 (t, 3H); 0.99 (t, 3H); 1.24 (t, 3H); 1.40 (t, 3H); 1.52 (m, 2H); 1.83 (m, 2H); 4.23 (m, 4H); 4.39 (q, 2H); 5.10 (t, 2H); 5.18 (s, 1H); 7.26 (m, 5H); 7.34 (m, 5H); 8.89 (s, 1H).

EXAMPLE PKC19

Z-Leu-DL-Abu-COOEt. This compound was prepared from Z-Leu-Abu-OH in 33 % yield by the procedure described in Example PKC1; oil, one spot on tlc, R_f = 0.66 ($CHCl_3$:MeOH = 20:1). NMR ($CDCl_3$) δ: 0.96 (t, 9H), OCH_2CH_3 and $(CH_3)_2CH$; 1.26 (t, 3H), $CH_2CH_2CH_3$; 1.37 (q, 2H), OCH_2CH_3; 1.66 (q, 2H), $(CH_3)_2CHCH_2CH$; 2.00 (m, 1H), $CH(CH_3)_2$; 4.12 (q, 2H) $CHCH_2CH_3$; 4.34 (m, 1H) $NHCH(CONH)CH_2CH(CH_3)_2$; 5.12 (q, 3H) CH_2Ph and CONH(Et)CHCOCOO; 5.29 (t, 1H) NH; 6.79 (d, 1H) NH; 7.35 (s, 5H) Ph.

Z-Leu-DL-Abu-enol ester, the precursor of Z-Leu-DL-Abu-COOEt was synthesized by the same procedure as described in Example PKC1 and purified by column chromatography, oil, one spot on tlc. NMR ($CDCl_3$) δ: 0.98 (t, 6H); 1.12 (t, 3H); 1.24 (t, 3H); 1.41 (t, 3H); 1.73 (m, 4H); 2.86 (q, 2H); 4.20 (q, 2H); 4.31 (m, 1H); 4.42 (q, 2H); 5.15 (q, 2H); 5.21 (s, 1H); 7.34 (m, 5H); 11.29 (s, 1H).

EXAMPLE PKC20

Ala-DL-Lys-COOEt·HCl. To a solution of N-carbobenzyloxyalanyl-N^e-carbobenzyloxylysine (1.88 g, 3.9 mmol), 4-dimethylaminopyridine (21 mg, 0.17 mmol), and pyridine (1.0 mL, 12.4 mmol) in THF (7 mL) was added ethyl oxalyl chloride (0.9 mL, 8.0 mmol) at a rate sufficient to start refluxing. The mixture was refluxed gently for 3 hr, treated with water (4 mL), and stirred vigorously at room temperature for 30 min. The mixture was extracted with ethyl acetate, the organic extracts were washed with water, dried over $MgSO_4$ and evaporated to give an oily residue (1.56 g). To a solution of the enol ester (1.56 g, 2.7 mmol) in anhydrous ethanol was added dropwise a solution of sodium ethoxide in ethanol at room temperature until the solution turned clear yellow. Ethanol was removed and the residue was dissolved in ethyl acetate. The organic solution was washed with water, dried over MgSO4, and evaporated to give a residue. This residue was then purified by column chromatography and the product was eluted with chloroform-methanol. The solvent was removed and Z-Ala-DL-Lys(Z)-CO_2Et was obtained as a hygroscopic powder (328 mg, 16 %), single spot on tlc, R_f^2 = 0.53 ($CHCl_3$:MeOH = 9:1); MS, m/e = 542 ($M^+ + 1$).

N-Carbobenzoxyalanyl-DL-N^ecarbobenzoxylysine keto ethyl ester, Z-Ala-DL-Lys(Z)-CO_2Et (328 mg, 0.61 mmol) was deprotected with liquid HF containing anisole at 0 °C for 30 min. The HF was removed under reduced pressure. The residual oil was dissolved in absolute ethanol. HCl/ethanol was added to the solution, and ethanol was removed *in vacuo*. The residue was washed by decantation with ether to give a semi solid (216 mg, 100 %); single spot on tlc (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2).

EXAMPLE PKC21

Bz-DL-Lys-COOEt·HCl. This compound was prepared from Bz-DL-Lys(Z)-COOEt in 62% yield by the procedure described in Example PKC20; one spot on tlc, R_f^4 = 0.57 (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2). The precursor, Bz-DL-Lys(Z)-COOEt was prepared from Bz-Lys(Z)-OH in 100% yield by the procedure described in Example PKC1; powder, one spot on tlc, R_f^2 = 0.75 ($CHCl_3$:MeOH = 9:1); MS, m/e = 440 (M^+). Anal. Calcd. for $C_{24}H_{28}O_6N_2 \cdot 2/3\ H_2O$: C, 63.70; H, 6.53; N, 6.19. Found: C, 63.49; H, 6.51; N, 5.92.

EXAMPLE PKC22

Bz-DL-Arg-COOEt·HCl. This compound was prepared from Bz-DL-Arg(Z)-COOEt in 99% yield by the procedure described in Example PKC20; one spot on tlc, R_f^4 = 0.71 (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2), Sakaguchi reagent positive. Bz-DL-Arg(Z)-COOEt was prepared from Bz-DL-Arg(Z)-OH in 19% yield by the procedure described in Example PKC20, R_f^2 = 0.38 ($CHCl_3$:MeOH = 9:1); mp 140-142 °C; MS, m/e = 468 (M^+). Anal. Calcd. for $C_{24}H_{28}O_6N_4$: C, 61.53; H, 6.02; N, 11.96. Found: C, 61.96; H, 6.48; N, 12.34.

EXAMPLE PKC23

H-Gly-DL-Lys-COOEt·2HCl. This compound was prepared from Z-Gly-DL-Lys(Z)-COOEt in 92% yield by the procedure described in Example PKC20; R_f^4 = 0.21 (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2). Z-Gly-DL-Lys(Z)-COOEt was prepared from Z-Gly-Lys(Z)-OH in 9% yield by the procedure described in Example PKC20, one spot on tlc, R_f^1 = 0.68 ($CHCl_3$:MeOH = 5:1); MS, m/e = 528 ($M^+ + 1$).

EXAMPLE PKC24

H-Pro-DL-Lys-COOEt·2HCl. This compound was prepared from Z-Pro-DL-Lys(Z)-COOEt in 100% yield by the procedure described in Example PKC20; one spot on tlc (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2). Z-Pro-DL-Lys(Z)-COOEt was prepared from Z-Pro-Lys(Z)-OH in 15% yield by the procedure described in Example PKC20; R_f2 = 0.73 ($CHCl_3$:MeOH = 9:1); MS, m/e 568 ($M^+ + 1$).

EXAMPLE PKC25

H-Phe-DL-Lys-COOEt·2HCl. This compound was prepared from Z-Phe-DL-Lys(Z)-COOEt in 39% yield by the procedure described in Example PKC20; one spot on tlc (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2). Z-Phe-DL-Lys(Z)-COOEt was prepared from Z-Phe-Lys(Z)-OH as previously described in 9% yield, R_f^2 = 0.68 ($CHCl_3$:MeOH = 9:1); MS, m/e = 482 (M^+).

EXAMPLE PKC26

H-Leu-Ala-DL-Lys-COOEt·2HCl. This compound was prepared from Z-Leu-Ala-DL-Lys(Z)-COOEt in 52% yield by the procedure described in Example PKC20; one spot on tlc (*n*-butanol:acetic acid:pyridine:H_2O = 4:1:1:2).

Z-Leu-Ala-DL-Lys(Z)-COOEt was prepared from Z-Leu-Ala-DL-Lys(Z)-OH in 5% yield by the previously described Dakin West reaction, R_f^3 = 0.34 ($CHCl_3$:MeOH = 19:1); MS, m/e = 609 (M^+-OCH_2CH_3).

EXAMPLE PKC27

Simple Amino Acid, Di- and Tripeptide Enol Esters (General Procedure). A modified Dakin-West procedure was used (Charles et al., *supra*) and is illustrated with the synthesis of Z-Leu-DL-Phe-EE. To a stirred solution of Z-Leu-Phe-OH (6.19 g, 15.0 mmol), 4- dimethylaminopyridine (0.183 g; 1,5 mmol) and pyridine (4.75 g, 4,85 ml, 60 mmol) in tetrahydrofuran (45 ml) warmed 50 °C was added ethyl oxalyl chloride (4.30 g, 3.52 ml, 31.5 mmol) at a rate sufficient to initiate refluxing. The mixture was then heated at a gentle reflux for 4 h. After cooling to room temperature the mixture was treated with water (25 ml) and stirred vigorously at room temperature for 30 min. The mixture was extracted with ethyl acetate (150 ml) and after separation of the organic layer, the water layer was saturated with solid $(NH_4)_2SO_4$ and re-extracted 2-times with 25 ml ethyl acetate. The combined organic phases were washed 2-times with 75 ml water, 2-times with 50 ml of satd. NaCl, decolorized with carbon and dried over $MgSO_4$. After evaporation of the solvent, the crude enol ester (8,36 g, 98%) was flash-chromatographed on silica gel and the product was eluted with a AcOEt. The solvent was evaporated in vacuo (rotavaporator) and the pure enol ester was obtained as a oil (7.22 g, 85%); single spot on TLC, R_f = 0.84, A; 0.68, C.

Z-Leu-Nva-EE. This compound was prepared from Z-Leu-Nva- OH using the general procedure and purified by flash chromatography on silica gel using $CHCl_3$:MeOH = 50:1 v/v as eluent. Yield 95%, single spot on TLC, R_f = 0.92, C; 0.28, L.

Z-Leu-Abu-EE. This compound was prepared from Z-Leu-Abu- OH in 78% yield the general procedure described above. Purification by flash obramatography on silica gel. Eluent, $CHCl_3$:MeOH = 50:1 v/v, single spot on TLC, Rf = 0.86, A.

PhCO-Abu-EE. This compound was prepared from PhCO-Abu-OH in 26% yield by the general procedure as described above. Purification by flash chromatography on silica gel. Eluent $CHCl_3$, single spot on TLC, R_f = 0.60, M.

$(CH3)_2CH(CH_2)_2CO$-Abu-EE. This compound was prepared from $(CH_3)_2CH(CH_2)_2CO$-Abu-OH in 82% yield by the general procedure as described

above. Purification by flash chromatography on silica gel. Eluent AcOEt, single spot on TLC, R_f = 0.72, C.

$(CH_3CH_2CH_2)_2$ CH CO-Abu-EE. This compound was prepared from $(CH_3CH_2CH_2)_2CH$ CO-Abu-OH in 100% yield by the general procedure described above. Purification by flash chromatography on silica gel. Eluent AcOEt, single spot on TLC, R_f = 0.78, C; 0.81, K.

$Ph(CH_2)_6CO$-Abu-EE. This compound was prepared from $Ph(CH_2)_6CO$-Abu-OH in 86% yield by the general procedure described above. Purification by flash chromatography on silica gel. Eluent AcOEt. Single spot on TLC, R_f =0.74, C.

Z-Leu-4-Cl-Phe-EE. This compound was prepared from Z-Leu-4-Cl-Phe-OH in 69% yield by the general procedure described above. Purification by flash chromatography on silica gel. Eluent AcOEt, single spot on TLC, R_f = 0.77, C; 0.78, K.

Z-Leu-Leu-Abu-EE. This compound was prepared from Z2-Leu-Leu-Abu-OH in 62% yield by the general procedure described above. Purification by flash chromatography on silica gel. Element $CHCl_3$:MeOH = 50:1 v/v. Single spot on TLC, R_f = 0.89, A; 0.75, M.

Z-Leu-Leu-Phe-EE. This compound was prepared from Z-Leu-Leu-Phe-OH in 60% yield by the general procedure described above. Purification by flash chromatography on silica gel. Eluent $CHCl_3$:MeOH = 50:1 v/v. Single spot on TLC, R_f = 0.80, K; 0.70, M.

2-$NapSO_2$-Leu-Leu-Abu-EE. This compound was prepared from 2-$NapSO_2$-Leu-Abu-OH in 73% yield by the general procedure described above. Purification by flash chromatography on silica gel. Eluent AcOEt, single spot on TLC, R_f = 0.71, K; 0.54, C.

2-$NapSO_2$-Leu-Leu-Abu-EE. This compound was prepared from 2-$NapSO_2$-Leu-Leu-Abu-OH in 74% yield by the general procedure described above. Purification by flash chromatography on silica gel. Eluent AcOEt: AcOH = 200:1 v/v. Single spot on TLC, R_f = 0.69. K.

EXAMPLE PKC28

Z-Leu-Phe-COOEt. *Single Aminoacid, Di-and Tripeptide- ketoesters (General Procedure).* To a stirred solution of 8.53 g (15.0 mmol) of Z-Leu-Phe-EE in 40 ml anhydrous ethanol at room temperature was added dropwise a solution of sodium ethoxide (0.204 g; 3.0 mmol) in 20.0 ml anhydrous ethanol. The color of the reaction mixture change from colorless or pall yellow to deep yellow or orange dependent on enol-ester. Then the reaction mixture was stirred at room temperature for 4-5 hours, the ethanol was then evaporated in vacuo (rotavaporator) and the residue treated with 200 ml ethyl ether (or 200 ml ethyl acetate in the case of the tripeptide). The ether (ethyl acetate) solution was washed with 2 x 75 ml H_2O, 2 x 75 ml satd. NaCl, decolorized with carbon and dried over $MgSO_4$. After evaporation of solvent, the crude product 6.09 g (89.7%) was flash chromatographed on silica gel using $CHCl_3$: MeOH = 50:1 v/v. Evaporation of solvent give pure Z-Leu-Phe-COOEt (4.08 g; 58.0%) as a thick oil. Single spot on TLC, R_f = 0.60, A; 0.47, M. Mass spectrum, FB-MS [(M+1)/Z] = 469.

EXAMPLE PKC29

Z-Leu-Nva-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$: MeOH = 100:1 v/v, yield 86.6%, thick, colorless oil, single spot on TLC, R_f = 0.49, A; 0.37, M. Mass spectrum FB-MS [(M+1)/Z] = 421.

EXAMPLE PKC30

Z-Len-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$, yield 82%, thick, pale yellow oil, single spot on TLC, R_f = 0.66, A. Mass spectrum, CI-MS [(M+1)/Z] = 407.

EXAMPLE PKC31

PhCO-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$:MeOH = 50:1 v/v, yield 83%, oil, single spot on TLC, R_f = 0.44, M. Mass spectrum, M/Z 263 (M^+); CI-MS, 264 ((M+1)/Z).

EXAMPLE PKC32

$(CH_3)_2CH(CH_2)_2CO$-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent AcOEt, yield 43%, oil, single spot on TLC, R_f = 0.56, C. Mass spectrum EI-MS M/Z 257 (M^+); FB-MS, [(M+1)/Z] = 258.

EXAMPLE PKC33

$CH_3CH_2CH)_2CHCO$-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$:MeOH = 50: 1 v/v, thick, yellowish oil, yield 66%, single spot on TLC, R_f = 0.80, C; 0.66, M. Mass spectrum EI-MS M/Z = 285 (M^+); CI-MS, [(M+1)/Z] = 286.

EXAMPLE PKC34

$Ph(CH_2)_6CO$-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$:MeOH = 50:1 v/v, yield 64%, pale yellow oil, single spot on TLC, R_f = 0.29, M. Mass spectrum EI-MS M/Z = 347 (M^+), FB-MS, [(M+1)/Z] = 348.

EXAMPLE PKC35

Z-Leu-4-Cl-Phe-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent AcOEt, yield 100%, colorless oil, single spot on TLC, R_f = 0.71, C. Mass spectrum FB-MS M/Z = 503(M^+).

EXAMPLE PKC36

Z-Leu-Leu-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$:MeOH = 50:1 v/v, yield 79.2%, very thick, colorless oil, single spot on TLC, R_f = 0.28. M. Mass spectrum FB-MS, [(M+1)/Z] = 520.

EXAMPLE PKC37

Z-Leu-Leu-Phe-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$:MeOH = 50:1 v/v, yield 33%, oil, single spot on TLC, R_f = 0.56, M. Mass spectrum, FB-MS, [(M+1)/Z] = 582.

EXAMPLE PKC38

2-NapSO_2-Leu-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent $CHCl_3$:MeOH = 50:1 v/v, yield 38%, thick oil, single spot on TLC, R_f = 0.71, K; 0.54, A. Mass spectrum FB-MS, [(M+1)/Z] = 463.

EXAMPLE PKC39

2-NapSO_2-Leu-Leu-Abu-COOEt. This was prepared by the preceding general procedure. Purification by flash chromatography on silica gel, eluent AcOEt:AcOH = 200:1 v/v, yield 61%, semi-solid, single spot on TLC, R_f = 0.67, K. Mass spectrum FB-MS, [(M+1)/Z] = 576.

EXAMPLE PKC40

Z-Leu-Met-CO_2Et. This compound was prepared by the above procedure. Yellow oil, single spot on TLC, R_f = 0.52 ($CHCl_3$:CH_3OH=50:1), yield 46% (from dipeptide), MS (FAB) 454 (m+1).

EXAMPLE PKC41

Z-Leu-NLeu-CO_2Et. This compound was prepared by the above procedure. Pale yellow oil, single spot on TLC, R_f = 0.57 ($CHCl_3$:CH_3OH = 50:1), yield 53% (from dipeptide), MS (FAB) 434 (m+1).

EXAMPLE PKC42

Synthesis of n-Butyl Oxalyl Chloride. This was prepared by a literature procedure (Warren and Malee, *supra*). N-Butanol (0.1 mol. 7.41 g) was added dropwise to oxalyl chloride (0.5 mol. 63.5 g) at -10 °C. After the addition was completed, the reaction mixture was stirred for 20 min. at r.t. and distilled, giving 15.0 g (91.18 mol. 91%) of the product n-butyl oxalyl chloride, bp 58-60 °C (0.6 mm Hg).

Z-Leu-Phe-CO_2Bu. This compound was prepared from Z-Leu- Phe-OH and butyl oxalyl chloride in 43% yield by the procedure described for the synthesis of Z-Leu-Phe-CO_2Et, except that butyl oxalyl chloride was used in place of ethyl oxalyl chloride and sodium butyloxide in butanol was used for enol ester hydrolysis. Single spot on TLC, R_f = 0.54 ($CHCl_3$:CH_3OH = 50:1) MS(FAB) m/e = 497 (m+1), ^1H NMR ($CDCl_3$) ok.

EXAMPLE PKC43

Z-Leu-Abu-CO_2Bu. This compound was prepared by the above procedure. Single spot on TLC, R_f = 0.53 ($CHCl_3$:CH_3OH = 50:1), yield = 36%, pale yellow oil, MS (FAB) m/e = 435 (M+1), ^1H NMR ($CDCl_3$) ok.

EXAMPLE PKC44

Synthesis of Benzyl Oxalyl Chloride. Benzyl alcohol (0.15 mol. 16 g) was added dropwise to oxalyl chloride (0.75 mol. 95 g) at 5-10 °C. After the addition was complete, the reaction was stirred for 20 min. at r.t. The excess oxalyl chloride was distilled and recycled. Then the mixture was distilled under vacuo, giving 26 g (0.12 mol. 86%) of benzyl oxalyl chloride, bp. 110-112 °C (0.6 mm-Hg). H^1NMR ($CDCl_3$) 7.39 (s, 5H), 5,33 (s.2H).

Z-Leu-Phe-CO_2Bzl. This compound was prepared from Z-Leu- Phe-OH and benzyl oxalyl chloride in 17% yield by the procedure described in the synthesis of Z-Leu-Phe-CO_2Et, except that benzyl oxalyl chloride was used in place of ethyl oxalyl chloride and sodium benzyloxide in benzyl alcohol was used for enol ester hydrolysis. Single spot on TLC, R_f = 0.63 ($CHCl_3$:CH_3OH = 50:1). Pale yellow solid, mp 117-119 °C. MS(FAB) m/e = 532 (m+1). H^1NMR ok.

EXAMPLE PKC45

Z-Leu-Abu-CO_2Bzl. This compound was prepared by the above procedure. Single spot on TLC. R_f = 0.51 ($CHCl_3$:CH_3OH = 50:1), pale yellow oil, MS(FAB) m/e = 469 (m+1), yield = 26%.

EXAMPLE PKC46

Z-Leu-Phe-COOH. Dipeptide Ketoacids (General Procedure). To a stirred solution of 0.53g (1,13 mmol) Z-Leu-Phe-COOEt in 6.0 ml methanol was added 1.27 ml (1.27 mmol) 1M NaOH. The color of the reaction mixture turned dark yellow and a small amount of solid was deposited. The reaction was run at room temperature and progress of the hydrolysis was checked on TLC. After 24 h. no more substrate was detected. The reaction mixture was chilled in one ice bath at 5 °C, acidified with 1M HCl to pH = 3 and extracted with AcOEt (2 x 50 mL). The organic extract were washed with 2 x 50 ml H_2O and if necessary, decolorized with carbon and dried over $MgSO_4$. After evaporation of the solvent (rotavaporator), the residue (thick oil) were titurated with 2 x 25 ml n-hexane and dried in vacuo. Yield 0.39 g (78%) of colorless,

very thick oil. TLC, main spot at R_f = 0.24, trace of impurity at R_f = 0.78, I. Mass spectrum, FB-MS [(M+1)/Z] = 441.

EXAMPLE PKC47

Z-Leu-Abu-COOH. This compound was prepared from Z-L-Leu- Abu-COOEt in 83% yield by the general procedure as described above; TLC, main spot at R_f = 0.14, trace of impurity at R_f = 0.73, I. Mass spectrum, FB-MS [(M+1)/Z] = 379.

EXAMPLE PKC48

Z-Leu-Phe-CONH-Et. To a stirred solution of Z-Leu-Phe-OH (20 g, 48.5 mmole), 4-dimethylaminopyridine (0.587 g, 4.8 mmole),and pyridine (15.7 ml, 194 mmole) in anhydrous THF (100 ml) was added ethyl oxalyl chloride (11.4 ml, 101.8 mmole) at a rate sufficient to initiate refluxing. The mixture was gently refluxed for 4 hours, cooled to room temperature, and water (80 ml) was added. The reaction mixture was stirred vigorously for 30 min, and extracted with ethyl acetate (3 x 100 ml). The combined organic layers were washed with water (2 x 100 ml), saturated sodium chloride (2 x 100 ml), decolorized with decolorizing carbon, dried over magnesium sulfate, and concentrated, leaving a dark orange oil. Chromatography on a silica gel column with $CHCl_3/CH_3OH$ (50:1 v/v) afforded 14.63 g (y = 53 %) of Z-Leu-Phe-enolester. The product was a yellow oil. Single spot on TLC, R_f = 0.77 ($CHCL_3/CH_3OH$ 50:1). NMR ($CDCl_3$) ok.

To a stirred pale yellow solution of the Z-Leu-Phe-enolester (14.63 g, 25.73 mmole) in anhydrous ethanol (50 ml) was added a solution of sodium ethoxide (0.177 g, 2.6 mmole) in ethanol (5 ml). The orange solution was stirred for 3 hours at room temperature, then the ethanol was evaporated and the residue was treated with ethyl ether (300 ml). The ether layer was washed with water (2 x 100 ml), saturated sodium chloride (2 x 100 ml), dried over magnesium sulfate, and concentrated, leaving a orange oil. Chromatography on a silica gel column with $CHCl_3/CH_3OH$ (50:1 v/v) afforded 7.76 g (y = 64 %) of the α-ketoester Z-Leu-Phe-COOEt. The product was a yellow oil. Single spot on TLC, R_f = 0.44 ($CHCl_3/CH_3OH$ 50:1). NMR ($CDCl_3$) ok. MS (FAB, calcd. for $C_{26}H_{32}N_2O_6$: 468.6), m/e = 469 (M+1).

The α-carbonyl group of Z-Leu-Phe-COOEt was protected by following procedure. A solution of Z-Leu-Phe-COOEt (1 g, 2.13 mmole) in 5 ml of CH_2Cl_2 was added 1,2-ethanedithiol (0.214 ml, 2.55 mmole), followed by 0.5 ml of boron trifluoride

etherate. The solution was stirred overnight at room temperature. Water (20 ml) and ethyl ether (20 ml) were added. The organic layer was separated, washed with water (2 x 10 ml), saturated sodium chloride (2 x 10 ml), dried over magnesium sulfate, and evaporated to afford 0.98 g (y = 84 %) yellow semisolid.

The protected α-ketoester (0.98 g, 1.8 mmole) was dissolved in ethanol (5 ml), cooled to 0-5 °C in a ice bath, and ethylamine was bubbled through the solution until 2.43 g (54 mmole) had been added. The reaction mixture was allowed to warm to room temperature slowly, and stirred overnight. The mixture was filtered, a white precipitate was removed, leaving a yellow semisolid. Chromatography on a silica gel column with $CHCl_3/CH_3OH$ (30:1 v/v) afford 0.63 g (y = 75 %) of Z-Leu-Phe-CONH-Et. The product was a pale yellow solid. Single spot on TLC, R_f = 0.60 ($CHCl_3/CH_3OH$ 20:1); mp 145-147 °C. Anal. calcd. for $C_{26}H_{33}N_3O_5$: 467.56; C, 66.79; H, 7.11; N,8.99; found: C, 66.59; H, 7.09; N, 8.95. NMR ($CDCl_3$) ok. MS (FAB) m/e = 468 (M+1).

EXAMPLE PKC49

Z-Leu-Phe-CONH-nPr. This compound was synthesized from the protected α-ketoester and propylamine in 92 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.50 ($CHCl_3/CH_3OH$ 50:1); mp 152-153 °C. Anal. calcd. for $C_{27}H_{35}N_3O_5$: 481.57; C, 67.33; H, 7.33; N, 8.72. Found: C, 67.21; H, 7.38; N, 8.64. NMR ($CDCl_3$) ok. MS (FAB) m/e = 482 (M+1).

EXAMPLE PKC50

Z-Leu-Phe-CONH-nBu. This compound was synthesized from the protected α-ketoester and butylamine in 67 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.50.($CHCl_3/CH_3OH$ 50:1); mp 152-153 °C. Anal. calcd. for $C_{28}H_{37}N_3O_5$: 495.59; C, 67.85; H, 7.52; N, 8.48. Found: C, 67.70; H, 7.57; N, 8.43. NMR ($CDCl_3$) ok. MS (FAB) m/e = 496 (M+1).

EXAMPLE PKC51

Z-Leu-Phe-CONH-iBu. This compound was synthesized from the protected α-ketoester and isobutylamine in 53 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 50:1); mp 152 °C. Anal. calcd. for $C_{28}H_{37}N_3O_5$: 495.59; C, 67.85; H, 7.52; N, 8.48. Found: C, 67.77; H, 7.56; N, 8.40. NMR ($CDCl_3$) ok. MS (FAB) m/e = 496 (M+1).

EXAMPLE PKC52

Z-Leu-Phe-CONH-Bzl. This compound was synthesized from the protected α-ketoester and benzylamine in 40 % yield by the procedure described in Example PKC48. After reacting overnight, ethyl acetate (60 ml) was added. The mixture was filtered to remove a white precipitate. The solution was washed with cooled 1 N HCl (3 x 25 ml), water (1 x 20 ml), saturated sodium chloride (2 x 20 ml), and dried over magnesium sulfate. The solution was evaporated leaving a yellow solid. Chromatography on a silica gel column with $CHCl_3/CH_3OH$ 30:1 v/v) afforded a yellow solid. Single spot on TLC, R_f = 0.45 ($CHCl_3/CH_3OH$ 30:1); mp 160-162 °C. Anal. calcd. for $C_{31}H_{35}N_3O_5$: 529.61; C, 70.30; H, 6.66; N, 7.93. Found: C, 70.18; H, 6.67; N, 7.99. NMR ($CDCl_3$) ok. MS (FAB) m/e = 530 (M+1).

EXAMPLE PKC53

Z-Leu-Phe-CONH-$(CH_2)_2$Ph. This compound was synthesized from the protected α-ketoester and phenethylamine in 50 % yield by the procedure described in Example PKC52. Single spot on TLC, R_f = 0.50 ($CHCl_3/CH_3OH$ 30:1); mp 151-153 °C. Anal. calcd. for $C_{32}H_{37}N_3O_5$: 543.66; C, 70.70; H, 6.86; N, 7.73. Found: C, 70.54; H, 6.88; N, 7.74. NMR ($CDCl_3$) ok. MS (FAB) m/e = 544 (M+1).

EXAMPLE PKC54

Z-Leu-Abu-CONH-Et. This compound was synthesized from protected α-ketoester derived from Z-Leu-Abu-CO_2Et and ethylamine in 64 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.36 ($CHCl_3/CH_3OH$ 50;1); mp 130-132 °C. Anal. calcd. for $C_{21}H_{31}N_3O_5$: 405.45; C, 62.20; H, 7.71; N, 10.36. Found: C, 61.92; H, 7.62; N, 10.31. NMR ($CDCl_3$) ok. MS (FAB) m/e = 406 (M+1).

EXAMPLE PKC55

Z-Leu-Abu-CONH-nPr. This compound was synthesized from the corresponding protected α-ketoester and propylamine in 47 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.28 ($CHCl_3/CH_3OH$ 50:1); mp 134-135 °C. Anal. calcd. for $C_{22}H_{33}N_3O_5$: 419.50; C, 62.98; H, 7.93; N, 10.02. Found: C, 62.84; H, 7.97; N, 9.94. NMR ($CDCl_3$) ok. MS (FAB) m/e = 420 (M+1).

EXAMPLE PKC56

Z-Leu-Abu-CONH-nBu. This compound was synthesized from the corresponding protected α-ketoester and butylamine in 42 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 50:1); mp 135-136 °C. Anal. calcd. for $C_{23}H_{35}N_3O_5$: 433.53; C, 63.71; H, 8.13; N, 9.69. Found: C, 63.48; H, 8.07; N, 9.67. NMR ($CDCl_3$) ok. MS (FAB) m/e = 434 (M+1).

EXAMPLE PKC57

Z-Leu-Abu-CONH-iBu. This compound was synthesized from the corresponding protected α-ketoester and isobutylamine in 65 % yield by the procedure described in Example PKC48. Single spot on TLC, R_f = 0.25 ($CHCl_3/CH_3OH$ 50:1); mp 133-135 °C. Anal. calcd. for $C_{23}H_{35}N_3O_5$: 433.52; C, 63.72; H, 8.14; N, 9.69. Found: C, 63.46; H, 8.10; N, 9.60. NMR ($CDCl_3$) ok. MS (FAB) m/e = 434 (M+1).

EXAMPLE PKC58

Z-Leu-Abu-CONH-Bzl. This compound was synthesized from the corresponding protected α-ketoester and benzylamine in 29 % yield by the procedure described in Example PKC52. Single spot on TLC, R_f = 0.56 ($CHCl_3/CH_3OH$ 30:1); mp 140-141 °C. Anal. calcd. for $C_{26}H_{33}N_3O_5$: 467.54; C, 66.79; H, 7.11; N, 8.99. Found: C, 66.65; H, 7.07; N, 8.93. NMR ($CDCl_3$) ok. MS (FAB) m/e = 468 (M+1).

EXAMPLE PKC59

Z-Leu-Abu-CONH-$(CH_2)_2$Ph. This compound was synthesized from the corresponding protected α-ketoester and phenethylamine in 51 % yield by the procedure described in Example PKC52. Single spot on TLC, R_f = 0.44 ($CHCl_3/CH_3OH$ 30:1); mp 156-157 °C. Anal. calcd. for $C_{27}H_{35}N_3O_5$: 481.59; C, 67.34; H, 7.33; N, 8.72. Found: C, 67.38; H, 7.33; N, 8.78. NMR ($CDCl_3$) ok. MS (FAB) m/e = 482 (M+1).

EXAMPLE PKC60

Z-Leu-Abu-CONH-$(CH_2)_3$-N$(CH_2CH_2)_2$O. This compound was synthesized from protected α-ketoester and 4(3-aminopropyl)morpholine in 33 % yield by the procedure described in Example PKC48. After reacting overnight, ethyl acetate (80 ml) was added. The mixture was filtered to remove a white precipitate. The solution was washed with water (3 x 20 ml), saturated sodium chloride (2 x 20 ml), and dried over magnesium sulfate. The solution was evaporated leaving a yellow oil. Chromatography

on a silica gel column with $CHCl_3/CH_3OH$ (10:1 v/v) afforded a yellow semisolid, which was recrystallized from ethyl acetate/hexane to obtain a pale yellow solid. Single spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 10:1); mp 125-126 °C. Anal. calcd. for $C_{26}H_{40}N_4O_6$: 504.63; C, 61.88; H, 7.99; N, 11.10. Found: C, 61,69; H, 7.95; N, 11.07. NMR ($CDCl_3$) ok. MS (FAB) m/e = 505 (M+1).

EXAMPLE PKC61

Z-Leu-Abu-CONH-$(CH_2)_7CH_3$. This compound was synthesized from the corresponding protected α-ketoester and octylamine in 67 % yield by the procedure described in Example PKC52. It was white solid. Single spot on TLC, R_f = 0.55 ($CHCl_3/CH_3OH$ 30:1); mp 134-135 °C. Anal. calcd. for $C_{27}H_{43}N_3O_5$: 489.66; C, 66.23; H, 8.85; N, 8.58. Found: C, 66.19; H, 8.81; N, 8.61. NMR ($CDCl_3$) ok. MS (FAB) m/e = 490 (M+1).

EXAMPLE PKC62

Z-Leu-Abu-CONH-$(CH_2)_2OH$. This compound was synthesized from the corresponding protected α-ketoester and ethanolamine in 29 % yield by the procedure described in Example PKC60. The product was a white sticky solid. Single spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 10:1); mp 151-153 °C. Anal: calcd. for $C_{21}H_{31}N_3O_6$: 421.49; C, 59.84; H, 7.41; N, 9.97. Found: C, 59.11; H, 7.44; N, 9.81. NMR ($CDCl_3$) ok. MS (FAB) m/e = 422 (M+1).

EXAMPLE PKC63

Z-Leu-Abu-CONH-$(CH_2)_2O(CH_2)_2OH$. This compound was synthesized from the corresponding protected α-ketoester and 2-(2-aminoethoxy)ethanol in 34 % yield by the procedure described in Example PKC60. The product was white sticky solid. Single spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 10:1); mp 103-105 °C. Anal.: calcd. for $C_{23}H_{35}N_3O_7$: 465.55; C, 59.34; H, 7.58; N, 9.03. Found: C, 59.23; H, 7.58; N, 9.01. NMR ($CDCl_3$) ok. MS (FAB) m/e = 466 (M+1).

EXAMPLE PKC64

Z-Leu-Abu-CONH-$(CH_2)_{17}CH_3$. This compound was synthesized from the corresponding protected α-ketoester and octadecylamine in 12 % yield by the procedure described in Example PKC52. The product was a pale yellow solid. Single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 30:1); mp 134-136 °C. Anal: calcd. for

$C_{37}H_{63}N_3O_5$: 629.92; C, 70.55; H, 10.08; N, 6.67. Found: C, 70.71; H, 10.14; N, 6.75. NMR ($CDCl_3$) ok. MS (FAB) m/e = 630.2 (M+1).

EXAMPLE PKC65

Z-Leu-Abu-CONH-CH_2-$C_6H_3(OCH_3)_2$. This compound was synthesized from the corresponding protected α-ketoester and 3,5-dimethoxybenzylamine in 45 % yield by the procedure described in Example PKC52. The product was yellow sticky solid. Single spot on TLC, R_f = 0.44 ($CHCl_3/CH_3OH$ 30:1); mp 153-155 °C. Anal.: calcd. for $C_{28}H_{37}N_3O_7$: 527.62; C, 63.74; H, 7.07; N, 7.96. Found: C, 63.66; H, 7.09; N, 7.92. NMR ($CDCl_3$) ok. MS (FAB) m/e = 528.8 (M+1).

EXAMPLE PKC66

Z-Leu-Abu-CONH-CH_2-C_4H_4N. This compound was synthesized from the corresponding protected α-ketoester and 4-(aminomethyl)pyridine in 45 % yield by the procedure described in Example PKC60. The product was greenish yellow solid. Single spot on TLC, R_f = 0.55 ($CHCl_3/CH_3OH$ 10:1); mp 124-126 °C. Anal: calcd. for $C_{25}H_{32}N_4O_5$: 468.55; C, 64.08; H, 6.88; N, 11.96. Found: C, 63.88; H, 6.87; N, 11.96. NMR ($CDCl_3$) ok. MS (FAB) m/e =469 (M+1).

EXAMPLE PKC67

Z-Leu-Abu-CONH-$(CH_2)_5OH$. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 5-amino-1-pentanol. To a solution of protected a-ketoester (1 mmol) in ethanol (3 mL) was added 5-amino-1-pentanol (3 mmol) and stirred overnight at r.t. To the mixture was added AcOEt (25 mL) and white precipitate was filtered. The filtrate was washed with cold 1N HCl (2 x 10 mL), water (1 x 10 mL), saturated NaCl (2 x 10 mL) and dried over $MgSO_4$. After evaporation of the solvent, chromatography on a silica gel column using solvent $CHCl_3/CH_3OH$ 10:1 followed by precipitation from AcOEt/hexane afforded a white solid (42% yield). Single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 10:1), mp 122-123 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 464 (M+1). Anal: calcd. for $C_{24}H_{37}N_3O_6$, 463; C, 62.18; H, 8.04; N, 9.06. Found: C, 61.52; H, 7.96; N, 8.98.

EXAMPLE PKC68

Z-Leu-Abu-CONH-$(CH_2)_2OH$. This is an alternative synthesis for the compound designated in Example PKC 62. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and ethanolamine by the procedure

described in Example PKC67, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (40% yield). White solid, single spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 10:1), mp 151-154 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 422 (M+1). Anal: calcd. for $C_{21}H_{31}N_3O_6$, 421; C, 59.84; H, 7.41; N, 9.97. Found: C, 59.11; H, 7.44; N, 9.81.

EXAMPLE PKC69

Z-Leu-Abu-CONH-$(CH_2)_2O(CH_2)_2OH$. This is an alternative synthesis for the compound designated in Example PKC 63. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 2-(2-aminoethoxy)ethanol by the procedure described in Example PKC67, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (34% yield). White solid, single spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 10:1), mp 103-105 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 466 (M+1). Anal: calcd. for $C_{23}H_{35}N_3O_7$, 465; C, 59.30; H, 7.58; N, 9.02. Found: C, 59.23; H, 7.58; N, 9.01.

EXAMPLE PKC70

Z-Leu-Abu-CONH-$CH_2CH(OCH_3)_2$. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and aminoacetaldehyde dimethylacetal by the procedure described in Example PKC67, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (25% yield). White solid, single spot on TLC, R_f = 0.47 ($CHCl_3/CH_3OH$ 20:1), mp 99-102 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 466 (M+1). Anal: calcd. for $C_{23}H_{35}N_3O_7$, 465; C, 59.30; H, 7.58; N, 9.02. Found: C, 58.95; H, 7.71; N, 9.00.

EXAMPLE PKC71

Z-Leu-Abu-CONH-$CH_2CH(OC_2H_5)_2$. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and aminoacetaldehyde diethylacetal, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (36% yield). White solid, single spot on TLC, R_f = 0.37 ($CHCl_3/CH_3OH$ 20:1), mp 100-103 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e =494 (12%, M+1), 448 (100%, M+1-45). Anal: calcd. for $C_{25}H_{39}N_3O_7$, 493; C, 60.83; H, 7.96; N, 8.51. Found: C, 60.73; H, 7.98; N, 8.42.

EXAMPLE PKC72

Z-Leu-Abu-CONH-CH_2-C_6H_8(1,3,3-$(CH_3)_3$-5-OH). This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 3-aminomethyl-3,5,5-trimethyl-cyclohexanol, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (51% yield). White solid, single spot on TLC, R_f = 0.55 ($CHCl_3/CH_3OH$ 30:1), mp 59-61 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 532 (M+1). Anal; calcd. for $C_{29}H_{45}N_3O_6$,531; C, 65.51; H, 8.53; N, 7.90. Found, C, 65.21; H, 8.55, N, 7.81.

EXAMPLE PKC73

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(4-OH). This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 4-(2-aminoethyl)phenol, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (60% yield). White solid, single spot on TLC, R_f = 0.56 ($CHCl_3/CH_3OH$ 30:1), mp 151-153 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 498 (M+1). Anal: calcd. for $C_{27}H_{35}N_3O_6$, 497; C, 65.17; H, 7.09; N, 8.45. Found, C, 65.16; H, 7.13, N, 8.52.

EXAMPLE PKC74

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(2-OCH_3). This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 2-methoxyphenethylamine, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 50:1 (71% yield). Yellow solid, single spot on TLC, R_f = 0.47 ($CHCl_3/CH_3OH$ 50:1), mp 101-103 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 512 (M+1). Anal; calcd. for $C_{28}H_{37}N_3O_6$, 511; C, 65.73; H, 7.29; N, 8.21. Found, C, 65.50; H, 7.31; N, 8.19.

EXAMPLE PKC75

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(3-OCH_3). This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 3-methoxyphenethylamine, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 50:1 (56% yield). Yellow solid, single spot on TLC, R_f = 0.46 ($CHCl_3/CH_3OH$ 50:1), mp 99-100 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e =512 (M+1). Anal: calcd. for $C_{28}H_{37}N_3O_6$, 511; C, 65.73; H, 7.29; N, 8.21. Found, C, 65.62; H, 7.34; N, 8.16.

EXAMPLE PKC76

Z-Leu-Abu-CONH-$(CH_2)_2C_6H_4$(4-OCH_3). This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 4-methoxyphenethylamine, and

purified by column chromatography using solvent $CHCl_3/CH_3OH$ 50:1 (50% yield). White solid, single spot on TLC, R_f = 0.46 ($CHCl_3/CH_3OH$ 50:1), mp 152-155 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 512 (M+1). Anal: calcd. for $C_{28}H_{37}N_3O_6$, 511; C, 65.73; H, 7.29; N, 8.21. Found, C, 65.64; H, 7.30; N, 8.19.

EXAMPLE PKC77

Z-Leu-Abu-CONH-$CH_2C_6H_3$(3,5-$(OCH_3)_2$. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 3,5-dimethoxyphenethylamine, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 50:1 (50% yield). White solid, single spot on TLC, R_f = 0.46 ($CHCl_3/CH_3OH$ 50:1), mp 153-155 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 528 (M+1). Anal: calcd. for $C_{28}H_{37}N_3O_7$, 527; C, 63.74; H, 7.07; N, 7.96. Found, C, 63.66; H, 7.09; N, 7.92.

EXAMPLE PKC78

Z-Leu-Abu-CONH-CH_2CH(OH)Ph. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 2-amino-1-phenylethanol by the procedure described in Example PKC67, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (50% yield). White solid, single spot on TLC, R_f = 0.48 ($CHCl_3/CH_3OH$ 10:1), mp 152-154 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 498 (M+1). Anal: calcd. for $C_{27}H_{35}N_3O_6$, 497; C, 65.17; H, 7.09; N, 8.44. Found, C, 65.06; H, 7.05; N, 8.50.

EXAMPLE PKC79

Z-Leu-Abu-CONH-CH_2CH(OH)C_6H_4(4-OCH_3). This compound was synthesized using 2-amino-1(4-methoxy)phenylethanol and purified by column chromatography using solvent AcOEt/hexane 3:2 (26% yield). Yellow solid, single spot on TLC, R_f = 0.56 (AcOEt/hexane 1:1), mp 128-129 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 528 (M+1). Anal: calcd. for $C_{28}H_{37}N_3O_7$, 527; C, 63.74; H, 7.07; N, 7.96. Found, C, 63.44; H, 7.08; N, 7.82.

EXAMPLE PKC80

Z-Leu-Abu-CONH-CH_2CH(OH)C_6H_2(2,4,6-$(OCH_3)_3$). This compound was synthesized using 2-amino-1(2,4,6-trimethoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 followed by $CHCl_3/CH_3OH$ 10:1 (29% yield). Yellow solid, single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 10:1), mp 170-172 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 588 (90%, M+1), 570 (100%,

M+1-18). Anal: calcd. for $C_{30}H_{41}N_3O_9$, 587; C, 61.31; H, 7.03; N, 7.15. Found, C, 60.86; H, 7.29; N, 6.95.

EXAMPLE PKC81

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4(4\text{-}N(CH_3)_2)$. This compound was synthesized using 2-amino-1(4-dimethylamino)phenylethanol and purified by column chromatography using solvent AcOEt/hexane 6:1 (23% yield). Yellow solid, single spot on TLC, R_f = 0.41 (AcOEt/hexane 6:1), mp 104 ⁞C (dec.). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 523 (M+1-18). Anal: calca. for $C_{29}H_{40}N_4O_6$, 540; C, 64.42; H, 7.45; N, 10.36. Found, C, 64.27, H, 7.42; N, 10.34.

EXAMPLE PKC82

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6F_5$. This compound was synthesized using 2-amino-1-pentafluorophenylethanol and purified by column chromatography using solvent $CHCL_3/CH_3OH$ 10:1 (66% yield). White solid, single spot on TLC, R_f = 0.28 ($CHCl_3/CH_3OH$ 10:1), mp 167-171 ⁞C. ^1H NMR (DMSO-d6) ok, MS (FAB) m/e = 570 (M+1-18). Anal: calcd. for $C_{27}H_{30}N_3O_6F_5$, 587; C, 55.19; H, 5.14; N, 7.15. Found, C, 56.13; H, 5.58; N, 7.20.

EXAMPLE PKC83

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4(3\text{-}CF_3)$. This compound was synthesized using 2-amino-1(3-trifluoromethyl)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (72% yield). Dark yellow semisolid, single spot on TLC, R_f = 0.48 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 566 (M+1). Anal: calcd. for $C_{28}H_{34}N_3O_6F_3$, 565; C, 59.46; H, 6.06; N, 7.42. Found, C, 59.12; H, 6.18; N, 7.14.

EXAMPLE PKC84

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4(3\text{-}OPh)$. This compound was synthesized using 2-amino-1(3-phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (67% yield). Yellow oil, single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 590 (53%, M+1), 572 (100%, M+1-18). Anal: Calcd. for $C_{33}H_{39}N_3O_7$, 589; C, 67.21; H, 6.66; N, 7.12. Found, C, 66.76; H, 6.25; N, 7.06.

EXAMPLE PKC85

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(4-OPh). This compound was synthesized using 2-amino-1(4-phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (48% yield). Yellow semisolid, single spot on TLC, R_f = 0.22 ($CHCl_3/CH_3OH$ 20:1), mp 55-60 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 590 (47%, M+1),572 (100%, M+1-18). Anal: calcd. for $C_{33}H_{39}N_3O_7$, 589; C, 67.21; H, 6.66; N, 7.12. Found, C, 67.30; H, 6.67; N, 7.10.

EXAMPLE PKC86

Z-leu-Abu-CONH-$CH_2CH(OH)C_6H_4$(4-OCH_2Ph). This compound was synthesized using 2-amino-1(4-benzyloxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (39% yield). Yellow solid, single spot on TLC, R_f = 0.40 ($CHCl_3/CH_3OH$ 20:1), mp 59-62 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 604 (M+1). Anal: calcd. for $C_{34}H_{41}N_3O_7$, 603; C, 67.64; H, 6.84; N, 6.96. Found, C, 67.50; H, 6.87; N, 6.90.

EXAMPLE PKC87

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$-3-OC_6H_4(3-CF_3). This compound was synthesized using 2-amino-1(3-(3'-trifluoromethyl)phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (57% yield). Yellow solid, single spot on TLC, R_f = 0.40 ($CHCl_3/CH_3OH$ 30:1), mp 97-101 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 658 (M+1). Anal: calcd. for $C_{34}H_{38}N_3O_7F_3$, 657; C, 62.09; H, 5.82; N, 6.39. Found, C, 62.05; H, 5.84; N, 6.42.

EXAMPLE PKC88

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_4$-3-OC_6H_3(3,4-Cl_2). This compound was synthesized using 2-amino-1(3-(3',4'-dichloro)phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (55% yield). Yellow solid, single spot on TLC, R_f = 0.28 ($CHCl_3/CH_3OH$ 20:1), mp 63-67 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 659 (M+1). Anal: calcd. for $C_{33}H_{37}N_3O_7Cl_2$, 658; C, 60.18; H, 5.66; N, 6.38. Found, C, 59.37; H, 5.12; N, 6.16.

EXAMPLE PKC89

Z-Leu-Abu-CONH-$CH_2CH(OH)C_6H_3$(3,4-(OCH_2Ph)$_2$). This compound was synthesized using 2-amino-1(3,4-dibenzyloxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (60% yield). White solid, single

spot on TLC, R_f = 0.48 ($CHCl_3/CH_3OH$ 10:1), mp 101-104 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 710 (M+1). Anal: calcd. for $C_{41}H_{47}N_3O_8$, 709; C, 69.37; H, 6.67; N, 5.92. Found, C, 68.23; H, 6.70; N, 6.08.

EXAMPLE PKC90

Z-Leu-Abu-CONH-CH_2CH(OH)-1-$C_{10}H_7$. This compound was synthesized using 2-amino-1(1-naphthyl)phenylethanol and purified by column chromatography using solvent AcOEt/hexane 1:1 (15% yield). Pale orange solid, single spot on TLC, R_f = 0.48 (AcOEt/hexane 1:1), mp 63-71 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 548 (M+1). Anal: calcd. for $C_{31}H_{37}N_3O_6$, 547; C, 67.99; H, 6.81; N, 7.67. Found, C, 67.73; H, 7.03; N, 7.40.

EXAMPLE PKC91

Z-Leu-Abu-CONH-CH_2CH(OH)-2-$C_{10}H_7$. This compound was synthesized using 2-amino-1(2-naphthyl)phenylethanol and purified by column chromatography using solvent AcOEt/hexane 3:2 (17% yield). Orange solid, single spot on TLC, R_f = 0.39 (AcOEt/hexane 3:1), mp 137-140 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 548 (M+1). Anal: calcd. for $C_{31}H_{37}N_3O_6$, 547; C, 67.99; H, 6.81; N, 7.67. Found, C, 68.15; H, 6.83; N, 7.43.

EXAMPLE PKC92

Z-Leu-Phe-CONH-CH_2CH(OH)Ph. This compound was synthesized using 2-amino-1-phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (46% yield). White solid, single spot on TLC, R_f = 0.72 ($CHCl_3/CH_3OH$ 10:1), mp 164-166 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 560 (M+1). Anal: calcd. for $C_{32}H_{37}N_3O_6$, 559; C,68.67; H, 6.66; N, 7.51. Found, C, 68.46, H, 6.68, N, 7.50.

EXAMPLE PKC93

Z-Leu-Phe-CONH-CH_2CH(OH)C_6H_4(4-N$(CH_3)_2$). This compound was prepared using 2-amino-1(4-dimethylamino)phenylethanol and purified by column chromatography with solvent $CHCl_3/CH_3OH$ 10:1 (22% yield). Yellow solid, single spot on TLC, R_f = 0.68 ($CHCl_3/CH_3OH$ 10:1), mp 130 ⁰C (dec.). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 603 (35%, M+1), 585 (100%, M+1-18). Anal: calcd. for $C_{34}H_{42}N_4O_6$, 602; C, 67.75, H, 7.02, N, 9.29. Found, C, 66.43; H, 7.06; N, 9.22.

EXAMPLE PKC94

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6F_5$. This compound was prepared using 2-amino-1-pentafluorophenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (47% yield). White solid, single spot on TLC, R_f = 0.45 ($CHCl_3/CH_3OH$ 20:1), mp 191-192 ⁰C. ^1H NMR (DMSO-d_6) ok, MS (FAB) m/e = 632 (100%, M+1-18). Anal: calcd. for $C_{32}H_{32}N_3O_6F_5$, 649; C, 59.16; H, 4.96; N, 6.46. Found, C, 61.18; H, 5.37; N, 6.68.

EXAMPLE PKC95

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(3-CF_3). This compound was prepared using 2-amino-1(3-trifluoromethyl)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (42% yield). Dark yellow semisolid, single spot on TLC, R_f = 0.48 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 628 (M+1). Anal: calcd. for $C_{33}H_{36}N_3O_6F_3$, 627; C, 63.15; H, 5.78; N, 6.69. Found, C, 63.24; H, 5.82; N, 6.65.

EXAMPLE PKC96

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(3-OPh). This compound was prepared using 2-amino-1(3-phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (50% yield). Yellow semisolid, single spot on TLC, R_f = 0.25 ($CHCl_3/CH_3OH$ 20:1). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 652 (M+1). Anal: Calcd. for $C_{38}H_{41}N_3O_7$, 651; C, 70.02; H, 6.34; N, 6.44. Found, 69.67; H, 6.60; N, 6.23.

EXAMPLE PKC97

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(4-OPh). This compound was prepared using 2-amino-1(4-phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (30% yield). Yellow semisolid, single spot on TLC, R_f = 0.20 ($CHCl_3/CH_3OH$ 30:1), mp 146-149 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 652 (25%, M+1), 634 (100 %, M+1-18). Anal: calcd. for $C_{38}H_{41}N_3O_7$, 651; C, 70.02; H, 6.34; N, 6.44. Found, 70.14; H, 6.36; N, 6.38.

EXAMPLE PKC98

Z-Leu-Phe-CONH-$CH_2CH(OH)C_6H_4$(4-OCH_2Ph). This compound was prepared using 2-amino-1(4-benzyloxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (49% yield). Yellow solid, single

spot on TLC, R_f = 0.45 ($CHCl_3/CH_3OH$ 20:1), mp 133-134 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 666 (M+1). Anal: calcd. for $C_{39}H_{43}N_3O_7$, 665; C, 70.35; H, 6.51; N, 6.31. Found, 69.55; H, 6.46; N, 6.25.

EXAMPLE PKC99

Z-Leu-Phe-CONH-CH_2CH(OH)C_6H_4-3-OC_6H_4(3-CF_3). This compound was prepared using 2-amino-1(3-trifluoromethyl)phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (52% yield). Yellow solid, single spot on TLC, R_f = 0.23 ($CHCl_3/CH_3OH$ 20:1), mp 142-143 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 720 (M+1). Anal: calcd. for $C_{39}H_{40}N_3O_7F_3$, 719; C, 65.08; H, 5.60; N, 5.72. Found, C, 64.66; H, 5.58; N, 5.72.

EXAMPLE PKC100

Z-Leu-Phe-CONH-CH_2CH(OH)C_6H_4-3-OC_6H_3(3,4-Cl_2). This compound was prepared using 2-amino-1(3-(3',4'-dichloro)phenoxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (41% yield). Yellow solid, single spot on TLC, R_f = 0.40 ($CHCl_3/CH_3OH$ 20:1), mp 136-137 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 721 (M+1). Anal: calcd. for $C_{38}H_{39}N_3O_7Cl_2$, 720; C, 63.33; H, 5.45; N, 5.83. Found, C, 62.78; H, 5.09; N, 5.42.

EXAMPLE PKC101

Z-Leu-Phe-CONH-CH_2CH(OH)C_6H_3(3,4-(OCH_2Ph)$_2$). This compound was prepared using 2-amino-1(3,4-dibenzyloxy)phenylethanol and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (45% yield). Yellow solid, single spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 20:1), mp 149-152 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 772 (M+1). Anal: calcd. for $C_{46}H_{49}N_3O_8$, 771; C, 71.57; H, 6.39; N, 5.44. Found, C, 71.33; H, 6.45; N, 5.41.

EXAMPLE PKC102

Z-Leu-Abu-CONH-CH_2-2-Furyl. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 2-furfurylamine by the procedure described in Example PKC67, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (43% yield). White solid, single spot on TLC, R_f = 0.68 ($CHCl_3/CH_3OH$ 10:1), mp 138-139 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 458 (M+1). Anal: calcd. for $C_{24}H_{31}N_3O_6$, 457; C, 63.00; H, 6.83; N, 9.18. Found, C, 62.22; H, 6.72; N, 9.00.

EXAMPLE PKC103

Z-Leu-Abu-CONH-CH_2-2-Tetrahydrofuryl. This compound was synthesized using 2-tetrahydrofurfurylamine and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (35% yield). Yellow solid, single spot on TLC, R_f = 0.59 ($CHCl_3/CH_3OH$ 20:1), mp 126-128 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 462 (M+1). Anal: calcd. for $C_{24}H_{35}N_3O_6$, 461; C, 62.45; H, 7.64; N, 9.10. Found, C, 62.37; H, 7.63; N, 9.19.

EXAMPLE PKC104

Z-Leu-Abu-CONH-CH_2-2-Pyridyl. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 2-aminomethylpyridine. After reacting overnight at r.t., to the mixture was added AcOEt (25 mL) and white precipitate was filtered. The filtrate was washed with water (3 x 10 mL), saturated NaCl (2 x 10 mL) and dried over $MgSO_4$. After evaporation of the solvent, chromatography on a silica gel column using solvent $CHCl_3/CH_3OH$ 10:1 followed by precipitation from AcOEt/hexane afforded a yellow solid (50% yield). Single spot on TLC, R_f = 0.50 ($CHCl_3/CH_3OH$ 10:1), mp 117-119 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 469 (M+1). Anal: calcd. for $C_{25}H_32N_4O_5$, 468; C, 64.08; H, 6.88; N, 11.96. Found, C, 63.93; H, 6.86; N, 11.85.

EXAMPLE PKC105

Z-Leu-Abu-CONH-CH_2-3-Pyridyl. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 3-aminomethylpyridine by the procedure described in Example PKC104, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (35% yield). Yellow solid, single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 10:1), mp 122-123 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 469 (M+1). Anal: calcd. for $C_{25}H_{32}N_4O_5$, 468; C, 64.08; H, 6.88; N, 11.96. Found, C, 63.98; H, 6.91; N, 11.97.

EXAMPLE PKC106

Z-Leu-Abu-CONH-CH_2-4-Pyridyl. This compound was synthesized using 4-aminomethyl-pyridine and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (45% yield). Yellow solid, single spot on TLC, R_f = 0.55 ($CHCl_3/CH_3OH$ 10:1), mp 124-126 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 469

(M+1). Anal: calcd. for $C_{25}H_{32}N_4O_5$, 468; C, 64.08; H, 6.88; N, 11.96. Found, C, 63.88; H, 6.87; N, 11.96.

EXAMPLE PKC107

Z-Leu-Abu-CONH-$(CH_2)_2$-2-Pyridyl. This compound was synthesized using 2-(2-aminoethyl)pyridine and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (53% yield). Yellow solid, single spot on TLC, R_f = 0.60 ($CHCl_3/CH_3OH$ 10:1), mp 128-130 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 483 (M+1). Anal: calcd. for $C_{26}H_{34}N_4O_5$, 482; C, 64.71; H, 7.10; N, 11.61. Found, C, 64.04; H, 7.05; N, 11.49.

EXAMPLE PKC108

Z-Leu-Abu-CONH-$(CH_2)_2$-2-(N-Methylpyrrole). This compound was synthesized from protected Z-Leu-Abu-COOEt and 2(2-aminoethyl)-1-methylpyrrole by the procedure described in Example PKC104, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (16% yield). Orange semisolid, single spot on TLC, R_f = 0.34 ($CHCl_3/CH_3OH$ 30:1), mp 120-123 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 485 (M+1). Anal: calcd. for $C_{26}H_{36}N_4O_5$, 484; C, 64.44; H, 7.48; N, 11.56. Found, C, 64.02; H, 7.26; N, 11.21.

EXAMPLE PKC109

Z-Leu-Abu-CONH-(CH_2)3-1-Imidazolyl. his compound was synthesized using 1(3-aminopropyl)imidazole by the procedure described in Example PKC104, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (27% yield). Yellow semisolid, single spot on TLC, R_f = 0.33 ($CHCl_3/CH_3OH$ 10:1), mp 52-55 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 486 (M+1). Anal: calcd. for $C_{25}H_{35}N_5O_5$, 485; C, 61.83; H, 7.26; N, 14.42. Found, C, 60.90; H, 7.21; N, 13.87.

EXAMPLE PKC110

Z-Leu-Abu-CONH-$(CH_2)_2$-4-Morpholinyl. This compound was synthesized using 4-(2-aminoethyl)morpholine and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (55% yield). Yellow semisolid, single spot on TLC, R_f = 0.49 ($CHCl_3/CH_3OH$ 10:1), mp 124-126 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 491 (M+1). Anal: calcd. for $C_{25}H_{38}N_4O_6$, 490; C, 61.15; H, 7.81; N, 11.42. Found, C, 61,08; H, 7.86; N, 11.34.

EXAMPLE PKC111

Z-Leu-Abu-CONH-(CH_2)3-4-Morpholinyl. This compound was synthesized using 4-(3-aminopropyl)morpholine and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (42% yield). Yellow semisolid, single spot on TLC, R_f = 0.50 ($CHCl_3/CH_3OH$ 10:1), mp 125-126 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 505 (M+1). Anal: calcd. for $C_{26}H_{40}N_4O_6$, 504; C, 61.88; H, 7.99; N, 11.10. Found, C, 61,69; H, 7.95; N, 11.07.

EXAMPLE PKC112

Z-Leu-Abu-CONH-(CH_2)3-1-Pyrrolidinyl-2-one. This compound was prepared from Z-Leu-Abu-COOH and 1-(3-aminopropyl)2-pyrrolidinone, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (33% yield). White semisolid, single spot on TLC, R_f = 0.51 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB), m/e = 503 (M+1). Anal: calcd. for $C_26H_{38}N_4O_6$, 502; C, 62.13; H, 7.62; N, 11.14. Found, C, 62.02; H, 7.71; N, 10.52.

EXAMPLE PKC113

Z-Leu-Abu-CONH-(CH_2)$_2$-3-Indolyl. This compound was prepared from Z-Leu-Abu-COOH and 3-(2-aminoethyl)indole and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (18% yield). White semisolid, single spot on TLC, R_f = 0.47 ($CHCl_3/CH_3OH$ 30:1). ^1H NMR ($CDCl_3$) ok, MS (exact FAB), m/e = 521 2745.

EXAMPLE PKC114

Z-Leu-Abu-CONH-CH_2-2-Quinolinyl. This compound was prepared from 1,3-dithiolane derivative of Z-Leu-Abu-COOEt and 2-aminomethylquinoline by the procedure described in Example PKC104, and purified by column chromatography using solvent AcOEt/hexane 2:1 (16% yield). Yellow solid, single spot on TLC, R_f = 0.27 (AcOEt/hexane 2:1), mp 135-138 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 519 (M+1). Anal: calcd. for $C_{29}H_{34}N_4O_5$, 518; C, 67.16; H, 6.60; N, 10.80. Found, C, 66.89; H, 6.68; N, 10.61.

EXAMPLE PKC115

Z-Leu-Abu-CONH-CH_2-1-Isoquinolinyl. This compound was prepared using 1-aminomethylisoquinoline and purified by column chromatography using solvent AcOEt/hexane 2:1 (12% yield). Yellow solid, single spot on TLC, R_f = 0.34

(AcOEt/hexane1:1), mp 121-125 ⁰C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 519 (M+1). Anal: calcd. for $C_{29}H_{34}N_4O_5$, 518; C, 67.16; H, 6.60; N, 10.80. Found, C, 67.11; H, 6.61; N, 10.83.

EXAMPLE PKC116

Z-Leu-Abu-CONH-(CH_2)3-1-Tetrahydroquinolinyl. This compound was synthesized using N-aminopropyltetraquinoline and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (40% yield). Oil, single spot on TLC, R_f = 0.26 ($CHCl_3/CH_3OH$ 20:1). ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 551 (M+1). Anal: calcd. for $C_{31}H_{42}N_4O_5$, 550; C, 67.61; H, 7.69; N, 10.17. Found, C, 67.15; H, 7.42; N, 10.02.

EXAMPLE PKC117

Z-Leu-Abu-CONH-(CH_2)3-2-Tetrahydroisoquinolinyl. This compound was synthesized using N-aminopropylisotetraquinoline and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (20% yield). Yellow semisolid, single spot on TLC, R_f = 0.51 ($CHCl_3/CH_3OH$ 20:1). ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 551 (M+1). Anal: calcd. for $C_{31}H_{42}N_4O_5$, 550; C, 67.61; H, 7.69; N, 10.17. Found, C, 67.23; H, 7.32; N, 9.98.

EXAMPLE PKC118

Z-Leu-Abu-CONH-CH_2-8-Caffeine. This compound was synthesized using 8-aminomethylcaffeine and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (30% yield). Yellow solid, single spot on TLC, R_f = 0.35 ($CHCl_3/CH_3OH$ 10:1), mp 171-177 ⁰C (dec.). ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 556 (16%, M+1-28), 471 (100%, M+1-113). Anal: calcd. for $C_{28}H_{37}N_7O_7$, 583; C, 57.62; H, 6.39; N, 16.79. Found, C, 57.70; H, 6.48; N, 16.69.

EXAMPLE PKC119

Z-Leu-Abu-CONH-CH_2-2-(4-Methyl-2-thiazolyl). This compound was prepared using synthesized 2-aminomethyl-4-methylthiazole and purified by column chromatography using solvent AcOEt/hexane 6:1 (26% yield). Orange semisolid, single spot on TLC, R_f = 0.40 (AcOEt/hexane 6:1). ^{1}H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{24}H_{32}N_4O_5S$, 488) m/e = 489 (3%, M+1), 376 (100%, M+1-113).

EXAMPLE PKC120

Z-Leu-Abu-CONH-$(CH_2)_2$NH-Biotinyl. This compound was prepared from Z-Leu-Abu-COOH and biotinylethylenediamine hydrochloride. Biotin (1 g, 4.1 mmole) was dissolved in 20 mL of DMF at 70 _C and cooled to 40 _C, CDI (0.97 g, 6 mmole) in 3 mL of DMF was then added and white precipitate were appeared. After stirring at r.t. for two hours, ethylenediamine (1.34 mL, 20 mmole) in 10 mL of DMF was added and stirred for another 3 hours. After evaporating DMF, the semisolid residue was dissolved in 50 mL of refluxed methanol and the unreacted biotin was removed by filtration. The solution was evaporated to dryness, the residue was washed with $CHCl_3$ to remove the imidazole, dissolved in 6 mL of water, acidified to pH 3.0 with 1N HCl, and evaporated to dryness. The crude product was crystallized from methanol to give 1.04 g of biotinylethylenediamine hydrochloride (79% yield). Long spot on TLC, R_f = 0.21 (butanol:AcOH:H_2O = 4:1:1), mp 241-242 _C. ^1H NMR is consistent with the structure.

To a stirred solution of Z-Leu-Abu-COOH (0.6 g, 1.58 mmol) in DMF (15 mL) was added HOBt (0.22 g, 1.58 mmol), DCC (0.49 g, 2.38 mmol), and stirring continued for 2 hours at r.t.(mixture A). To a stirred solution of biotinylethylenediamine hydrochloride (0.6 g, 1.85 mmol) in DMF (10 mL) was added TEA (0.28 mL, 2.03 mmol) at 0-5 ⁞C and stirred for 2 hours at r.t.(mixture B). To the stirred mixture A was added mixture B and stirred 3 days. After filtering, the filtrate was evaporated to get a semisolid which was washed with H_2O (30 mL), 1M HCl (30 mL), H_2O (30 mL) and dried under vacuum. Chromatography on a silica gel column using solvent $CHCl_3/CH_3OH$ 5:1 afforded a yellow solid (42 % yield). Long spot on TLC, R_f = 0.41 ($CHCl_3/CH_3OH$ 5:1), mp 188-192 ⁞C (dec.). ^1H NMR (DMSO-d_6) ok, MS (FAB) m/e = 647 (M+1). Anal: calcd. for $C_{31}H_{46}N_6O_7S$, 646; C, 57.56; H, 7.17; N, 12.99. Found, C, 57.04; H, 7.21; N, 13.29.

EXAMPLE PKC121

Z-Leu-Abu-CONH-CH_2-3-Pyridyl-N-oxide. This compound was prepared from Z-Leu-Abu-COOH and 3-aminomethylpyridine-N-oxide, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (15% yield). Yellow oil, long spot on TLC, R_f = 0.40 ($CHCl_3/CH_3OH$ 5:1). ^1H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{25}H_{32}N_4O_6$, 484) m/e = 485 (2%, M+1), 372 (100%, M+1-113).

EXAMPLE PKC122

Z-Leu-Abu-CONH-CH_2-6-Uracil. This compound was prepared from Z-Leu-Abu-COOH and 6-aminomethyluracil and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (1.5% yield). Brown oil, long spot on TLC, R_f = 0.28 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{24}H_{31}N_5O_7$, 501) m/e = 389 (100%, M+1-113).

EXAMPLE PKC123

Z-Leu-Phe-CONH-CH_2-2-Pyridyl. This compound was prepared from 1,3-dithiolane derivative of Z-Leu-Phe-COOEt and 2-aminomethylpyridine by the procedure described in Example PKC104, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1(41% yield) . Yellow solid, long spot on TLC, R_f = 0.40 ($CHCl_3/CH_3OH$ 20:1), mp 144-146 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 531 (M+1). Anal: calcd. for $C_{30}H_{34}N_4O_5$, 530; C, 67.91; H, 6.46; N, 10.56. Found, C, 67.64; H, 6.50; N, 10.64.

EXAMPLE PKC124

Z-Leu-Phe-CONH-(CH_2)3-4-Morpholinyl. This compound was prepared from 1,3-dithiolane derivative of Z-Leu-Phe-COOEt and 4-(3-aminopropyl)morpholine, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1(40% yield) . Yellow solid, long spot on TLC, R_f = 0.55 ($CHCl_3/CH_3OH$ 10:1), mp 155-156 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 581 (M+1). Anal: calcd. for $C_{31}H_{42}N_4O_6$, 566; C, 65.70; H, 7.47; N, 9.89. Found, C, 65.64; H, 7.49; N, 9.84.

EXAMPLE PKC125

Z-Leu-Phe-CONH-CH_2-2-Quinolinyl. This compound was prepared using 2-aminomethylquinoline and purified by column chromatography using solvent AcOEt/hexane 1:1 (33% yield) . Yellow solid, long spot on TLC, R_f = 0.30 (AcOEt/hexane 1:1), mp 131-135 °C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 581 (M+1). Anal: calcd. for $C_{34}H_{36}N_4O_5$, 580; C, 70.32; H, 6.25; N, 9.65. Found, C, 70.31; H, 6.27; N, 9.63.

EXAMPLE PKC126

Z-Leu-Phe-CONH-CH_2-1-Isoquinolinyl. This compound was prepared using 1-aminomethylisoquinoline and purified by column chromatography using solvent AcOEt/hexane 1:1 (7% yield). Yellow solid, single spot on TLC, R_f = 0.45

(AcOEt/hexane 1:1), mp 169-173 ⁰C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 581 (M+1). Anal: calcd. for $C_{34}H_{36}N_4O_5$, 580; C, 70.32; H, 6.25; N, 9.65. Found, C, 70.05; H, 6.29; N, 9.47.

EXAMPLE PKC127

Z-Leu-Phe-CONH-(CH_2)3-1-Tetrahydroquinolinyl. This compound was prepared using N-aminopropyltetraquinoline and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (40% yield). Yellow solid, single spot on TLC, R_f = 0.58 ($CHCl_3/CH_3OH$ 20:1), mp 115-120 ⁰C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 613 (M+1). Anal: calcd. for $C_{36}H_{44}N_4O_5$, 612; C, 70.56; H, 7.24; N, 9.14. Found, C, 70.46; H, 7.26; N, 9.19.

EXAMPLE PKC128

Z-Leu-Phe-CONH-(CH_2)3-2-Tetrahydroisoquinolinyl. This compound was prepared using N-aminopropylisotetraquinoline and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 20:1 (51% yield). Yellow solid, single spot on TLC, R_f = 0.62 ($CHCl_3/CH_3OH$ 10:1), mp 107-111 ⁰C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 613 (M+1). Anal: calcd. for $C_{36}H_{44}N_4O_5$, 612; C, 70.56; H, 7.24; N, 9.14. Found, C, 69.61; H, 7.25; N, 9.05.

EXAMPLE PKC129

Z-Leu-Phe-CONH-(CH_2)$_2$NH-biotinyl. This compound was prepared from Z-Leu-Phe-COOH and synthesized biotinylethylenediamine hydrochloride by the procedure described for Example PKC120, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 5:1 (35% yield). White solid, long spot on TLC, R_f = 0.42 ($CHCl_3/CH_3OH$ 5:1), mp 204-206 ⁰C (dec.). ^{1}H NMR (DMSO-d_6) ok, MS (FAB) m/e = 709 (M+1). Anal: calcd. for $C_{36}H_{48}N_6O_7S$, 708; C, 60.99; H, 6.82; N, 11.85. Found, C, 61.03; H, 6.83; N, 11.77.

EXAMPLE PKC130

Z-Leu-Nva-CONH-CH_2CH(OH)Ph. This compound was synthesized from 1,3-dithiolane derivative of Z-Leu-Nva-COOEt and 2-amino-1-phenylethanol by the procedure described in Example PKC67, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (54% yield). White solid, single spot on TLC, R_f = 0.56 ($CHCl_3/CH_3OH$ 10:1), mp 75-77 ⁰C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_28H_{37}N_3O_6$, 511) m/e = 512 (M+1).

EXAMPLE PKC131

Z-Leu-Nva-CONH-CH_2-2-Pyridyl. This compound was prepared from 1,3-dithiolane derivative of Z-Leu-Nva-COOEt and 2-aminomethylpyridine by the procedure described in Example PKC104, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1(50% yield) . Yellow solid, long spot on TLC, R_f = 0.55 ($CHCl_3/CH_3OH$ 10:1), mp 65-70 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{26}H_{34}N_4O_5$,482) m/e = 483 (M+1).

EXAMPLE PKC132

Z-Leu-Nva-CONH-(CH_2)3-4-Morpholinyl. This compound was prepared from 1,3-dithiolane derivative of Z-Leu-Nva-COOEt and 4-(3-aminopropyl)morpholine, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1(37% yield) . Yellow solid, long spot on TLC, R_f = 0.23 ($CHCl_3/CH_3OH$ 10:1), mp 108-110 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{27}H_{42}N_4O_6$, 518) m/e = 519 (M+1).

EXAMPLE PKC133

$CH_3OCO(CH_2)_2CO$-Leu-Abu-CONHEt. To a solid Z-Leu-Abu-CONHEt (1 g, 2.47 mmol) was added a solution of hydrogen bromide in acetic acid (30 wt%, 1.52 mL, 7.40 mmol) at r.t. The mixture was vigorously stirred for 1 hour during this time all of the ketoamide dissolved in acetic acid. The reaction was quenched with Et_2O (30 mL) then separated. The semisolid was triturated and washed successively with Et2O (5 x 30 mL). After removing solvent, the residue was dried under vacuum, leaving a very hydroscopic solid. ^1H NMR ($CDCl_3$) showed loss of Z group. The yield was 70-80%.

To a stirred solution of mono-methylsuccinate (0.28 g, 2.13 mmol) in DMF (10 mL) was added DCC (0.44 g, 2.13 mmol) and HOBt (0.29 g, 2.13 mmol). The mixture was stirred for 2 hours at r.t.(mixture A). To a stirred solution of Leu-Abu-CONHEt.HBr (0.5 g, 1.42 mmol) in DMF (5 mL) was added TEA (0.2 mL, 1.42 mmol) at 0-5 ⁰C and stirred for 30 min (mixture B). To the stirred mixture B was added mixture A at 0-5 ⁰C and the reaction was stirred overnight at r.t. After evaporation of the solvent, AcOEt (40 mL) was added, the precipitate was filtered, the filtrate was washed with 0.25 N HCl (10 mL), H_2O (20 mL), 10% Na_2CO_3 (3 x 20 mL), H_2O (20 mL), satd. NaCl (2 x 20 mL), dried over $MgSO_4$, and concentrated. Chromatography on a silica gel column with solvent $CHCl_3/CH_3OH$ 10:1 afforded a

yellow semisolid (42% yield). Single spot on TLC, R_f = 0.43 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{18}H_{31}N_3O_6$, 385) m/e = 386 (M+1).

EXAMPLE PKC134

2-Furyl-CO-Leu-Abu-CONHEt. This compound was synthesized using 2-furoic acid by the procedure described for compound 67 and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (39% yield). Yellow solid, single spot on TLC, R_f = 0.51 ($CHCl_3/CH_3OH$ 10:1), mp 58-59 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 366 (M+1). Anal: calcd. for $C_{18}H_{27}N_3O_5$, 365; C, 59.16; H, 7.44; N, 11.50. Found, C, 58.12; H, 7.53; N, 11.64.

EXAMPLE PKC135

2-Tetrahydrofuryl-CO-Leu-Abu-CONHEt. This compound was synthesized using 2-tetrahydrofuroic acid and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (41% yield). Yellow oil, single spot on TLC, R_f = 0.54 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{18}H_{31}N_3O_5$, 369) m/e = 370 (M+1).

EXAMPLE PKC136

3-Pyridyl-CO-Leu-Abu-CONHEt. This compound was synthesized using nicotinic acid and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (49% yield). Yellow solid, single spot on TLC, R_f = 0.56 ($CHCl_3/CH_3OH$ 10:1), mp 57-61 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 377 (M+1). Anal: calcd. for $C_{19}H_{28}N_4O_4$, 376; C, 60.58; H, 7.49; N, 14.92. Found, C, 60.05; H, 7.51; N, 14.58.

EXAMPLE PKC137

2-Pyrazinyl-CO-Leu-Abu-CONHEt. This compound was synthesized using 2-pyrazinecarboxylic acid and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (18% yield). Yellow solid, single spot on TLC, R_f = 0.33 ($CHCl_3/CH_3OH$ 10:1), mp 51-56 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 378 (M+1). Anal: calcd. for $C_{18}H_{27}N_5O_4$, 377; C, 57.29; H, 7.16; N, 18.56. Found, C, 56.74; H, 7.28; N, 18.32.

EXAMPLE PKC138

2-Quinolinyl-CO-Leu-Abu-CONHEt. This compound was synthesized using quinaldic acid and purified by column chromatography using solvent AcOEt/hexane 1:1 (45% yield). Orange solid, single spot on TLC, R_f = 0.48 (AcOEt/hexane 1:1), mp

56-59 °C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 427 (M+1). Anal: calcd. for $C_{23}H_{30}N_4O_4$, 426; C, 64.79; H, 7.09; N, 13.13. Found, C, 64.98; H, 7.45; N, 12.48.

EXAMPLE PKC139

1-Isoquinolinyl-CO-Leu-Abu-CONHEt. This compound was synthesized using 1-isoquinoline carboxylic acid and purified by column chromatography with solvent AcOEt/hexane 1:1 (46% yield). Red solid, single spot on TLC, R_f = 0.47 (AcOEt/hexane 1:1), mp 104-106 °C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 427 (M+1). Anal: calcd. for $C_{23}H_{30}N_4O_4$, 426; C, 64.79; H, 7.09; N, 13.13. Found, C, 65.00; H, 7.31; N, 12.96.

EXAMPLE PKC140

4-Morpholinyl-CO-Leu-Abu-CONHEt. This compound was synthesized from 4-morpholinecarbonyl chloride (1 mmol), Leu-AbuCONH-EtHBr (1 mmol) and TEA (2.5 mmol), and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (33% yield). Yellow oil, single spot on TLC, R_f = 0.45 ($CHCl_3/CH_3OH$ 10:1). ^{1}H NMR ($CDCl_3$) ok, MS (FAB, calcd. for $C_{18}H_{32}N_4O_5$, 384) m/e = 385 (M+1).

EXAMPLE PKC141

$Ph(CH_2)_2CO$-Leu-Abu-CONHEt. This compound was synthesized from 1,3-dithiolane derivative of $Ph(CH_2)_2CO$-Leu-Abu-COOEt and $EtNH_2$, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (72% yield). Yellow solid, single spot on TLC, R_f = 0.23 ($CHCl_3/CH_3OH$ 30:1), mp 134-136 °C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 404 (M+1). Anal: calcd. for $C_{22}H_{33}N_3O_4$, 403; C, 65.48; H, 8.24; N, 9.60. Found, C, 65.52; H, 8.30; N, 9.42.

EXAMPLE PKC142

1-$C_{10}H_7CH_2CO$-Leu-Abu-CONHEt. This compound was synthesized from 1,3-dithiolane derivative of 1-$C_{10}H_7CO$-Leu-Abu-COOEt and $EtNH_2$, and purified by column chromatography using solvent $CHCl_3/CH_3OH$ 30:1 (67% yield). Yellow solid, single spot on TLC, R_f = 0.47 ($CHCl_3/CH_3OH$ 30:1), mp 201-203 °C. ^{1}H NMR ($CDCl_3$) ok, MS (FAB) m/e = 440 (M+1). Anal: calcd. for $C_{25}H_{33}N_3O_4$, 439; C, 68.31; H, 7.57; N, 9.56. Found, C, 68.19; H, 7.52; N, 9.49.

EXAMPLE PKC143

Ph_2CHCO-Leu-Abu-CONHEt. This compound was synthesized from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Abu-COOEt and $EtNH_2$, and purified by

column chromatography using solvent $CHCl_3/CH_3OH$ 10:1 (24% yield). Yellow solid, single spot on TLC, R_f = 0.40 ($CHCl_3/CH_3OH$ 10:1), mp 78-83 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 467 (M+1). Anal: calcd. for $C_{27}H_{35}N_3O_4$, 466; C, 69.65; H, 7.58; N, 9.02. Found, C, 70.04; H, 7.72; N, 8.72.

EXAMPLE PKC144

Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)$Ph. This compound was synthesized from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Abu-COOEt and 2-amino-1-phenylethanol, and purified by column chromatography using $CHCl_3$ followed by solvent $CHCl_3/CH_3OH$ 30:1 (30% yield). Yellow solid, single spot on TLC, R_f =0.40 (AcOEt/hexane 1:1), mp 178-180 ⁰C. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 558 (M+1). Anal: calcd. for $C_{33}H_{39}N_3O_5$, 557; C, 71.07; H, 7.05; N, 7.53. Found, C, 70.93; H, 7.10; N, 7.46.

EXAMPLE PKC145

Ph_2CHCO-Leu-Abu-CONH-2-CH_2-Pyridyl. This compound was prepared from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Abu-COOEt and 2-aminomethylpyridine, and purified by column chromatography using $CHCl_3$ following by solvent $CHCl_3$/AcOEt 7:3 (9% yield), mp 161-163. Yellow solid, single spot on TLC, R_f = 0.30 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 529 (M+1). Anal: calcd. for $C_{31}H_{36}N_4O_4$, 528; C, 70.43; H, 6.86; N, 10.60. Found, C, 70.42; H, 6.91; N, 10.47.

EXAMPLE PKC146

Ph_2CHCO-Leu-Abu-CONH-N-(CH_2)3-Morpholinyl. This compound was prepared from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Abu-COOEt and N-aminopropylmorpholine, and purified by column chromatography using $CHCl_3$ followed by solvent $CHCl_3$/AcOEt 7:3 (25 % yield), mp 170-174. Yellow solid, single spot on TLC, R_f = 0.25 ($CHCl_3/CH_3OH$ 10:1). ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 565 (M+1). Anal: calcd. for $C_{32}H_{44}N_4O_5$, 564; C, 68.06; H, 7.85; N, 9.92. Found, C, 67.22; H, 7.77; N, 9.75.

EXAMPLE PKC147

Ph_2CHCO-Leu-Phe-CONH-$CH_2CH(OH)$Ph. This compound was prepared from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Phe-COOEt and 2-amino-1-phenylethanol, and purified by crystallization from $CHCl_3$/ether (16% yield).

Yellow solid, single spot on TLC, R_f = 0.41 (AcOET/CH_3OH 9:1), mp 192-196 ℃. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 620 (M+1). Anal: calcd. for $C_{38}H_{41}N_3O_5$, 619; C, 73.64; H, 6.67; N, 6.78. Found, C, 72.00; H, 6.62; N, 6.41.

EXAMPLE PKC148

Ph_2CHCO-Leu-Phe-CONH-CH_2-2-Pyridyl. This compound was synthesized from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Phe-COOEt and 2-aminomethylpyridine, and purified by column chromatography using $CHCl_3$ following by solvent $CHCl_3$/AcOEt 9:1 (9% yield). Yellow solid, single spot on TLC, R_f = 0.33 (AcOET/CH_3OH 9:1), mp 160-162 ℃. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 591 (M+1). Anal: calcd. for $C_{36}H_{38}N_4O_4$, 590; C, 73.20; H, 6.48; N, 9.48. Found, C, 69.91; H, 6.29; N, 8.98.

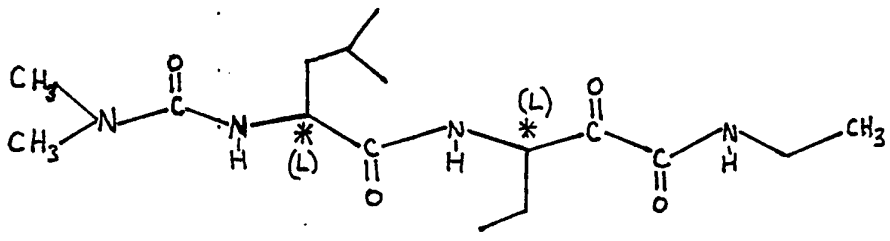
EXAMPLE PKC149

Ph_2CHCO-Leu-Phe-CONH-(CH_2)3-4-Morpholinyl. This compound was synthesized from 1,3-dithiolane derivative of Ph_2CHCO-Leu-Phe-COOEt and N-aminopropylmorpholine, and purified by column chromatography using AcOEt following by crystallization from AcOEt/ether (20 % yield). Yellow solid, single spot on TLC, R_f = 0.45 (AcOET/CH_3OH 9:1), mp 158-160 ℃. ^1H NMR ($CDCl_3$) ok, MS (FAB) m/e = 627 (M+1). Anal: calcd. for $C_{37}H_{46}N_4O_5$, 626; C, 70.90; H, 7.40; N, 8.94. Found, C, 70.05; H, 7.43; N, 8.68.

A variety of techniques for certain synthetic steps in the synthesis of the Peptide Keto-Compounds can be used. Additional synthetic procedures are provided in the following two Examples.

EXAMPLE PKC150

Dimethylurea-(L)-Leu-(L)-Abu-CONH-Et. The structure of Dimethylurea-(L)-Leu-(L)-Abu-CONH-Et is shown below:



This compound was produced through synthesis of the reactant Dimethylurea-(L)-Leu-(L)-Abu hydroxy ethyl amide.

(L)-Leucine (1.31 g (10 mmoles)) was placed in a 3 neck round-bottomed flask, equipped with two pressure equalizing dropping funnels. 12.5 mL of 1.0 N NaOH (12.5 mmoles) was added to the flask and then the mixture was cooled on ice, 12.5 mL of 1.0 N NaOH was placed in one dropping funnel and 1.15 mL (12.5 mmoles) of dimethylcarbamoyl chloride was placed in the other. The contents of the addition funnels were added to the flask simultaneously over ten minutes. The mixture was allowed to react for an additional fifteen minutes. The reaction was then washed twice with 15 mL of ethyl acetate. The aqueous layer was cooled on ice and acidified to a pH of 2 with 1.0 N HCl. The aqueous layer was extracted three times with 15 mL of ethyl acetate. The combined organics were dried over magnesium sulfate, filtered and concentrated in vacuo. There remained 0.10 g of a white solid (5%) which possessed an Rf value of 0.31 using 91:8:1 chloroform:methanol:acetic acid as the eluent.

Boc-Abu hydroxy ethyl amide (0.233 g, 0.894 mmoles) was dissolved in 5 mL of dioxane followed by the addition of 20 mL of 4N HCl/dioxane. The reaction mixture was allowed to react for two hours. After this time, the reaction mixture was concentrated in vacuo and used immediately in the next step. The HCl·Abu hydroxy ethyl amide was dissolved in 30 mL of anhydrous DMF and cooled on an ice bath for ten minutes. To this solution was added 0.217 g (1.07 mmoles) of morpholineleucine urea, 0.46 mL (2.68 mmoles) of diisopropylethylamine and 0.133 g (0.984 mmoles) of 1-hydroxybenzotriazole (HOBt) and allowed to equilibrate for thirty minutes. After this time, 0.188 g (0.984 mmoles) of EDC suspended in 10 mL of anhydrous DMF was added and the reaction mixture was allowed to react overnight. The reaction mixture was concentrated in vacuo and the resulting residue was purified by silica gel column chromatography employing 90:10 chloroform:methanol as the eluent. There remained 0.2044 g (66.56% yield) of a white solid with Rf value of 0.38 in the solvent system detailed above.

0.100 g (0.291 mmoles) of Dimethylurea-Leu-Abu hydroxy ethyl amide was dissolved in 10 mL of methylene chloride and cooled in an ice bath. To this mixture was added 0.487 mg (.003 mmoles) of 2,2,6,6-tetramethyl-1-piperidinyloxy, free radical (TEMPO) and .014 mL (0.291 mmoles) of an aqueous KBr solution (5.95 g of KBr

dissolved in 25 mL of water). The reaction mix was stirred vigorously while four 80 microliter portions of a 1M aqueous sodium hypochlorite (pH 9.5) were added at 15 minute intervals. After this time the reaction mixture was analyzed by TLC employing 90:10 chloroform:methanol to check for completeness of the reaction. If the reaction was not complete another portion of TEMPO and another regimen of the sodium hypochlorite solution should be added. This reaction required three additional regimens of TEMPO and sodium hypochlorite.

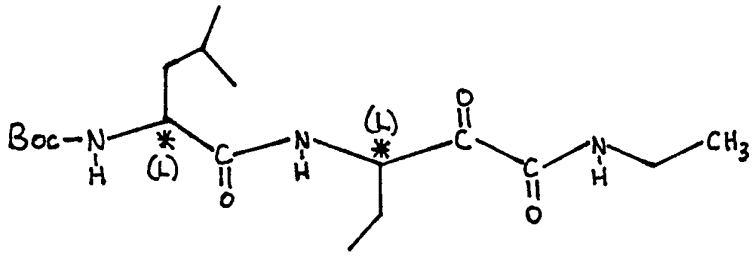
When the reaction was deemed complete by TLC, the layers were separated. The aqueous layer was extracted with methylene chloride (3 x 10 mL). The combined organic layer was washed with 10% HCl (1 x 10 mL), 30 mL of a 100 mL stock solution of 10% HCl containing 1.6 g of KI, 10% sodium thiosulfate (2 x 30 mL) and brine (1 x 30 mL). The organic layer was then dried over magnesium sulfate, filtered, and concentrated in vacuo. The crude material was triturated with petroleum ether to give an off-white solid which was recrystallized from ethyl acetate:hexane. There remained 0.048 g (48.5% yield) of a white solid with an Rf value of 0.43 in the solvent system detailed above.

TLC analysis of the product on silica gel gave an Rf value of 0.43 in the solvent system detailed above. HPLC analysis was performed on a Vydac C4 column (4.6 x 250 mm) at 60°C using a gradient of 15-25% B/30 minutes (A=0.1% TFA in water, B=0.1% TFA in acetonitrile). The product had a retention time of 14.49 minutes and a purity of 97%.

Analyses of the final product provided the following results: Mass spectrum analysis found $(M+H)^+$ at m/z 343. Elemental analysis for $C_{16}H_{30}N_4O_4$ found 55.80 C, 8.70 H and 15.97N while calculated values were 56.12 C, 8.83 H and 16.36N. For ^{1}HNMR (600 MHz, d6-DMSO) analysis, the shifts observed were 8.65(t,1H), 8.10(d,1H), 6.07(d,1H), 4.85(m,1H), 4.20(m,1H), 3.12(m,2H), 2.77(s,6H), 1.77(m,1H), 1.63(m,1H), 1.48(m,2H), 1.40(m, 1H), 1.02(t,1H), 0.85(m,9H).

EXAMPLE PKC151

Boc-(L)-Leu-(L)-Abu-CONH-Et. The structure of Boc-(L)-Leu-(L)-Abu-CONH-Et is shown below:



This compound was produced by synthesis of the reactant Boc-(L)-Leu-(L)-Abu hydroxy ethyl amide.

Boc-Abu hydroxy ethyl amide (0.233 g, 894 mmoles) was dissolved in 5 mL of dioxane followed by the addition of 20 mL of 4N HCl/dioxane. The reaction mixture was allowed to react for two hours. After this time, the reaction mixture was concentrated in vacuo and used immediately in the next step. The HCl·Abu hydroxy ethyl amide prepared above, was dissolved in 25 mL of anhydrous DMF and cooled on an ice bath for ten minutes. To this solution was added 0.267 g (1.07 mmoles) of morpholineleucine urea, 0.46 mL (2.68 mmoles) of diisopropylethylamine and 0.133 g (0.984 mmoles) of 1-hydroxybenzotriazole (HOBt) and allowed to equilibrate for thirty minutes. After this time 0.188 g (0.984 mmoles) of EDC suspended in 10 mL of anhydrous DMF was added and the reaction mixture was allowed to react overnight. The reaction mixture was concentrated in vacuo and the resulting residue redissolved in 100 mL of chloroform. The solution was washed twice with 50 mL of both saturated sodium bicarbonate and brine. The organic layer was dried over magnesium sulfate, filtered and concentrated in vacuo. The crude material was purified by silica gel column chromatography employing 90:10 chloroform:methanol as the eluent. There remained 0.1841 g (55.12% yield) of a white solid with and Rf value of 0.42 in the solvent system detailed above.

Boc-Leu-Abu hydroxy ethyl amide (.0823 g, 0.22 mmoles) was dissolved in 10 mL of methylene chloride and cooled in an ice bath. To this mixture was added 0.325 mg (.002 mmoles) of 2,2,6,6-tetramethyl-1-piperidinyloxy, free radical (TEMPO) and .011 mL of an aqueous KBr solution (5.95 g of KBr dissolved in 25 mL of water). The

reaction mix was stirred vigorously while four 60 microliter portions of an 1M aqueous sodium hypochlorite (pH 9.5) were added at 15 minute intervals. After this time the reaction mixture was analyzed by TLC employing 90:10 chloroform:methanol to check for completeness of the reaction. If the reaction was not complete another portion of TEMPO and another regimen of the sodium hypochlorite solution should be added. This reaction required one additional regiment of TEMPO and sodium hypochlorite.

When the reaction was deemed complete by TLC, the layers were separated. The aqueous layer was extracted with methylene chloride (3 x 10 mL). The combined organic layer was washed with 10% HCl (1 x 10 mL), 30 mL of a 100 mL stock solution of 10% HCl containing 1.6 g of KI, 10% sodium thiosulfate (2 x 30 mL) and brine (1 x 30 mL). The organic layer was then dried over magnesium sulfate, filtered and concentrated in vacuo. The crude material was triturated with petroleum ether to give an off-white solid which was recrystallized from ethyl acetate:hexane. There remained 0.067 g (82.3% yield) of a white solid with an Rf value of 0.52 in the solvent system detailed above.

TLC analysis of the product on silica gel gave an Rf value of 0.52 in the solvent system detailed above. HPLC analysis was performed on a Vydac C4 column (4.6 x 250 mm) at 60°C using a gradient of 25-35% B/30 minutes (A=0.1% TFA in water, B=0.1% TFA in acetonitrile). The product had a retention time of 21.05 minutes and a purity of 99.14%.

Analyses of the final product provided the following results: Mass spectrum analysis found $(M+H)^+$ at m/z 372. Elemental analysis for $C_{18}H_{33}N_3O_5$ found 57.84 C, 8.84 H and 11.05 N while calculated values were 58.20 C, 8.95 H and 11.05 N. For ^{1}HNMR (600 MHz, d6-DMSO) analysis, the shifts observed were 8.66(t,1H), 8.06(d,1H), 6.85(d,1H), 4.88(m,1H), 3.99(m,1H), 3.12(m,2H), 1.77(m,1H), 1.77(m,1H), 1.60(m,1H), 1.51(m,1H), 1.35(br s,11H), 1.02(t,3H), 0.86(m,9H).

Morpholine Peptide Keto-Compounds

As is clear from the foregoing description of the Peptide Keto-Compounds, the term Peptide Keto-Compound as used herein also includes the morpholine Peptide Keto-Compounds. These morpholine compounds can be classified in any of the various classes or subclasses and types of Peptide Keto-Compounds referred to hereinabove. Thus, for example these compounds include the morpholine Peptide Ketoacids, the

morpholine Peptide Ketoamides and the morpholine Peptide Ketoesters. The morpholine Peptide Keto-Compounds can include either N-terminal or C-terminal morpholine groups. In the N-terminal morpholine Peptide Keto-Compounds, the M group (or M_1, M_2, M_3, M_4 group) includes a morpholine ring that can, in some circumstances, include the nitrogen of the N-terminal amino acid. The C-terminal morpholine Peptide Keto-Compounds include a morpholine ring that is part of the C-terminal R (or R_1 etc.) group of the compound. In certain examples of these compounds, the R-group includes an alkyl morpholine, as in the compound described above in Example PKC140.

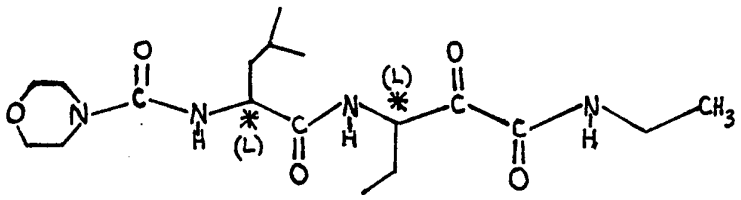
The morpholine Peptide Keto-Compounds can be produced using synthesis techniques generally similar to those used for synthesis of other Peptide Keto-Compounds.

The C-terminal morpholine Peptide Keto-Compounds can be produced using the general method of production of Peptide Ketoamides, which are derived from the corresponding Peptide Ketoesters. In the case of C-terminal Peptide Keto-Compounds, the Peptide Ketoesters can be reacted with N-amino alkyl morpholine to produce the C-terminal N-alkyl morpholine derivative of the Peptide Ketoester. One such procedure is shown hereinabove as Example PKC140.

The N-terminal morpholine Peptide Keto-Compounds can be produced using the general scheme outlined above, wherein a morpholine compound is substitued for other N-terminal blocking groups. However, other methods of synthesis can also be used. The following Example shows one exemplary procedure for production of N-terminal morpholine compounds.

Example PKC152

Morpholineurea-(L)-Leu-(L)-Abu-CONH-Et. The structure of Morpholineurea-(L)-Leu-(L)-Abu-CONH-Et is shown below:



Ten grams of N-t-butyloxycarboxy-(L)-α-aminobutric acid (N-Boc-(L)-Abu) was dissolved in 100 ml of anhydrous tetrahydrofuran (THF). To this solution was added 9.4 mL of diisopropylethylamine and 25.61 g (49.2 mmoles) of PyBOP. The solution was allowed to equilibrate for 10 minutes. Following equilibration, a solution of 5.28 g (54.1 mmoles) of N,O-dimethylhydroxylamine hydrochloride dissolved in 5 mL of acetonitrile and containing 25.6 mL of N,N-diisopropylethylamine (54.1 mmoles) was added. The reaction was stirred overnight at room temperature.

The reaction mixture was then concentrated in vacuo and redissolved in 200 mL of ethyl acetate. The ethyl acetate layer was washed three times with 1.0 N HCl (100 mL), three times with saturated sodium bicarbonate (100 mL) and three times with brine (100 mL). The reaction mixture was dried over magnesium sulfate, filtered and concentrated in vacuo giving a yellow oil. The crude product was purified by silica gel chromatography using 2:1 ethyl acetate:hexane as the eluent. The product was isolated as a white solid (77% yield) with an Rf of 0.77 on silica employing the same solvent system used above.

Anhydrous ethyl ether (75 mL) and 0.9 g (23.7 mmoles) of lithium aluminum hydride were placed in a 500 mL round-bottomed flask. The suspension was cooled in an ice bath for ten minutes. A pressure equalizing dropping funnel, containing 4.5 g (18.4 mmoles) of Boc-Abu hydroxamate dissolved in 75 mL of anhydrous ethyl ether, was attached to the round bottom flask and the contents were added dropwise over one

hour, with continued cooling. The reaction mixture was allowed to react for an additional two hours at room temperature.

The reaction mixture was then cooled in an ice bath and a cold solution of potassium hydrogen sulfate (5.4 g in 230 mL of water) was slowly added to the reaction flask and allowed to react for an additional 10 minutes. The aqueous and organic layers were separated and the aqueous layer was extracted with anhydrous ethyl ether (3 x 100 mLs). The combined organic layer was washed 3 x 100 mLs each with 1.0 N HCl, saturated sodium bicarbonate and brine and then dried over magnesium sulfate, filtered and concentrated in vacuo. The product was isolate as a white solid (63% yield), with an Rf of 0.90 on silica, using 2:1 ethyl acetate:hexane as the eluent.

N-Boc abuinal (4.00 g (21.39 mmoles)) was dissolved in 26 mL of methanol and cooled on ice. To this was added a cold solution of 2.67 g of sodium bisulfite dissolved in 54 mL of water. This reaction was stirred overnight at 4°C. 265 mL of ethyl acetate was then added to the above reaction mix followed by a solution of 1.08 g (22 mmoles) of sodium cyanide dissolved in 80 mL of water, and then stirred overnight at 4°C. The aqueous and organic layers were separated and the aqueous layer was extracted twice with 50 mL of ethyl acetate. The combined organics were dried over magnesium sulfate, filtered and evaporated in vacuo leaving a clear colorless oil (70% yield). TLC analysis on silica using 1:1 ethyl acetate:hexane as the eluent showed the product to have an Rf of 0.69. The Boc-Abu cyanohydrin was used without further purification.

The Boc-Abu cyanohydrin isolated was dissolved in 120 mL of 4N HCl/dioxane. 60 mL of water was then added to the reaction mixture and it was refluxed overnight. The reaction mixture was rotavapped to dryness leaving a brown solid. The solid was dissolved in water and extracted three times with 100 mL of ethyl acetate. The aqueous layer was then concentrated in vacuo and rotavapped from ethyl ether three times. This material was used without further purification.

HCl·Abu hydroxy acid (2.9 g (17.16 mmoles)) was dissolved in 51 mL of 2:1 dioxane:water and placed in an ice bath. To this was added 42.5 mL (42.5 mmoles) of 1N sodium hydroxide. The reaction was allowed to cool and 6.12 g (28.04 mmoles) of di-tert-butyl dicarbonate was then added. The pH of the reaction was maintained between 9.5 and 10 by the addition of base. Following an overnight reaction time it

was worked up as follows. The dioxane was rotavapped off and an additional 15 mL of water was added to the reaction mixture. The water was covered with a layer of ethyl acetate and cooled on ice. The pH of the aqueous layer was adjusted to 2.5 with 3N HCl. The organic and aqueous layers were separated and the aqueous layer was extracted twice with 50 mL of ethyl acetate. The combined organic layers were dried over magnesium sulfate, filtered, and evaporated in vacuo leaving a brown viscous oil. The crude material was purified by silica gel chromatography using 91:8:1 chloroform:methanol:acetic acid as the eluent. There remained 1.140 g Boc-Abu hydroxy acid (26.3% yield from Boc-Abuinal). TLC analysis on silica using the same system detailed above showed the product to be one spot with an Rf value of 0.22.

Boc-Abu hydroxy acid (0.96 g (4.13 mmoles)) was dissolved in 35 mL of dimethylformamide (DMF) and cooled in an ice bath. 0.78 mL (12.4 mmoles) of 70% triethylamine and 0.84 g (6.2 mmoles) of 1-hydroxybenzotriazole (HOBT) were added and allowed to equilibrate for thirty minutes. After this time 1.0 g (5.22 mmoles) of 1-(3-dimethylaminopropy)-3-ethylcarbodiimide hydrochloride (EDC) suspended in 10 mL of DMF was added. The reaction was allowed react at room temperature overnight.

The reaction was then rotavapped to dryness and redissolved in 100 mL of chloroform and washed three times with 35 ml of saturated sodium bicarbonate and then brine. The mixture was dried over magnesium sulfate, filtered and concentrated in vacuo. The crude material was purified by silica gel column chromatography employing 9:1 ethyl acetate:hexane. 0.938 g (85% yield) of product was isolated which possessed and Rf value of 0.55 in the above solvent system.

Boc-Abu hydroxy ethylamide (0.233 g, 0.894 mmoles) was dissolved in 5mL of dioxane followed by the addition of 20 mL of 4N HCl/dioxane. The reaction mixture was allowed to react for two hours. After this time, the reaction mixture was concentrated in vacuo and used immediately in the next step.

(L)-Leucine (1.31 g (10 mmoles)) was placed in a 3-neck round-bottom flask, equipped with two pressure equalizing dropping funnels. 12.5 mL of 1.0N NaOH (12.5 mmoles) was added to the flask and then the mixture was cooled on ice. 12.5 mL of 1.0 N NaOH was placed in one dropping funnel and 1.46 mL (12.5 mmoles) of morpholinecarbonyl chloride was placed in the other. The contents of the addition

funnels were added to the flask simultaneously over ten minutes. The mixture was allowed to react for an additional twenty minutes. The reaction mixture was then washed twice with 15 mL of ethyl acetate. The aqueous layer was cooled on ice and acidified to a pH of 2 with 1.0 N HCl. The aqueous layer was extracted three times with 15 mL of ethyl acetate. The combined organics were dried over magnesium sulfate, filtered and concentrated in vacuo. There remained 0.48 g of a white solid (20% yield) which possessed an Rf value of 0.45 using 91:8:1 chloroform:methanol:acetic acid as the eluent.

Boc-Abu hydroxy ethyl amide (0.266g) was dissolved in 5 mL of dioxane followed by the addition of 20 mL of 4N HCl/dioxane. The reaction mixture was allowed to react for two hours. After this time, the reaction mixture was concentrated in vacuo and used immediately in the next step. The HCl·Abu hydroxy ethyl amide was dissolved in 30 mL of anhydrous DMF and cooled on an ice bath for ten minutes. To this solution was added 0.30 g (1.23 mmoles) of morpholineleucine urea, 0.55 mL (3.07 mmoles) of diisopropylethylamine and 0.152 g (1.13 mmoles) of 1-hydroxybenzotriazole and allowed to equilibrate for thirty minutes. After this time, 0.218 g (21.13 mmoles) of EDC suspended in 10 mL anhydrous DMF was added and the reaction mixture was allowed to react overnight. The reaction mixture was concentrated in vacuo and the resulting residue was purified by silica gel column chromatography employing 90:10 chloroform:methanol as the eluent. There remained 0.2414 g (61.04% yield) of a white solid with an Rf value of 0.36 in the solvent system detailed above.

Boc-(L)-Leu-(L)-Abu hydroxy ethyl amide (0.1225 g (0.317 mmoles)) was dissolved in 10 mL of methylene chloride and cooled in an ice bath. To this mixture was added 0.5 mg (.00317 mmoles) of 2,2,6,6-tetramethyl-1-piperidinyloxy, free radical (TEMPO) and .0159 mL (.0317 mmoles) of an aqueous KBr solution (5.95 g of KBr dissolved in 25 mL of water). The reaction mix was stirred vigorously while four 87 microliter portions of a 1M aqueous sodium hypochlorite (pH 9.5) were added at 15 minute intervals. After this time the reaction mixture was analyzed by TLC employing 90:10 chloroform:methanol to check for completeness of the reaction. If the reaction was not complete another portion of TEMPO and another regimen of the sodium

hypochlorite solution should be added. This reaction required three additional regimens of TEMPO and sodium hypochlorite.

When the reaction was deemed complete by TLC, the layers were separated. The aqueous layer was extracted with methylene chloride (3 x 10 mL). The combined organic layer was washed with 10% HCl (1 x 10 mL), 30 mL of a 100 mL stock solution of 10% HCl containing 1.6 g of KI, 10% sodium thiosulfate (2 x 30 mL) and brine (1 x 30 mL). The organic layer was then dried over magnesium sulfate, filtered and concentrated in vacuo. The crude material was triturated with petroleum ether to give an off-white solid which was recrystallized from ethyl acetate hexane. There remained 0.048 g (39.6% yield) of a white solid with an Rf value of 0.32 in the solvent system detailed above.

TLC analysis of the product on silica gel gave an Rf value of 0.32 in the solvent system detailed above. HPLC analysis was performed on a Vydac C4 column (4.6 x 250 mm) at 60°C using a gradient of 15-25% B/30 minutes (A=0.1% TFA in water, B=0.1% TFA in acetonitrile). The product had a retention time of 14 minutes and a purity of 97.8%. Analyses of the final product provided the following results: Mass spectrum analysis (FABMS) found $(M+H)^+$ at m/z 385. Elemental analysis for $C_{18}H_{32}N_4O_5$ found 56.14 C, 8.24 H and 14.36 N while calculated values were 56.23 C, 8.39 H and 14.57 N. For ^{1}HNMR (600 MHz, d6-DMSO) analysis, the shifts observed were 8.65(t,1H), 8.10(d,1H), 6.41(d,1H), 4.85(m,1H), 4.20(m,1H), 3.51(m,4H), 3.26(m,4H), 3.12(m,2H), 1.75(m,1H), 1.62(m,1H), 1.48(m,2H), 1.40(m,1H), 1.02(t,3H), 0.85(m,9H).

D. HALO-KETONE PEPTIDES

Halomethyl ketone peptides are irreversible inhibitors for serine proteases and cysteine proteases. This class of compounds includes peptides having a variety of halomethyl groups at their C-terminus. These halomethyl groups include $-CH_2X$, $-CHX_2$ and CX_3, where X represents any halogen. A number of analogous compounds have been synthesized, including the amino-halo ketones and the diazo-ketone peptides. Although these analogous compounds are chemically distinguishable, all of these

haloketone compounds are believed to have a similar mechanism of action. Accordingly, for simplicity, all of the foregoing compounds will be referred to collectively herein as the "Halo-Ketone Peptides."

The reactivity of haloketones has generally been found to be in the order I > Br > Cl > F. However, increasing the reactivity of the haloketone in this way can lead to acceleration of competing side effects. Thus, it is preferable to increase the reactivity of the halomethyl ketone peptides by altering the peptide structure.

In selecting a proper inhibitor for Calpain, the same basic peptide structure selection techniques as used for the Peptide Keto-Compounds can be used. Once a peptide structure has been identified, the most effective C-terminus grouping can be empirically determined through kinetic inhibition studies of each of the compounds with Calpain.

Many of the Halo-Ketone Peptides are available commercially. For example, Leu-CH_2Cl, Phe-CH_2Cl, Z-lys-CH_2Cl, Tosyl-LysCH_2Cl (TLCK), Tosyl-PheCH_2Cl (TPCK), Z-Gly-Leu-Phe-CH_2Cl, Z-Phe-Ala-CH_2Cl, z-Phe-Phe-CH_2Cl, D-Phe-Pro-Arg-CH_2Cl, MeoSuc-Phe-Gly-Gly-Ala-CH_2Cl, MeoSuc-Ala-Ala-Pro-Ala-CH_2Cl, MeoSuc-Ala-Ala-Pro-Val-CH_2Cl, Ala-Ala-Pro-Val-CH_2Cl, Ala-Ala-Phe-CH_2Cl, Suc-Ala-Ala-Pro-Phe-CH_2Cl and D-Val-Leu-Lys-CH_2Cl are all available from suppliers such as Enzyme Systems Products of Livermore, California. From the same suppliers, the following diazomethyl ketone peptides are available: Leu-CHN_2, Z-Phe-Phe-CHN_2, Z-Phe-Ala-CHN_2, Z-Phe-Pro-CHN_2, Z-Lys-CHN_2 and Gly-Phe-CHN_2. In addition, the production of α-amino fluoro ketone peptides has been described in United States Patent No. 4,518,528 to David W. Rasnick, the disclosure of which is hereby incorporated by this reference.

The preparation of various Halo-Ketone Peptides is reviewed in *Methods in Enzymology*, **46**:197-208 (1977), the disclosure of which is hereby incorporated by reference. Briefly, halomethyl ketone derivatives of blocked amino acids are readily prepared by the reaction of mineral acids (hydrohalic) with the corresponding diazomethyl ketone. Iodomethyl ketones are prepared by reaction of a halo-ketone with NaI, since reaction with HI with a diazomethyl ketone yields the methyl ketone. A number of different blocking groups can be used, including benzyloxycarbonyl (Z) and t-butyloxycarbonyl (Boc). The diazomethyl ketone is prepared by reaction of

diazomethane with the appropriate acid activated by means of dicyclohexylcarbodiimide (DCCI), by the mixed anhydride method.

Unblocked amino acid chloromethyl ketones can be prepared by reaction of benzyloxycarbonyl blocked derivatives with HBr or HOAc, trifluoroacetic acid, or by hydrogenation.

Synthesis of peptide chloromethyl ketones can be accomplished simply by coupling an appropriate peptide or amino acid with an unblocked amino acid chloromethyl ketone. A few dipeptides can be converted directly to the chloromethyl ketone using the mixed anhydride and CH_2N_2 followed by HCl.

Various synthetic problems are encountered in the preparation of chloromethyl ketone derivatives of basic amino acids. The side chain usually must be blocked during synthesis, and difficulties are often encountered during removal of the blocking group. Use of trifluoroacetic acid or HF was eventually found to give a good conversion to product.

A number of examples of the preparation of Halo-Ketone Peptides have been reported in the literature, including a comprehensive review of over 100 amino acid derivatives and approximately 60 peptide derivatives listed in J.C. Powers, in "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Vol. 4, Dekker, New York (1977), the disclosure of which is hereby incorporated by reference. Those of skill in the art will recognize how to locate a multitude of examples of the production of the Halo-Ketone Peptides. Accordingly, no additional examples are provided herein.

E. IN VITRO USES

In addition to the foregoing classes of compounds now discovered to possess Calpain inhibitory activity, we believe that a large number of other such compounds exist. In view of the large number of inhibitors of Calpain of different classes we disclose herein, all of the known, newly discovered and yet undiscovered inhibitors of Calpain shall be referred to hereinafter collectively, using the term "Calpain Inhibitor."

The Calpain Inhibitors may be used *in vitro* for a variety of purposes to inhibit unwanted Calpain activity. For example, the Calpain Inhibitors may be used *in vitro* to prevent proteolysis that occurs in the process of production, isolation, purification, storage or transport of peptides and proteins.

The Calpain Inhibitors described herein can also be used *in vitro* to prevent further degradation of tissue samples from occurring after preparation of the samples. This *in vitro* prevention of degradation can be especially useful in the preparation of assays for neurodegeneration wherein the assay comprises a test for the products of Calpain activity in the tissues, such as assays for breakdown products (BDP's) of cytoskeletal components such as spectrin, MAP2, actin binding protein and tau. P. Seubert *et al.*, *Neuroscience*, **31**:195 (1989), the disclosure of which is hereby incorporated by reference, disclose an exemplary method of quantitating the amount of spectrin BDP's as an indication of Calpain activity.

The Calpain Inhibitors of this invention are also useful in a variety of other experimental procedures where proteolysis due to Calpains is a significant problem. For example, inclusion of the Calpain Inhibitors in radioimmunoassay experiments can result in higher sensitivity. The use of the Calpain Inhibitors in plasma fractionation procedures can result in higher yields of valuable plasma proteins and can make purification of the proteins easier. The Calpain Inhibitors disclosed here can be used in cloning experiments utilizing recombinant or transfected bacterial or eukaryotic cell cultures in order to increase yield of purified recombinant product.

To use the Calpain Inhibitors *in vitro*, the Calpain Inhibitors are dissolved in an organic acid, such as dimethylsulfoxide (DMSO) or ethanol, and are added to an aqueous solution containing the protease which is to be inhibited, such that the final concentration of organic solvent is 25% or less. The Calpain Inhibitors may also be added as solids or in suspension.

F. TREATMENT OF NEURODEGENERATION

We have discovered that the Calpain Inhibitors are useful *in vivo* to treat pathologies in which excess proteolysis by Calpains is involved. Such pathologies are believed to include neuropathologies such as neurodegeneration resulting from excitotoxicity, HIV-induced neuropathy, ischemia, denervation, injury, subarachnoid hemorrhage, stroke, multiple infarction dementia, Alzheimer's Disease (AD), Huntington's Disease, surgery-related brain damage, Parkinson's Disease, and other pathological conditions.

In additional *in vivo* uses, peptide α-ketoamide can be used to control protein turnover, muscular dystrophy, myocardial tissue damage, and bone resorption as shown

in Tables PKC2, PKC3, and PKC4 by effective inhibition of lysosomal cathepsin B. Peptide α-ketoamides can also be used as neuroprotectants or for the treatment of ischemia, stroke, restenosis or Alzheimer's disease as shown in Tables PKC2, PKC3, and PKC4 by effective inhibiton of calpain I and calpain II.

1. **Identification of Inhibitors**

In order to identify Calpain Inhibitors that are useful in the practice of the present invention for treatment or inhibition of neurodegenerative conditions and diseases, it is important to identify those inhibitors posessing significant Calpain inhibitory activity. It is also important to identify those Calpain Inhibitors having a high degree of specificity for inhibition of Calpain, in order to avoid interference with other biological processes when the Calpain Inhibitor is introduced into a mammal requiring treatment for neurodegeneration. Because all thiol proteases are believed to exert their effect through a similar mechanism of action, our primary concern was to identify those Calpain Inhibitors having substantial inhibitory activity against Calpain, but relatively weak or no activity against other thiol proteases. Accordingly, in order to identify such Calpain Inhibitors, we tested a variety of Calpain Inhibitors for their ability to inhibit calpains I and II, and compared this data with the ability of the same Calpain Inhibitors to inhibit Cathepsin B, another thiol protease. Those Calpain Inhibitors with high *in vitro* inhibitory activity against Calpain and a relatively lower activity against Cathepsin B are believed to be most useful for *in vivo* therapy. Examples 1A through 1C show the results of these studies for a variety of Calpain Inhibitors.

EXAMPLE 1A

Inhibition by Substituted Heterocyclic Compounds

The isocoumarins are irreversible inhibitors of Calpain. We obtained IC_{50} values for a variety of these Calpain Inhibitors as a kinetic analysis of these compounds. Purified Calpains can be assayed using the fluorogenic substrate succinyl-leucine-tyrosine-methylaminocoumarin (available commercially) or by measuring the release of acid-soluble peptides from casein because we have found that the isocoumarins inhibit casein proteolysis by Calpain.

Calpains I and II were purified by the method of (Yoshimura, et al. 1983). (Kitahara, *supra*) provides an alternative purification scheme. Calpain II may

alternatively be purchased from Sigma Chemical Co. as "Calcium Activated Neutral Protease." In this assay, purified Calpain was incubated with ^{14}C-methylated casein in the presence of various Heterocyclic Compounds and the amount of acid-soluble radioactivity released by the action of Calpain was measured. The IC_{50} values were determined as the concentration of Heterocyclic Compound compound at which 50% of the Calpain activity was inhibited. Table 1A shows IC_{50} values for various Isocoumarin Compounds.

TABLE 1A

INHIBITION OF CALPAINS BY SUBSTITUTED ISOCOUMARINS

	IC_{50} (μM)	
	Calpain I	Calpain II
CiTPrOIC	100	70
NH_2-CiTPrOIC (ACITIC)	10	120
$PhCH_2NHCONH$-CiTPrOIC	80	30
CH_3CONH-CiTPrOIC	700	80
L-Phe-NH-CiTPrOIC		30
BOC-L-Phe-NH-CiTPrOIC	no inhibition	>200
$PhCH_2NHCONH$-CiTEtOIC	90	
$PhCH_2CONH$-CiTEtOIC	30	
D-Phe-NH-CiTEtOIC	200	

Thus, it can be seen from Table 1A that a variety of the Isocoumarin Compounds have significant Calpain inhibitory activity at low concentrations.

EXAMPLE 1B(i)

Protease Inhibition by Peptide Keto-Compounds

The Peptide Keto-Compounds are reversible inhibitors of Calpains and other thiol proteases. The K_i values for the inhibition of calpain I, calpain II and Cathepsin B were determined for several Peptide Keto-Compounds. Inhibition of calpain I from human erythrocytes and calpain II from rabbit muscle were assayed using Suc-Leu-Tyr-amidomethylcoumarin as substrate in an assay buffer of 20mM HEPES

pH=7.2, 10mM $CaCl_2$, 10mM ß-mercaptoethanol. Cathepsin B from bovine spleen was assayed using Z-Lys-4-nitrophenylphosphate as substrate.

Table 1B(i) shows the results of the studies of Example 1B(i). The Ki value for the inhibition of Calpains and cathepsin B by several Peptide Keto-Compounds are shown in µM (micromolar). The values for leupeptin, which is commercially available from Calbiochem of La Jolla, California, are shown for comparison.

Table 1B(i)

K_i VALUES FOR PEPTIDE KETO-COMPOUNDS

Inhibitor	Calpain I	Calpain II	Cathepsin B
Leupeptin	0.32	0.43	6
Z-Ala-Ala-Ala-CO_2Me	200	----	1.5
Z-Ala-Ala-Abu-CO_2Et	50	200	0.9
Z-Leu-Phe-CO_2Et	0.23	0.4	>50
Z-Leu-Nle-CO_2Et	0.12	0.18	18
Z-Leu-Abu-CO_2Et	0.04	0.4	14
Z-Leu-Nva-COOEt		1.2	30

It can be seen from the results in Table 1B(i) that the Peptide Keto-Compounds inhibit Calpain with Ki values similar or superior to leupeptin. In particular, Z-Leu-Phe-CO_2Et, Z-Leu-Nle-CO_2Et and Z-Leu-Abu-CO_2Et were found to possess greater Calpain inhibitory activity than leupeptin. In addition, these particular compounds were highly specific to Calpain, with lower inhibitory activity toward Cathepsin B than leupeptin.

EXAMPLE 1B(ii)

Protease Inhibition of Peptide Keto-Compounds

We tested the ability of an additional group of Peptide Keto-Compounds to inhibit several proteases in order to evaluate their specificity for Calpain. The results of these studies are shown in Table 1B(ii).

Table 1B(ii). Inhibition of Calpain I, Calpain II, Cathepsin B (CathB), Chymotrypsin (Chym), PP Elastase and Papain

Inhibitor	**K_i(μM)**					
	Calpain I	**Calpain II**	**CathB**	**Chym**	**elastase**	**papain**
Z-Leu-Abu-COOEt	4.5	0.4	30	>100	>100	220

Inhibitor	K_i(μM)					
	Calpain I	Calpain II	CathB	Chym	elastase	papain
Z-Leu-Abu-COOnBu	1.8		4	>100	25	10
Z-Leu-Abu-COOBz	9.5	0.47	4	40	>100	40
Z-Leu-Leu-Abu-COOEt	1.8	2.6	22	>100	25	
2-NapSO_2-Leu-Leu-Abu-COOEt	16	1.4	25	35	47	
2-NapCO-Leu-Leu-Abu-COOEt		0.09		>300	28	
Tos-Leu-Leu-Abu-COOEt	33		69	25	28	
Ph-$(CH_3)_2$-CO-Leu-Abu-COOEt		1.2				
Z-Leu-Abu-COOH	0.075	0.022	1.5	>150	>150	
Z-Leu-Abu-CONHEt	0.5	0.23	2.4	>150	65	
Z-Leu-Abu-CONHnPr		0.25	8	>300	2	
Z-Leu-Abu-CONHnBu	0.2		13	>300	5	
Z-Leu-Abu-CONHiBu		0.14	4	>300	40	
Z-Leu-Abu-CONHBz		0.35	2	>300		
Z-Leu-Abu-CONH-$(CH_2)_2$-Ph		0.022				
Z-Leu-Abu-CONH-$(CH_2)_3$-Mpl		0.041				
Z-Leu-Abu-CONH-$(CH_2)_7CH_3$		0.019				
Z-Leu-Abu-CONH-$(CH_2)_2OH$		0.078				
Z-Leu-Abu-CONH-$(CH_2)_2O(CH_2)_2OH$	0.16					
Z-Leu-Phe-COOEt	1.8	0.4	340	0.025	>100	75

Inhibitor	$K_i(\mu M)$					
	Calpain I	Calpain II	CathB	Chym	elastase	papain
Z-Leu-Phe-COOnBu	5.0	1.1	15	0.15	>100	15
Z-Leu-Phe-COOBz	3.4	1.6	45	1.6	>100	45
Z-Leu-Leu-Phe-COOEt	1.4	1.9	42	0.26	>100	15
Z-Leu-Phe-COOH	0.0085	0.0057	4.5	76	>150	
Z-Leu-Phe-CONHEt	7.0	0.32	6	73	>150	
Z-Leu-Phe-CONHnPr	15.0	0.05	3	18	>300	
Z-Leu-Phe-CONHnBu		0.028	3	8	>100	
Z-Leu-Phe-CONHiBu		0.065	4	24		
Z-Leu-Phe-CONHBz		0.046				
Z-Leu-Phe-CONH$(CH_2)_2$Ph		0.024		(2)		
Z-Leu-Nle-COOEt		0.18	20		2.2	190
Z-Leu-Nva-COOEt	1.4	1.2	25	160	2.3	150
Z-Leu-Met-COOEt	1.0	1.5	55	1.75	>100	140
Z-Leu-4-Cl-Phe-COOEt	<4.0	0.4	50	0.9	>100	150

Table 1B(ii) shows the inhibition constants (K_I) for cathepsin B, calpain I, and calpain II with peptide ketoamides. Dipeptide Ketoamides with Abu and Phe in the P_1 site and Leu in the P_2 site are potent inhibitors of calpain I and calpain II. Z-Leu-Abu-CONH-Et is a better inhibitor of calpain I than Z-Leu-Phe-CONH-Et by 14 fold. Replacement of the Z group ($PhCH_2OCO$-) by similar groups such as $PhCH_2CH_2CO$-, $PhCH_2CH_2SO_2$-, $PhCH_2NHCO$-, and $PhCH_2NHCS$- would also result in good inhibitor

structures. One good inhibitor of calpain II is Z-Leu-Abu-CONH-$(CH_2)_2$-Ph. Changing the R_3 and R_4 groups significantly improves the inhibitory potency toward calpain II. The best Dipeptide Ketoamide inhibitors are those which have long alkyl side chains (e.g. Z-Leu-Abu-CONH-$(CH_2)_7CH_3$), alkyl side chains with phenyl substituted on the alkyl group (e.g. Z-Leu-Abu-CONH-$(CH_2)_2$-Ph), or alkyl groups with a morpholine ring substituted on the alkyl group (e.g. Z-Leu-Abu-CONH-$(CH_2)_3$-Mpl, Mpl = -$N(CH_2CH_2)_2O$). Dipeptide α-ketoamides with a small aliphatic amino acid residue or a Phe in the P_1 site are also good inhibitors for cathepsin B. The best inhibitor is Z-Leu-Abu-CONHEt and replacement of the Z ($PhCH_2OCO$-) by $PhCH_2CH_2CO$-, $PhCH_2CH_2SO_2$-, $PhCH_2NHCO$-, and $PhCH_2NHCS$- would also result in good inhibitor structures.

EXAMPLE 1B(iii)

Stability of Peptide Keto-Compounds

We determined the half-life in minutes of several Peptide Keto-Compounds in both plasma and liver homogenates. The results of the determinations of stability of the compounds in plasma and liver homogenates are shown in Table 1B(iii).

Table 1B(iii). Stability in Plasma and in Liver of Peptide Keto-Compounds.

Inhibitor	$t_{1/2}$	
	plasma	liver
Z-Leu-Abu-COOEt	2.8	
2-NapSO_2-Leu-Leu-Abu-COOEt	> 60	
2-NapCO-Leu-Leu-Abu-COOEt	25	
Tos-Leu-Leu-Abu-COOEt	30	
Z-Leu-Abu-COOH	> 60	> 60
Z-Leu-Abu-CONHEt	> 60	> 60
Z-Leu-Abu-CONHnPr	> 60	> 60
Z-Leu-Abu-CONHnBu	> 60	> 60
Z-Leu-Abu-CONHiBu	> 60	
Z-Leu-Abu-CONHBz	> 60	> 60
Z-Leu-Phe-COOEt	7.8	
Z-Leu-Phe-COOnBu	7.7	
Z-Leu-Phe-COOBz	1.9	
Z-Leu-Phe-COOH	> 60	> 60
Z-Leu-Phe-CONHEt	> 60	> 60
Z-Leu-Phe-CONHnPr	> 60	> 60
Z-Leu-Phe-CONHnBu	> 60	> 60
Z-Leu-Phe-CONHiBu	> 60	
Z-Leu-Phe-CONH$(CH_2)_2$Ph	> 60	

Inhibitor	$t_{1/2}$	
	plasma	liver
Z-Leu-Nle-COOEt	3.7	
Z-Leu-Nva-COOEt	2.8	
Z-Leu-Met-COOEt	8	

It can be seen from the data in Table 1B(iii) that the Peptide Keto-Compounds are generally quite stable in plasma and liver homogenates. However, it is also shown that the Peptide α-ketoamides were substantially more stable in both plasma and liver than the corresponding peptide α-ketoesters

EXAMPLE 1C

Protease Inhibition by Halo-Ketone Peptides

The Halo-Ketone Peptides, like the substituted isocoumarins, are irreversible inhibitors of Calpain. We determined the $K_{app}/[I]$ values for various members of this class of compounds against Calpains I and II. For comparison, we also determined these values against the additional thiol proteases Papain and Cathepsin B for at least one Halo-Ketone Peptide. These K_{app} values are not directly comparable to the K_i or IC_{50} values determined above for other classes of inhibitors.

We assayed Calpain I and II using Suc-leu-tyr-amidomethylcoumarin. Papain was assayed using benzoyl-arg-4-nitroanilide, and Cathepsin B (bovine) was assayed using CBZ-lys-4-nitrophenyl ester. We followed the progress curve method of Tian and Tsou, *Biochemistry*, **21**:1028-1032 (1982), the disclosure of which is hereby incorporated by reference, to derive kinetic data. Briefly, this method makes use of the equation:

$$[P_\infty] = \frac{V[S]/K}{(1 + [S]/K)A[Y]}$$

where $[P_\infty]$ represents the concentration of product formed at a time approaching infinity, A is the K_{app} in the presence of substrate (S), K is the Michaelis constant and [Y] is the concentration of the inhibitor. Since [S] and [Y] are known and V and K can be determined, K_{app} can be readily determined.

The $K_{app}/[I]$ for various Halo-Ketone Peptides are shown in Table 1C.

TABLE 1C

KINETIC PARAMETERS OF HALO-KETONE PEPTIDES

Inhibitor	CI	CII[3]	P	CB
Z-Gly-Leu-Phe-CH_2Cl	284000[1]	946000		
Boc-Gly-Leu-Phe-CH_2Cl		902000	540000	290000
Z-Leu-Phe-CH_2Cl	225000[2]	585000		
Z-Gly-Leu-Ala-CH_2Cl		210000		
Ac-Leu-Phe-CH_2Cl	25900[1]	33400		
Z-Val-Phe-CH_2Cl		27200		
Z-Ala-Phe-CH_2Cl		2400		
Ac-Ala-Ala-Ala-Ala-CH_2Cl		1300		

CI = Calpain I
CII = Calpain II
P = Papain
CB = Cathepsin B

[1] - Rat
[2] - Human
[3] - Rabbit

It can be seen from the results in Table 1C that the Halo-Ketone Peptides inhibit Calpain with relatively high $K_{app}/[I]$ values. In particular, Z-gly-leu-phe-CH_2Cl, Boc-gly-leu-phe-CH_2Cl, Z-leu-phe-CH_2Cl and Z-gly-leu-ala-CH_2Cl were found to possess significant Calpain inhibitory activity. In addition, Boc-gly-leu-phe-CH_2Cl was shown to be somewhat specific to Calpain, with lower inhibitory activity toward Cathepsin B or Papain than toward Calpain. The results shown in the table reveal that Z-gly-leu-phe-CH_2Cl and Boc-gly-leu-phe-CH_2Cl produce similar inhibitory effects. Thus, the blocking group is not shown to have a great effect on Calpain inhibitory activity.

The kinetic constants of other irreversible Calpain Inhibitors include the following with $K_{app}/[I]$ in parentheses: E-64 (7500), E64-d (23000) and Z-leu-leu-tyr-CHN_2 (230000). E-64 is commercially available from Sigma Chemical Co., and is shown here to be a poor inhibitor of Calpain. Z-leu-leu-tyr-CHN_2 is a diazomethyl peptide compound, here shown to possess significant Calpain inhibitory activity.

2. **Inhibition of Calpain in Neural Tissues**

In order to evaluate the inhibition of Calpain by the various Calpain Inhibitors in neural tissues, we assayed the Calpain Inhibitors using the known ability of Calpain to cleave spectrin, a protein component of neuronal and other tissue, into BDP's. In this assay, more effective Calpain Inhibitors will prevent the conversion of spectrin into BDP's. Example 2 is one example of such an assay.

EXAMPLE 2

Inhibition of Calpain in
Crude Brain Extracts by Calpain Inhibitors

The activity of Calpain in crude brain extracts was measured by examining the Ca^{2+}-stimulated proteolysis of the endogenous substrate spectrin. Brain tissue was homogenized in 10mM Tris pH=7.4, 0.32M sucrose, 1mM EGTA, 1mM dithiothreitol a nd the nuclei and debris removed by low speed centrifugation. Various Calpain Inhibitors were added to the supernatant in a DMSO vehicle and a calcium salt (final effective concentration about 1.2mM) added to start the reaction. Proteolysis of spectrin was evaluated by western blot as described by Seubert, et al., *Brain Res.*, **459**:226-232 (1988), the disclosure of which is hereby incorporated by reference. Briefly, a known quantity of a spectrin-containing sample treated with Calpain is separated by SDS-PAGE and immunoblotted with anti-spectrin antibody. The amount of spectrin immunoreactivity found corresponding to the characteristic BDP's is indicative of the amount of spectrin activity present in the sample. An examplary method for quantitating BDP's is to assay Spectrin BDP's by homogenizing brain parts in 20mM Tris pH=7.2, .32M sucrose, 50μM Ac-Leu-Leu-nLeu-H on ice. Homogenates are then mixed 1:1 with 10% SDS, 5% ß-mercaptoethanol, 10% glycerol, 10mM Tris pH=8.0, 0.5% bromophenolblue, heated to 95°C, and subjected to electrophoresis in 4-1/2 % polyacrylamide gels. The proteins in the gels are transferred to nitrocellulose and the spectrin and BDP's detected using a rabbit polyclonal anti-spectrin antibody and established immunodetection methods. The amount of spectrin and BDP's in each sample can be quantitated by densitrometric scanning of the developed nitrocellulose.

Due to Calpain's requirement for Ca^{2+}, in the absence of Ca^{2+} little or no spectrin proteolysis occurred, regardless of the presence of inhibitor, while in the

presence of Ca^{2+} the spectrin was >95% cleaved to BDP's within 40 min. if no Calpain Inhibitor is added.

Both leupeptin and CI1 showed inhibition in the system of Example 2. In addition, the following compounds of the Substituted Heterocyclic Compounds were found to produce significant inhibition at 100 μM:

3-chloroisocoumarin

3,4-dichloroisocoumarin

3-benzyloxy-4-chloroisocoumarin

7-(acetylamino)-4-chloro-3-(propoxy)-isocoumarin

4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(benzylcarbamoylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(phenylcarbamoylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(acetylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(3-phenylpropionylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(phenylacetylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(L-phenylalanylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(benzylcarbamoylamino)-4-chloro-3-(3-isothiureidoethoxy)isocoumarin

7-(phenylcarbamoylamino)-4-chloro-3-(3-isothiureidoethoxy)isocoumarin

7-(D-phenylalanylamino)-4-chloro-3-(3-isothiureidoethoxy)isocoumarin.

The following compounds of the Halo-Ketone Peptides were also found to produce significant inhibition at 100 μM:

Z-Leu-Phe-CH_2Cl

Ac-Leu-Phe-CH_2Cl

Z-Gly-Leu-Phe-CH_2Cl

Boc-Gly-Leu-Phe-CH_2Cl

Ac-Val-Phe-CH_2Cl

Z-Gly-Leu-Ala-CH_2Cl.

In addition, the following compounds of the Peptide Keto-Compounds were found to produce significant inhibition at 100 μM:

Bz-DL-Phe-COOEt

Z-Leu-Nva-COOEt

Z-Leu-Nle-COOEt

Z-Leu-Phe-COOEt

Z-Leu-Abu-COOEt

Z-Leu-Met-COOEt

Z-Ala-Ala-DL-Abu-COOEt

MeO-Suc-Val-Pro-DL-Phe-COOMe

Z-Ala-Ala-Ala-DL-Ala-COOEt

MeO-Suc-Ala-Ala-Pro-DL-Abu-COOMe.

Z-Leu-Phe-COOEt

Thus, the Substituted Heterocyclic Compounds, Peptide Keto-Compounds and Halo-Ketone Peptides, in addition to leupeptin and CI1, provide inhibition in brain homogenates.

3. **<u>In vivo</u> Inhibition of Neurodegeneration through Infusion Techniques**

In order to demonstrate that the inhibition of Calpain activity alone is sufficient to inhibit neurodegeneration *in vivo*, we tested the ability of the Calpain Inhibitor, leupeptin, to inhibit neurodegeneration in gerbils subjected to transient ischemia.

As stated above, leupeptin is poorly membrane permeant. Therefore, leupeptin is not expected to cross the blood-brain barrier ("BBB") very well. Accordingly, in order to provide the brain with sufficient leupeptin to adequately inhibit Calpain activation, we used brain infusion techniques. Through the use of these techniques we

were able to subject brain tissues to intimate contact with leupeptin for sustained periods of time. Example 3A is provided to show the *in vivo* protection from neurodegeneration found during one such study.

EXAMPLE 3A

In Vivo Protection Against Neurodegeneration

A small cannula was implanted in the right lateral ventricle of adult gerbils, and secured to the skull with dental cement. An Alzet micro-osmotic pump was attached to the cannula for intracerebroventricular perfusion. The pump was filled with either saline alone (control) or leupeptin (20 mg/ml in saline). After three days perfusion with either the control solution or with the leupeptin solution, transient ischemia was induced by bilaterally clamping the carotid arteries for a period of ten minutes. Core temperatures were taken during and following ischemia, with no differences noted between control and leupeptin treated animals. Fourteen days later, the animals were sacrificed by Nembutal overdose and transcardial perfusion of a 10% solution of paraformaldehyde in PBS. Coronal sections of the brain were stained with cresyl violet and were examined for the extent of neuronal loss. The control gerbils exhibited the typical damage found in the CA1 field following ischemia, with a 72% loss of neurons. However, the leupeptin treated gerbils showed far less neurodegeneration, with only a 15 % loss of neurons.

The results of Example 3A cannot be explained by changes in thermoregulation, since core temperatures did not differ between the groups. Accordingly, we believe that the Calpain inhibitory activity of leupeptin is responsible for the observed differences in neuronal cell loss. In order to further quantitate the differences, and verify that leupeptin produced a Calpain inhibitory effect within the observed regions of the brain, we performed a related series of experiments. In this series of experiments, spectrin BDP's were measured in the leupeptin treated and control animals. As discussed above, these BDP's are indicative of the amount of Calpain activity occurring within the tissue. Example 3B is provided to demonstrate the results of these experiments.

EXAMPLE 3B

In Vivo Inhibition of Calpain Activity

Implantation surgeries and clamping of the carotid arteries were performed as above with a control-ischemia group (n=4) and a leupeptin-ischemia group (n=5). A third group of animals (n=4) received implantation with pumping of saline, but was not subjected to ischemia. Animals were sacrificed by decapitation 30 minutes after clamping of the arteries. The brains were rapidly removed and placed in cold homogenization buffer (0.32 M sucrose, 10 mM Tris-HCl, 2 mM EDTA, 1 mM EGTA, 100 μM leupeptin and 1 μg/ml of the Halo-Ketone Compound, tos-phe-CH_2Cl (TPCK)). The CA1 region of the hippocampus was then dissected. The samples from both control and leupeptin treated animals were then prepared for SDS-PAGE and immunoblotting with labeled anti-spectrin antibody, as described above in connection with *in vitro* uses of the Calpain Inhibitors. The control animals exhibited a marked increase in the levels of BDP's relative to the gerbils not subjected to ischemia. These BDP's co-migrated with BDP's observed after *in vitro* proteolysis of spectrin with Calpain. The brain tissue from the leupeptin treated gerbils exhibited approximately 25% of the BDP's observed in the control ischemia treated gerbils.

Another group of gerbils (n=3) were sacrificed immediately after ischemia without leupeptin treatment in order to observe the effects of ischemia without reoxygenation. These gerbils exhibited a similar amount of increase of BDP's as the control-ischemic gerbils observed after a 30 minute reperfusion period.

Thus, the results of Example 3B indicate that leupeptin exerts its neuroprotective effect through the inhibition of Calpain activation. The results also indicate that the observed proteolysis of spectrin was an effect of ischemia, and not secondary to the reoxygenation. Accordingly, the results indicate that inhibition of Calpain activity *in vivo* produces a neuroprotective effect.

Although the foregoing studies demonstrate that leupeptin can inhibit neurodegeneration *in vivo*, leupeptin is not the therapeutic drug of choice because of the need to infuse the drug directly into the brain for an extended period of time to exert its neuroprotective effect. This is due to the relatively poor ability of this compound to cross the BBB. Accordingly, it is believed that a more therapeutically

practical way to inhibit neurodegeneration would be to use more membrane permeant Inhibitor of Calpain.

4. **Platelet Permeability**

In accordance with our discoveries demonstrated in Examples 3 and 3A, we believe that having a compound cross the BBB and enter CNS tissue is a key characteristic of a therapeutically useful approach to treat or inhibit neurodegeneration within the CNS. Use of Calpain inhibitors that have enhanced membrane permeability is one such approach. Thus, we measured the ability of various Calpain inhibitors to penetrate the platelet membrane and inhibit Calpain that is normally contained in platelets. As shown below in the following examples, our results indicate that particular compounds of the Heterocyclic Compounds, Peptide Keto-Compounds and Halo-Ketone Peptides, in addition to the Peptide Aldehyde, CI1, exhibit good membrane permeability.

As an indication of the membrane permeability of the various Calpain Inhibitors, we measured the ability of various Calpain Inhibitors to penetrate platelet membranes and inhibit the Calpain normally found within platelets. The membrane of platelets is believed to have many similarities to the BBB and accordingly, such experiments are believed to provide a good indication of the ability of the various Calpain Inhibitors to cross the BBB. Example 4 shows the results of some of these platelet experiments using the Calpain Inhibitors of the present invention.

EXAMPLE 4A

Membrane Permeation of Calpain Inhibitors

Platelets were isolated by a modification of the method of Ferrell and Martin, *J. Biol. Chem.*, **264**:20723-20729 (1989), the disclosure of which is hereby incorporated by reference. Blood (15-20 ml) was drawn from male Sprague-Dawley rats into 100mM EDTA-citrate containing 10 units heparin, and centrifuged 30 minutes at 1600 rpm at room temperature. The plasma was resuspended in 15ml buffer 1 (136mM NaCl, 2.7mM KCl, 0.42mM NaH_2PO_4, 12mM $NaHCO_3$, 2mM $MgCl_2$, 2 mg/ml BSA (Sigma), 5.6mM glucose, 22mM Na_3Citrate pH 6.5) and platelets were isolated at 2200 rpm at room temperature of 25 minutes. Platelets were resuspended to 10^7 cells/ml in buffer 2 (136mM NaCl, 2.7mM KCl, 0.42 NaH_2PO_4, 12mM $NaHCO_3$, 2mM MgCl, 1 mg/ml

BSA, 5.6mM glucose, 20mM HEPES pH 7.4) and allowed to "rest" for a minimum of 10 minutes at room temperature before use.

Platelets were incubated for 5 minutes in the presence of inhibitor. In order to provide sufficient intracellular calcium to activate Calpain, the calcium ionophore A23187 was added to a final concentration of 1μM. After a further 5 minute incubation, the platelets were harvested by centrifugation (1 min 10,000 x g) and resuspended in 10% sodium dodecyl sulfate, 10mM Tris pH=8.0, 5% ß-mercaptoethanol, 0.02% bromophenol blue, and heated to 95° C for 5 min. Samples were subjected to SDS-PAGE on 6% mini gels and transferred to nitrocellulose (Schleicher and Schuell BA83) for 2 hours at 100mA/gel in an LKB Novablot. Filters were blocked for 10 minutes in 0.25% gelatin, 1% BSA, 0.25% Triton X100, 0.9% NaCl, 10mM Tris-Cl pH 7.5, incubated overnight in the same solution containing antibody to rat spectrin, washed 3 X 10 minutes with 10mM Tris-Cl pH 7.5, 0.5% Triton X100, incubated 4 hours in wash buffer plus alkaline phosphatase conjugated goat anti-rabbit antibody (Biorad), and washed as above. Filters were developed using the Biorad AP conjugate substrate kit. Spectrin immunoreactivity on the filters was quantitated by densitometry.

The inhibition of Calpain within platelets as measured by the proteolysis of the endogenous Calpain substrate spectrin in the presence of inhibitors was assayed for a variety of Calpain Inhibitors. The poorly permeant inhibitors leupeptin and E-64 had little effect on intracellular Calpain. In contrast, the highly membrane permeant Heterocyclic Compounds, Peptide Keto-Compounds, and Halo-Ketone Peptides effectively inhibited platelet Calpain.

The following Heterocyclic Compounds were found to produce significant inhibition at 100 μM in the system of Example 4:

3-chloroisocoumarin

4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-amino-4-chloro-3-
(3-isothiureidopropoxy)isocoumarin

7-(benzylcarbamoylamino)-4-chloro-3-
(3-isothiureidopropoxy)isocoumarin

7-(phenylcarbamoylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(acetylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(3-phenylpropionylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(phenylacetylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(L-phenylalanylamino)-4-chloro-3-(3-isothiureidopropoxy)isocoumarin

7-(benzylcarbamoylamino)-4-chloro-3-(3-isothiureidoethoxy)isocoumarin

7-(phenylcarbamoylamino)-4-chloro-3-(3-isothiureidoethoxy)isocoumarin

7-(D-phenylalanylamino)-4-chloro-3-(3-isothiureidoethoxy)isocoumarin.

The following Halo-Ketone Peptides were found to produce significant inhibition at 100 μM in the system of Example 4:

Z-Leu-Phe-CH_2Cl

Ac-Leu-Phe-CH_2Cl

Z-Gly-Leu-Phe-CH_2Cl

Boc-Gly-Leu-Phe-CH_2Cl.

The following Peptide Keto-Compounds were found to produce significant inhibition at 100 μM in the system of Example 4:

Z-Ala-Ala-D,L-Abu-COOEt

Z-Ala-Ala-Ala-D,L-Ala-COOEt

MeO-Suc-Ala-Ala-Pro-D,L-Abu-COOMe

Z-Leu-Phe-COOEt

Z-Leu-Nle-COOEt

Z-Leu-Nva-COOEt

Z-Leu-Abu-COOEt

Z-Leu-4-Cl-Phe-COOEt

Z-Leu-Leu-Abu-COOEt

Z-Leu-Leu-Phe-COOEt

2-NapSO_2-Leu-Abu-COOEt

2-NapSO_2-Leu-Leu-Abu-COOEt

Z-Leu-Met-CO_2Et

Z-Leu-NLeu-CO_2Et

Z-Leu-Phe-CO_2Bu

Z-Leu-Abu-CO_2Bu

Z-Leu-Phe-CO_2Bzl

Z-Leu-Abu-CO_2Bzl

Z-Ala-Ala-D,L-Abu-COOBzl

Z-Leu-Phe-COOH

Z-Leu-Abu-COOH.

Among those compounds found to exhibit Calpain inhibitory activity in the homogenate system of Example 2, we found at least three compounds which failed to exhibit Calpain inhibitory activity in the platelet system of Example 4. These compounds are leupeptin, MeO-Suc-Val-Pro-D,L-Phe-COOMe and Bz-D,L-Phe-COOEt. Leupeptin is known to be poorly membrane permeant, thus confirming that the platelet assay will exclude known poorly membrane permeant compounds. Accordingly, the two Peptide Ketocompounds found not to provide Calpain inhibitory activity within platelets are also believed to be poorly membrane permeant, and would not be expected to cross the BBB.

EXAMPLE 4B

Quantitative Studies of Platelet Membrane Permeability

We performed additional quantitative or semi-quantitative studies on several Peptide Keto-Compounds using the assay of Example 4A, except that IC_{50} values were determined as the concentration at which 50% of the Calpain activation present in controls occurred. Results are shown in Table 4B. For the semi-quantitative assays, indicated with +'s in Table 4B, "+" indicates detectable inhibition at 100 μM, "++" indicates significantly more inhibition than "+", and "+++" indicates no detectable activation of Calpain detected.

TABLE 4B

Platelet Assay of Peptide Ketoamides, Ketoesters and Ketoacids

Inhibitor	IC_{50}
Z-Leu-Abu-COOEt	42
Z-Leu-Abu-COOnBu	28
Z-Leu-Abu-COOBz	+ +
Z-Leu-Leu-Abu-COOEt	40
2-NapSO_2-Leu-Leu-Abu-COOEt	100
Tos-Leu-Leu-Abu-COOEt	30
Z-Leu-Abu-COOH	8
Z-Leu-Abu-CONHEt	1.5
Z-Leu-Abu-CONHnPr	70
Z-Leu-Abu-CONHnBu	2.0
Z-Leu-Abu-CONHiBu	28
Z-Leu-Abu-CONHBz	1.5
Z-Leu-Phe-COOEt	42
Z-Leu-Phe-COOnBu	+ + +
Z-Leu-Phe-COOBz	+ +
Z-Leu-Leu-Phe-COOEt	+ +
Z-Leu-Phe-COOH	6.5
Z-Leu-Phe-CONHEt	1.7

Inhibitor	IC_{50}
Z-Leu-Phe-CONHnPr	24
Z-Leu-Phe-CONHnBu	38
Z-Leu-Phe-CONHiBu	22
Z-Leu-Phe-CONH$(CH_2)_2$Ph	3.0
Z-Leu-Nle-COOEt	20
Z-Leu-Nva-COOEt	40
Z-Leu-Met-COOEt	+
Z-Leu-4-Cl-Phe-COOEt	+

Table 4B shows that peptide α-ketoamides and ketoacids were much more effective than corresponding peptide ketoesters in this platelet assay. Extending the R_3 group to an alkyl group or an alkyl group substituted with a phenyl group increased the membrane permeability of the inhibitors as indicated by increased potency in the platelet assay. In view of these results, Applicants believe that extending the R group to include longer alkyl groups or alkyl groups substituted with phenyl groups would increase the membrane permeability of a given inhibitor.

In view of the foregoing, the results of Examples 4A and 4B support our belief that CI1 and the Substituted Heterocyclic Compounds, Peptide Keto-Compounds and Halo-Ketone Peptides are believed to be membrane permeant and therefore, are expected to be effective in crossing the BBB subsequent to *in vivo* administration of the compounds.

5. **Reduction of Glutamate Toxicity**

To further identify those Calpain Inhibitors likely to possess pharmacologically active neuroprotective ability, we tested the ability of the Calpain Inhibitors to protect against glutamate excitotoxicity. Excess extracellular glutamate is thought to play a key

role in the induction of neuropathology in ischemia, which is accompanied by Calpain activation. In support of this role for excess glutamate, cultured N18-RE-105 (a neuroblastoma-retinal hybrid) cells can be killed by the addition of glutamate into the culture medium. This glutamate-mediated cytotoxicity is calcium dependent and can be reduced through a number of mechanisms, including free radical scavengers, blockers of the N-type voltage-sensitive calcium channel, and quisqualate-subtype glutamate antagonists. Thus, glutamate-mediated killing of N18-RE-105 cells is an *in vitro* model for neuropathology.

Accordingly, we tested the ability of the Calpain Inhibitors to inhibit glutamate-induced cell death in these cells in order to establish that the Calpain Inhibitors can decrease or prevent glutamate-induced death Of N18-RE-105 cells. Some of these tests are shown in Example 5A.

EXAMPLE 5A

Inhibition of Glutamate-Induced Cell Death

Stock cultures of N18-RE-105 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and supplemented with hypoxanthine, aminopterin and thymidine (HAT). Subconfluent cultures were split and plated into 96-well plates. Twenty-four hours after plating the cells were exposed to fresh media containing glutamate and various concentrations of Calpain inhibitors. Control cells were not treated with glutamate. The treated cells received 5mM glutamate and leupeptin (5μg/ml) or the other Calpain Inhibitors listed in Figure 1 at 3μg/ml. Conversion of MTT was measured 19 hours later as described. Nineteen hours after the onset of exposure, cell viability was quantitated by measuring the extent to which the cells convert 3(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) to a blue formazan product, which occurs in the mitochondria of living but not dead cells (Pauwels et al., 1988). A higher absorbance is indicative of greater cell viability.

Figure 1 shows the percent of blue formazan product remaining after treatment with glutamate, relative to control where no glutamate was added. Thus, it can be seen that with vehicle plus glutamate but no inhibitor, less than 70% of the mitochondrial activity remains. However, Figure 1 shows that several Calpain inhibitors, including leupeptin, CI1 and representatives of the Heterocyclic Compounds, Peptide Keto-

Compounds and Halo-Ketone Peptides protect N18-RE-105 cells against glutamate toxicity. The Peptide Keto-Compound Calpain inhibitor, Z-Ala-Ala-Abu-CO_2Et, the Substituted Heterocyclic Compounds, CITPrOIC and ACITIC, and the Halo-Ketone Peptide, TPCK completely blocked the toxic effects of glutamate, resulting in 100% or greater of the formazan product as seen with cells not treated with glutamate. Thus, Example 5 shows that these Calpain Inhibitors effectively block cell death in an *in vitro* model for neuropathology. Accordingly, this data further supports our discovery that Calpain Inhibitors are neuroprotective *in vivo*.

We have discovered that glutamate-induced cell death in pheochromocytoma PC12 cells can be prevented by the membrane permeant calpain inhibitors Z-Leu-Phe-CONHCH$_2$CH$_3$ and Z-Leu-Nva-CONH(CH$_2$)$_3$ morpholine. These inhibitors rescue a greater proportion of the PC12 cells than calpain inhibitor 1 (Ac-Leu-Leu-Norleucinal) although higher concentrations can be required in certain instances. Z-Leu-Nva-CONH(CH$_2$)$_3$ morpholine also induces short processes in both the presence and absence of glutamate. We observed reduction in glutamate-induced cell death is observed even when calpain inhibitors are added several hours after glutamate. These observations are the first demonstration that Calpain inhibitors can rescue cells *in vitro* from glutamate toxicity and support the critical role of calpain activation in excitotoxicity. These experiments provide still further support that inhibition of calpain is useful in the treatment of neurodegeneration, such as stroke and ischemia.

The rat pheochromocytoma PC 12 is described by Greene et al, *Proc. Natl. Acad. Sci USA*, 73:2424-2427 (1976), the disclosure of which is hereby incorporated by reference. This tissue expresses the NMDA subtype of glutamate receptors. Treatment of PC12 cells with glutamate for 24 hours produces death of 80% of the cells as measured by conversion of Glutamic acid, 3-[4,5-dimethylthazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into its blue formazan product. When PC12 cells are exposed simultaneously to glutamate and calpain inhibitor, cell death is reduced. Thus, these cells can be used as an effective model to determine the effectiveness of the various calpain inhibitors to alleviate cell death. The experimental procedures we used to evaluate glutamate toxicity are described below in Example 5B.

Example 5B

Glutamate Toxicity Assay in Pheochromocytoma Cells

Cells of the RC72 subclone of the rat pheochromocytoma PC12 were grown in high glucose Dulbecco's modified Eagle's medium (DME) without glutamine with 10% fetal bovine serum and 5% horse serum and gentamycin. These cells are available from Dr. David Schubert of the Salk Institute. Media, horse serum, dialyzed fetal calf serum, and gentamycin were from Irvine Scientific. Fetal calf serum was from BioCell.

Prior to plating, cells were cultured for 2 passages (4 days) in the same media with glutamine. Cells were plated at 10,000 cells/well into 96 well plates coated with collagen and grown for 24 hours prior to experiments.

Exposure to glutamate was performed in DME without glutamine with 10% dialyzed fetal calf serum and 50 ug/ml gentamycin. Calpain inhibitors were added to cultures from DMSO stocks. Final DMSO concentrations did not exceed 0.1%.

After 24 hours exposure to glutamate and inhibitors, 20ul of 7.5 mg/ml Glutamic acid, 3-[4,5-dimethylthazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) in PBS was added to each well. MTT is available from Sigma. The cultures were incubated for 60 minutes and the media carefully removed. Detergent (10% Triton X-100, 0.4% concentrated HCl in isopropanol) was added and incubated for 10 minutes on a shaking table before the plates were read using a microplate reader. The difference between the absorbance at 655 and 595 nm was used as a measure of viability. All experiments were normalized to untreated cells in the same plate.

We tested a number of Calpain inhibitors for their ability to rescue PC12 cells from glutamate toxicity using the experimental protocol described in the foregoing example. The results are shown in Table 5B.

TABLE 5B

Inhibitor	Concentration μM	% of control
Ac-Leu-Leu-Arg-H (leupeptin)	5000	6.9
Ac-Leu-Leu-Nle-H (calpain inhibitor 1)	3	61
	30	34
E64	100	17
TPCK	100	12
Cystatin C	0.1IU/ml	23

Z-Leu-Phe-CONHCH2CH_3	100	77
Z-Leu-Abu-CONHCH_2CH_3	100	26
Z-Leu-Abu-CONH$(CH_2)_3$ morpholine	100	6.7
Z-Leu-Abu-CONH$(CH_2)_2$ phenyl	100	28
Z-Leu-Abu-$CONH_2$	100	11
Z-Leu-Nva-CONHCH_2CH_3	50	69
Z-Leu-Phe-CONH$(CH_2)_3$ morpholine	100	50
Z-Leu-Nva-CONH$(CH_2)_3$ morpholine	100	89

Reduction of glutamate-mediated cell death is produced by several different calpain inhibitors. Z-Leu-Phe-CONHCH_2CH_3 and Z-Leu-Nva-CONH$(CH_2)_3$ morpholine appeared to provide the best results in rescuing cells from glutamate toxicity. Inhibitors related to Z-Leu-Phe-CONHCH_2CH_3 also rescue PC12 cells from glutamate toxicity, although with varying efficacy. Substitution of Abu for Phe or Nva in the P1 position decreases the efficacy of the compounds. Several calpain inhibitors, including leupeptin and E64, did not rescue the cells. Leupeptin and E64 are known to be poorly cell-penetrating, providing further support for membrane-permeance as an important factor in the pharmacological effectiveness of the calpain inhibitors as used in the present invention. The poor calpain inhibitors cystatin C and TPCK also did not rescue the cells. This result is consistent with our conclusion that cell death is specifically the result of calpain activation and does not involve another protease with related specificity.

We further studied the more effective compounds, Z-Leu-Phe-CONHCH_2CH_3 and Z-Leu-Nva-CONH$(CH_2)_3$ morpholine. Figure 2 shows the results obtained using the procedures of the foregoing Example using different concentration of these two compounds along with calpain inhibitor 1 (CI1), a peptide aldehyde. The concentrations we used are as indicated. CI1 prevents glutamate-induced cell death at concentrations as low as 3uM but its efficacy does not increase but rather decreases with increasing concentration. We have observed toxicity by CI1 at higher concentrations; however, CI1 is not toxic by itself at concentrations below 10uM. Thus, we believe that this toxicity explains the observed increase in cell death at higher concentrations of CI1. The calpain inhibitors Z-Leu-Phe-CONHCH_2CH_3 and Z-Leu-Nva-CONH$(CH_2)_3$ morpholine exhibit

typical sigmoidal dose-response curves for cell rescue and produce nearly complete rescue at high concentrations.

We observed no toxicity of Z-Leu-Phe-$CONHCH_2CH_3$ or Z-Leu-Nva-$CONH(CH_2)_3$ morpholine at any concentration tested. This reduction of cell death by these compounds is dose-dependent with IC_{50} values of 20-50uM. The IC_{50} values of 20-50uM for Z-Leu-Phe-$CONHCH_2CH_3$ and Z-Leu-Nva-$CONH(CH_2)_3$ morpholine are significantly above the K_i's for calpain I or calpain II for these compounds. The K_i values for Z-Leu-Phe-$CONHCH_2CH_3$ and Z-Leu-Nva-$CONH(CH_2)_3$ morpholine are 200 nM and 250nM, respectively, using human erythrocyte calpain I, and 22nM and 100nM, respectively, using rabbit muscle calpain II. There are two possible explanations for the difference between the IC_{50} values we measured and the K_i's for these compounds using purified Calpain: less than complete penetration of the cell or the metabolism of the inhibitors by the cells. As discussed above, the poorly permeant inhibitor leupeptin is ineffective at preventing cell death. Thus, we believe that the difference between the K_i and IC_{50} values is due to membrane permeance effects.

We also evaluated the effect of glutamate concentration on the ability of Z-Leu-Phe-$CONHCH_2CH_3$ or Z-Leu-Nva-$CONH(CH_2)_3$ morpholine to alleviate cell death. Figure 3 shows the results obtained when PC12 cells were incubated with the indicated concentration of glutamate and no inhibitor (circles), 20uM Z-Leu-Nva-$CONH(CH_2)_3$ morpholine (triangles). or 30uM Z-Leu-Phe-$CONHCH_2CH_3$ (squares) for 24 hours and cell viability was assayed by MTT, as described in the Example. Values are expressed as % of naive control ± sem. At submaximal concentrations of these compounds, the rescuing effect can be overcome by high concentrations of glutamate. Thus, it is clear that the rescue of PC12 cells from glutamate toxicity is related to both the concentration of glutamate and to the concentration of inhibitor. We believe that the dependence on glutamate concentration is the result of the activation of multiple pathways of cell damage by the high concentration of glutamate. There is ample evidence for calpain-independent mechanisms of excitotoxic cell death. Observations by others that antioxidants such as vitamin E also rescue these cells from glutamate toxicity provide support for the idea that such calpain-independent mechanisms are operative. However, we have shown that inhibition of calpain alone is sufficient to alleviate cell death. Thus,

the use of Calpain inhibitors in accordance with the present invention is a signficant unexpected finding.

We also studied the ability of Calpain inhibitors to alleviate cell death after the cells have been exposed to glutamate. The results of this analysis are shown in Figure 4. Glutamate at 7.5mM was added at 0 time and Z-Leu-Phe-$CONHCH_2CH_3$ (squares) or Z-Leu-Nva-$CONH(CH_2)_3$ morpholine (triangles) added at the indicated times to final concentrations of 100μM each. Cell viability was measured 24 hours after the addition of glutamate by the MTT assay. Values are expressed in the Figure as % of naive control ± sem. It can be seen that addition of calpain inhibitors after the cells have been exposed to glutamate is only partially effective. Advantageously, some rescue of cell death is still observed if inhibitor is added as long as 2 hours after glutamate. However, by 8 hours after the glutamate addition, inhibitors no longer have an effect. Accordingly, it is advantageous to administer the Calpain inhibitors of the present invention within two hours of glutamate activation. Interestingly, at 8 hours after the addition of glutamate, the cells are still largely normal in appearance and are 100% viable by MTT assay regardless of the presence of inhibitor. This suggests that Calpain cleaves one or more cellular proteins which are essential for cellular functioning, and this perturbation results in cell death some hours later. Thus, it is still desirable to administer Calpain inhibitors within two hours of glutamate release, even where cell or tissue morphology remains normal.

We also evaluated morphological changes produced by the Calpain inhibitors. We exposed cells to 100 μM Z-Leu-Nva-$CONH(CH_2)_3$ morpholine alone or to 100 μM Z-Leu-Nva-$CONH(CH_2)_3$ morpholine and 7.5 μM glutamate. Cells exposed to both treatments show an altered morphology with short processes extending from the cell body which are rarely seen in the absence of inhibitor but are occasionally seen with glutamate alone in surviving cells. Inhibitor at high concentrations does not prevent some of the glutamate-induced decrease in the number of adherent cells, but these non-adherent cells remain viable as measured by MTT conversion. Incubation of PC12 cells with Z-Leu-Nva-$CONH(CH_2)_3$ for longer times (up to 72 hours) does not cause the expansion of these short processes into longer neurites, nor does it cause cytotoxicity. This morphological effect is not seen consistently with different calpain inhibitors, and is not caused by Cl1.

Excitotoxicity *in vivo*, as well as other forms of neurodegeration, are accompanied by the breakdown of the cytoskeletal protein spectrin, which we believe is mediated by calpain. The breakdown of spectrin *in vivo*, as well as the digestion of spectrin by calpain *in vivo*, produces not only the reduction in the amount of intact spectrin but also a characteristic doublet of spectrin breakdown products (BDP's) of molecular weight 150 and 155kDa. These BDP's appear to be unusually persistent *in vivo*. The detection of spectrin BDP's can be used as an assay for cellular degeneration, especially neurodegeneration. See U.S. Patent No. 5,118,606 to Lynch et al., the disclosure of which is hereby incorporated by reference.

We exposed PC12 cells to glutamate for 24 hours. Cells were extracted with CHAPS and analyzed for spectrin breakdown by western assay, as described in U.S. Patent No. 5,118,606. Analysis of the PC12 cells after glutamate toxicity reveals a decrease in the amount of intact spectrin but no striking increase in the 150 and 155kDa BDP's. The decrease in the amount of spectrin immunoreactivity cannot be accounted for by loss of protein from the samples as equal amounts of protein were loaded in each lane. Thus, in this assay the BDP's that are usually seen upon proteolysis appear to be degraded into small fragments not recognized in the western assay either directly or through the SBDP's more rapidly than is observed *in vivo*. We also added either Z-Leu-Phe-$CONHCH_2CH_3$ or Z-Leu-Nva-$CONH(CH_2)_3$ morpholine to the samples exposed to glutamate, and included in the western assay. The loss of spectrin immunoreactivity was prevented by the addition of either calpain inhibitor.

Our results show that calpain inhibitors can rescue PC12 cells from glutamate toxicity. Thus, inhibition of calpain represents an exciting new approach to the amelioration of ischemic and excitotoxic damage in stroke and other neurodegenerative processes.

6. Reduction of Infarction upon MCA Occlusion

Stroke is a significant health problem in the human population. Strokes are occlusions of cerebral arteries producing a decreased blood flow to brain regions, which cause cell death through oxygen and nutrient deprivation. This type of lesion can be modeled in rats by surgical occlusion of the middle cerebral artery (MCA). Several models for MCA occlusion have been developed, and all give substantially similar results.

MCA occlusion produces a large volume of infarcted brain tissue 24 hours after occlusion. Previous studies have shown that the size of the infarct as judged by TTC staining does not increase after the first 24 hours post-occlusion. Thus, we used an MCA occlusion model in order to test the ability of Calpain inhibitors to prevent neurodegeneration. This model is described in Example 6.

EXAMPLE 6

MCA Occlusion Model for Neurodegeneration

Male Sprague-Dawley albino rats weighing approximately 250-300 grams were anesthetized with pentobarbital (70 mg/kg, i.p.). The neck region was shaven and a 2 cm incision was made. The superficial fascia was teased away with tissue forceps and blunt tip tissue scissors using a spread method. The right common carotid artery was isolated away from the vagus nerve and tied off with a single suture (3.0 silk). The external carotid was permanently occluded by suturing. The bifurcation of the internal carotid and pterygopalatine arteries was exposed and a single microaneurysm clip was placed on the pterygopalatine. Another microaneurysm clip was placed on the common carotid just proximal to the external/internal bifurcation. A suture was placed loosely around the common carotid and a lumen was made in the vessel with the tip of a 25g needle. A 40 mm nylon suture was prepared by melting the tip to smooth the pointed end and marked with a dot exactly 17.5 mm from the melted end. The suture was inserted into the lumen of the artery as far as the vessel clip, the clip is removed and the suture advanced until the marking was at the bifurcation of the internal and external carotid arteries. This places the end of the suture in the circle of Willis just beyond the source of the middle cerebral artery and occludes this artery. The loose suture around the carotid is tied lightly to keep the nylon suture in place. The microaneurysm clip on the pterygopalatine artery was removed, the incision is closed and the animals are allowed to recover in heated recovery cages.

Twenty-four hours after occlusion, the brains of these animals were removed and sliced into 2mm sections. The sections were stained using 2,3,5-triphenyltetrazolium chloride as in Lundy, et al., *J. Pharmacol. Meth.*, **16**:201-214 (1986). Absence of red color development indicated tissue damage or death. The sizes of the infarcted tissue zone (area with red stain) and impaired zone (area with partial development of red color) were evaluated using quantitative morphometry.

Drugs or vehicle were administered by infusion into the femoral vein. All animals received the same volume of drug or vehicle (20% dimethyl sulfoxide/80% propylene glycol) via a catheter attached to an Alzet osmotic minipump (24 hr pump, 8 µl/hr, 90 ul total volume).

The model of Example 6 was used to determine the size of infarcted area for control (vehicle, i.v.) and with administration of each of two Calpain inhibitors: Z-Leu-Phe-CONH-Et and Z-Leu-Abu-CONH-Et. These results are depicted graphically in Figure 5. It can be seen that administration of either of the Calpain inhibitors Z-Leu-Phe-CONH-Et or Z-Leu-Abu-CONH-Et produces a reduction in the size of the infarcted area.

7. **Inhibition of Anoxic and Hypoxic Damage**

The CA1 region of hippocampus is a brain area particularly vulnerable to ischemic damage and other insults involving excitatory amino acids. The hippocampus is also a major focus of cell degeneration in Alzheimer's disease. Neural cells in slices *in vitro* degenerate following hypoxia through the same chain of events (including reperfusion effects) observed *in vivo* during and after ischemia. We believe that studies of degeneration of neural slices in the presence of the various Calpain Inhibitors is an effective indicator of the membrane permeance of the Calpain Inhibitors. Accordingly, we believe that these studies provide a model for the treatment and inhibition of neurodegeneration *in vivo*. Similar studies for determining the efficacy of compounds useful in the treatment of neurodegeneration in accordance with the present invention can be performed using other models, such as protection against degeneration in platelets or cells in culture.

It is believed that hypoxia is a major cause of neurotoxicity in a variety of neurodegenerative diseases and conditions, such as stroke and head injury. Thus, we conducted further studies using hippocampal slices to show that the various Calpain inhibitors, advantageously, can increase survival of hippocampal nerve cells during exposure to hypoxic or anoxic conditions. An initial screening procedure was first used to qualitatively determine whether the various Calpain Inhibitors can provide neuroprotection from anoxia in hippocampal slices. An example of these initial screening procedures is shown by Example 7A.

EXAMPLE 7A

Initial Screen for Inhibition of Anoxic Damage

Hippocampal slices (400 um) were prepared from Sprague Dawley rats (6 to 7 weeks) and maintained in an interface chamber at 35° C using conventional techniques, i.e., the lower surface of the slice received a constant perfusion (0.5 ml/min) of ACSF, while the upper surface was exposed to a moist atmosphere of O_2:CO_2 (95%:5%) exchanged at a rate of 2 L/min. The ACSF medium contains (in mM): NaCl (124), KCl (3), $KHPO_4$ (2.5), $CaCl_2$ (3.4), $NaHCO_3$ (26) and D-Glucose (10). Field excitatory post-synaptic responses were recorded from stratum radiatum of CAlb in response to stimulation of Schaffer-commissural fibers in CAla or CAlc. The depth of the recording electrode was optimized and evoked responses were collected at a rate of one evoked response every 30 seconds.

For the initial screening procedure, 14 to 16 slices are harvested from the hippocampus of a single rat and placed in a common ACSF bath. Each slice is tested in sequence to determine the magnitude of its pre-anoxic evoked response. Five stimulation pulses (each 0.1 ms (millisecond) in duration) were presented over a 15 second interval. The largest evoked response was noted and recorded for each slice.

Following this, the slices were incubated for one hour, with either drug or vehicle alone added to the ACSF. After the one hour drug incubation period, the oxygen-enriched atmosphere of the chamber was made anoxic by substituting nitrogen for oxygen (N_2 = 95%; CO_2 = 5%). The slices were retained in this anoxic environment for 10 minutes, following which the oxygen-enriched atmosphere (O_2 = 95%; CO_2 = 5%) was reestablished.

The slices were given the opportunity to recover for 30 minutes following reoxygenation whereupon each was stimulated and the maximum evoked potential determined, as described above during the pre-anoxia period. Those slices which, after anoxia, produced a maximum evoked potential of greater than 50% of that observed prior to anoxia were defined as surviving slices.

Results of the studies of Example 7A are shown in Figure 6. Figure 6 shows the effects of Z-Leu-Abu-CO_2Et, a Peptide Keto-Compound, and CI1 relative to control slices on survival of hippocampal slices exposed to 10 minutes exposure of anoxic atmosphere. As seen in this figure, when the control slices are deprived of oxygen for 10 minutes in the

absence of drug, virtually all fail to survive, as measured by their ability to elicit 50% of their pre-anoxia evoked response. In accordance with this finding, few if any recover upon reoxygenation. Figure 6 also shows that when CI1 or Z-Leu-Abu-CO_2Et are added to the ACSF, the slices are protected from the effects of anoxia, evidenced by a substantial proportion of slices eliciting evoked potentials.

Finally, it can be seen that Z-Leu-Abu-CO_2Et is significantly more effective in protecting against anoxia and preventing degradation of slices at the minimal 1 hour incubation time, and at lower concentrations than CI1. This effect is believed to be due to the superior membrane permeance of the Peptide Keto-Compounds.

Table 7A shows further data from the studies of Example 7A.

TABLE 7A

PERCENT OF SLICES SURVIVING TEN MINUTES ANOXIA

Compound	Dose (μM)	Incubation Time	Survival
Control	----	1 hour	<1%
Leupeptin	1000	3 hours	50%
CI1	200	2 hours	53%
SHC	20	1 hour	50%
HKP	50	1 hour	50%
PKC	100	1 hour	70%

It can be seen from the data in Table 7A that all of the Calpain Inhibitors tested provide increased survival. SHC, a Substituted Heterocyclic Compound is ACITIC; HKP, a Halo-Ketone Peptide, is Boc-Gly-Leu-Phe-CH_2Cl; and PKC, a Peptide Keto-Compound, is Z-Leu-Abu-CO_2Et. All are shown to be highly effective in influencing survival times. Leupeptin is seen to be the least effective neuroprotectant. Thus, we believe that ACITIC, Boc-Gly-Leu-Phe-CH_2Cl and Z-Leu-Abu-CO_2Et are more effective in influencing survival because of their membrane permeability. Accordingly, the results shown in Table 7A support our belief that Calpain Inhibitors with membrane permeability are effective neuroprotectants.

To further elucidate the ability of Calpain Inhibitors to provide neuroprotection to hippocampal slices, and to provide a more quantitative indication of the membrane permeability of these Calpain Inhibitors, we measured the effect of various Calpain

Inhibitors on the evoked response on a single neuronal slice before, during and after anoxia. These studies are shown in Example 7B.

EXAMPLE 7B

Inhibition of Anoxic Damage

As in Example 7A, hippocampal slices (400 μm) were prepared from Sprague Dawley rats (6-7 weeks) and maintained in an interface chamber at 35°C using conventional techniques, i.e. the lower surface of the slice received a constant perfusion (0.5 ml/min) of an artificial cerebrospinal fluid (ACSF), while the upper surface was exposed to a moist atmosphere of O_2:CO_2 (95%:5%) exchanged at a rate of 2 L/min. The ACSF medium contains (in mM): NaCl (124), KCl (3), $KHPO_4$ (1.25), $MgSO_4$ (2.5), $CaCl_2$ (3.4), $NaHCO_3$ (26) and D-Glucose (10). Field excitatory post-synaptic responses were recorded from stratum radiatum of CA1b in response to stimulation of Schaffer-commissural fibers in CA1a or CA1c. The depth of the recording electrode was optimized and evoked responses were collected at a rate of one evoked response every 30 seconds.

After establishing a stable baseline of evoked responses (approximately 10 minutes), ACSF containing Calpain Inhibitor was washed into the chamber and slices were incubated for a period of one hour. After incubation, evoked responses were again recorded and the change in the amplitude of the responses from baseline levels was noted. No effect of the inhibitors tested on baseline evoked responses was observed.

For anoxia experiments, incubation in the drug-containing medium was followed by replacement of the O_2:CO_2 (95%:5%) atmosphere with N_2:CO_2 (95%:5%). Slices were exposed to this anoxic environment until disappearance of the pre-synaptic fiber volley and for two minutes (severe anoxia) longer (total time in anoxic environment approximately 7-8 minutes in control case). Effects of Calpain Inhibitors on the functional recovery of the slices after the anoxic episode were then measured. Recovery of the evoked potential (EPSP) slope and amplitude by the drug treated slices can be compared to control slices to determine the relative efficacy of various Calpain Inhibitors.

Figure 7 shows the EPSP amplitude in millivolts for control, CI1 treated and Z-Leu-Abu-CO_2Et (a Peptide Keto-Compound) treated hippocampal slices in the studies of Example 7B. The periods of anoxia are represented by the black bars under the graph. It can be seen in Figure 7 that the control slices deprived of oxygen in the absence of drug

display a gradual reduction of EPSP and abruptly lose fiber volley activity about 5-6 minutes after the beginning of anoxia. Reoxygenation at or before this point leads to complete functional recovery after about 20 minutes of reoxygenation, but reoxygenation after this point does not. In the latter case the recovered EPSP slope and amplitude become progressively reduced as the duration of anoxia post-fiber volley disappearance (post-FVD) increases. After severe anoxia (2 minutes post-FVD), slices recover only 15% of the EPSP slope.

In contrast to the control slices, recovery begins to occur shortly after the end of anoxia for the treated slices. Figure 7 shows a comparison of the effects on EPSP amplitude produced in the presence of no inhibitor; the Peptide Keto-Compound, Z-Leu-Abu-CO_2Et and CI1. Z-Leu-Abu-CO_2Et produces a recovery from severe anoxia superior to that seen with CI1.

Figure 8 shows the percent recovery of EPSP from severe hypoxia using the peptide ketoester Z-Leu-Phe-CO_2Et and its corresponding peptide ketoamide Z-Leu-Phe-CONH-Et. These studies were performed in a manner similar to that of Example 7B, except using a hypoxic environment in place of the anoxia of Example 7B. It can be seen that use of the peptide ketoamide results in essentially complete (near 100%) recovery from hypoxia while the peptide ketoester produces a partial recovery. The control slices experienced little or no recovery.

An interesting characteristic that we have discovered for certain Calpain Inhibitors is their ability to lengthen the period of exposure to anoxia required to produce fiber volley disappearance (FVD). Typically, under control anoxia conditions, fiber volley disappearance occurs in less than six minutes (Figure 9). The Peptide Keto-Compound, Z-Leu-Phe-CO_2Et, substantially lengthens the period of exposure to anoxia required to produce FVD. This is an important advantage of the use of this Peptide Keto-Compound for neuroprotection because slices can be expected to recover completely if reoxygenated before fiber volley disappearance. Thus, treatment with this Peptide Keto-Compound is expected to produce a greater percentage of recovery of cells from incipient neurodegenerative conditions. It is believed that other representatives of the Peptide Keto-Compounds as well as of other classes of Calpain Inhibitors also provide this effect.

Table 7B shows the perecentage of recovery of pre-anoxia synaptic transmission (evoked potential amplitude) of slices treated with various Calpain Inhibitors or of control

slices. All of these slices were exposed to ten minutes of anoxia according to the protocol of Example 7B.

TABLE 7B

PERCENT RECOVERY OF SYNAPTIC TRANSMISSION AFTER ANOXIA

Compound	Concentration	% Recovery
Control	---	15
CI1	200	35
SHC	20	60
HKP	50	30
PKC-1	100	38
PKC-2	100	55

The results shown in Table 7B provide further evidence that the peptide aldehyde, CI1, as well as the Substituted Heterocyclic Compounds (SHC) represented by ACITIC, Halo-Ketone Peptides (HKP) represented by Boc-Gly-Leu-Phe-CH_2Cl, and Peptide Keto-Compounds (PKC) represented by Z-Leu-Phe-CO_2Et (PKC-1) and Z-Leu-Abu-CO_2Et (PKC-2) are sufficiently membrane permeant to provide neuroprotection through Calpain inhibition.

CI1, which is at least partially membrane permeant, produces some effect, however, does not significantly lengthen the period of anoxia required to suppress electrical activity. For example, see Figure 9. Thus, compared to control, or even compared to leupeptin and CI1, the Substituted Heterocyclic Compounds, Peptide Keto-Compounds and Halo-Ketone Peptides can increase the degree of recovery after anoxic episodes while producing the additional advantage of extending the amount of time slices can tolerate anoxia and thereby recover completely.

An important effect of the Peptide Keto-Compounds and other membrane permeant Calpain Inhibitors is that they are significantly more effective in lower doses than less permeable Calpain Inhibitors such as CI1. Although CI1 is shown to be at least somewhat membrane permeant due to its ability to affect slice survival, the more membrane-permeant inhibitors provide significantly increased protection. Thus, the more highly membrane-permeant Calpain Inhibitors are believed to be especially effective in treating and inhibiting neurodegeneration.

The results of the studies of Examples 7A and 7B show that the Substituted Heterocyclic Compounds, Peptide Keto-Compounds and Halo-Ketone Peptides are membrane-permeant Calpain Inhibitors which are believed to be especially effective in treating and inhibiting neurodegeneration. The results also show that Peptide Keto-Compounds, and perhaps representatives of other classes, can extend the duration of anoxia required to suppress electrical activity in hippocampal slices. As discussed above, these effects are important advantages of these compounds.

8. **in vivo Neuroprotection by Calpain Inhibitors**

As discussed above, therapeutics useful for influencing the function of cells within the CNS must cross the BBB to reach their targets within the CNS. Non-BBB permeant compounds might, in addition to the brain infusion techniques described above, be administered via intraventricular administration, but this also severely limits their usefulness in practice. In order to test the *in vivo* effectiveness of the Calpain Inhibitors to cross the BBB and become therapeutically useful, we tested the ability of intraperitoneal injection of the Calpain Inhibitors to protect against excitotoxic damage *in vivo*. Protection was measured by evaluating changes in behavior of rats after injection with kainate. These studies are shown in Example 8A.

EXAMPLE 8A

Protection Against Behavioral Changes from Excitotoxic Damage by Peripherally Administered Calpain Inhibitors

Rats (male Sprague-Dawley, 200±5 gms) were injected intraperitoneally with 12mg/kg kainic acid in saline vehicle and either 200µl DMSO (dimethylsulfoxide) or 4.6mg calpain inhibitor dissolved in the same volume of DMSO. The rats were observed for six hours following the injections and the kainate-induced behavioral symptoms and convulsions scored on a scale of 0-6 (0=no symptoms; 1=wet dog shakes; 2=salivation and chewing; 3=at least one convulsive episode; 4=repeated or sustained convulsions; 5=convulsions, including rearing and falling; 6=convulsions followed by death within the 6 hrs post injection).

Figure 10 shows the effects of CI1 on the behavioral and convulsive effects of kainic acid. In the control group, over half the animals showed symptoms greater than mild behavioral symptoms, and many exhibited overt convulsions, presumably reflecting seizure activity within the brain. Unexpectedly, in the inhibitor treated group, the

incidence and severity of convulsions was reduced. Thus, this data suggests that Calpain Inhibitors have an anti-convulsive effect. This effect is a distinct advantage in the use of Calpain Inhibitors in epilepsy-related neurodegenerative conditions and in stroke, which is often accompanied by seizures.

In order to more clearly demonstrate that the behavioral and anti-convulsive effects seen with the Calpain Inhibitors result from inhibition of Calpain we tested the brain tissues of the rats from Example 8A for accumulation of spectrin BDP's. As discussed above, these BDP's are associated with Calpain activity and with the neurodegeneration associated therewith.

EXAMPLE 8B

Protection Against Spectrin Breakdown from Excitotoxic Damage by Peripherally Administered Calpain Inhibitors

Four days following the injection of kainate in the rats from Example 8A, the brains of the rats were removed and assayed for spectrin BDP's. Spectrin BDP's were assayed by homogenizing brain parts in 20mM Tris pH=7.2, .32M sucrose, 50μM Ac-Leu-Leu-nLeu-H on ice. Homogenates were mixed 1:1 with 10% SDS, 5% ß-mercaptoethanol, 10% glycerol, 10mM Tris pH=8.0, 0.5% bromophenolblue, heated to 95°C, and subjected to electrophoresis in 4-1/2% polyacrylamide gels. The proteins in the gels were transferred to nitrocellulose and the spectrin and BDP's detected using a rabbit polyclonal anti-spectrin antibody and established immunodetection methods. The amount of spectrin and BDP's in each sample was quantitated by densitometric scanning of the developed nitrocellulose.

Figure 11 shows the results of Example 8B. It can be seen that kainate stimulated the breakdown of spectrin in both Calpain Inhibitor treated and control rats. However, treated rats exhibited significantly less BDP's. These results verify that Calpain activity in the brains of the treated rats was reduced. An unexpected observation was that even those treated animals that exhibited severe seizures had significantly less spectrin breakdown than untreated animals subjected to kainate. Thus Calpain Inhibitor treatment reduced both the behavioral/convulsive effects of kainate and the activation of calpain in the most severely affected animals.

9. **Conclusion**

All of the foregoing studies support our discovery that Calpain Inhibitors provide *in vivo* protection against neurodegeneration associated with anoxia, excitotoxicity and other causes. Thus, these Calpain inhibitors possess neuroprotective activity against a variety of *in vivo* neurodegenerative diseases and conditions, including excitotoxicity, HIV-induced neuropathy, ischemia following denervation or injury, subarachnoid hemorrhage, stroke, multiple infarction dementia, Alzheimer's Disease (AD), Huntington's Disease, Parkinson's Disease, surgery-related brain damage and other pathological conditions.

Those Calpain Inhibitors which possess significant Calpain Inhibitory activity *in vitro* and also meet at least one of the foregoing or different tests for membrane permeability are excellent candidates for treatment of neurodegeneration.

G. TREATMENT OF NON-NEUROLOGICAL CONDITIONS

A number of medical conditions associated with increased Calpain activity can be treated with the Calpain Inhibitors of the present invention. These Inhibitors are administered to a mammal having a medical condition which is caused at least in part by the proteolytic activity of Calpain. Specific medical conditions are described below which benefit from the administration of Calpain Inhibitors.

1. **Treatment of Cardiac Muscle Tissue Damage**

Damage to the cardiac muscle tissue of a mammal can be slowed or prevented by the administration of Calpain Inhibitors. To treat a mammal, such as a human patient, who has cardiac muscle tissue damage with Calpain Inhibitors, that patient is first identified by screening patients for those with symptoms of having cardiac tissue damage. Examples of such patients include those who have experienced heart attacks. Other groups likely to have experienced cardiac tissue damage include victims of violent assault whose thoracic cavities have received a physical insult, as well as those who have suffered from viruses or other pathogenic agents known to attack the heart muscle. After identifying such people, routine tests, such as ultrasound and magnetic resonance imaging, are then used to determine whether or not they actually have suffered cardiac muscle tissue damage.

Calpain Inhibitors can be administered to people with damaged myocardial tissue in a number of ways. The most direct method of administration is the injection

of up to a liter of a Calpain Inhibitor solution directly into the damaged tissue, where the Calpain Inhibitor is at a concentration in the range of between 0.001 mg/ml and 10 mg/ml, and preferably 0.01 mg/ml, of a Calpain Inhibitor. Any pharmaceutically acceptable carrier vehicle may be used to carry the Calpain Inhibitor. This method of administration is normally used only when the myocardium is exposed and undergoing treatment, as during surgery. The direct infusion of a Calpain Inhibitor into the heart in the foregoing concentrations with a catheter, or the injection of such a Calpain Inhibitor into the pericardial space, in order to achieve the local administration of the Inhibitor is another way of treating the heart muscle when tissue damage is occurring.

The intravenous or intramuscular administration of a Calpain Inhibitor is preferred, however, when a patient is not undergoing surgery or other invasive treatment. A Calpain Inhibitor in a pharmaceutically acceptable solution is injected once daily in a dosage of between approximately 0.001 mg/kg of bodyweight and 100 mg/kg, and preferably between 0.01 mg/kg and 10 mg/kg, to treat cardiac muscle tissue damage. More preferably, a Calpain Inhibitor is administered several times per day at appropriately reduced dosages. More preferably still, a Calpain Inhibitor is infused slowly into a patient by drip infusion, to ensure that the Calpain Inhibitor is present in the bloodstream in a relatively constant concentration. Of course, oral formulations of a Calpain Inhibitor can also be administered, and other methods of administration known to the art can be used as well.

Calpain Inhibitors can also be used to treat a mammal at risk for suffering damage to that mammal's cardiac tissue. Thus, myocardial infarctions can be prevented or decreased in size through the administration of a Calpain Inhibitor to such a mammal. A mammal, such as a person, at risk for suffering damage to its myocardial tissue is identified by screening a population for people with symptoms that indicate a higher-than-average risk for suffering a heart attack, including shortness of breath, obesity, high blood pressure, and high levels of cholesterol in the blood. Preferably, people who have been diagnosed as having cardiac ischemia, who have experienced a mild heart attack, or who have other symptoms which indicate that they are at risk for suffering a serious heart attack in the near future are identified. Those at risk for suffering cardiac tissue damage can be at least partially protected from such damage by taking Calpain Inhibitors prophylactically. The screening of a population

for people who would benefit from Calpain Inhibitor therapy is normally done by a physician in the course of examining that physician's patients, but might also be done through a health screening program sponsored by an employer or a school.

Once an individual who would benefit from the administration of a Calpain Inhibitor has been identified, an Inhibitor can be administered in a number of ways. One method of administration is to inject an Inhibitor only once at a relatively large dosage in order to treat a person suffering from acute myocardial ischemia (a heart attack). In this case, between approximately 0.001 mg/kg and 100 mg/kg, and preferably between 0.01 mg/kg and 1 mg/kg, of an Inhibitor suspended or dissolved in an appropriate pharmaceutical carrier can be injected intravenously into such an individual. In this way, the acute damage to the myocardium suffered by heart attack victims can be avoided or reduced.

Inhibitors can also be injected several times over the course of a period of time, or they can be infused intravenously at a steady rate for a period of time in order to protect an individual at risk for suffering cardiac tissue damage. Individuals are administered between approximately 0.001 mg/kg and 100 mg/kg, and preferably between 0.01 mg/kg and 10 mg/kg, of a Calpain Inhibitor daily. This route of administration is preferred for individuals who are at high risk for suffering a myocardial infarction in the near future, and such administration may be continued as long as such a risk of having a heart attack remains.

In addition, other methods of administering a Calpain Inhibitor are possible to protect an individual from myocardial tissue damage. The oral administration of a Calpain Inhibitor in tablet or liquid form is preferable for long term Calpain Inhibitor therapy, because such routes of administration are easier for an individual to administer to him or herself. Between approximately 0.001 mg/kg and 100 mg/kg, and preferably between 0.01 mg/kg and 10 mg/kg, of a Calpain Inhibitor is administered daily in this form of Calpain Inhibitor therapy.

EXAMPLE 9

The effect of a Calpain Inhibitor is tested on rabbits by inducing a region of ischemic myocardium and administering a Calpain Inhibitor. Cardiac ischemia is induced in rabbit myocardium by ligating the coronary artery followed by ligation of the branches of the left circumflex artery adjacent to the ischemic (cyanotic) area when the

epicardial cyanotic area reaches 0.75 to 0.80 of the length of the long axis of the left ventricle (measured between the atrioventricular groove and the cardiac apex). This produces a coronary infarct of regular size. A Calpain Inhibitor is administered intravenously at 10 mg/kg/hour for two hours beginning either 5 minutes before or just after the ligations. In both cases, groups treated with a Calpain Inhibitor exhibit smaller infarcts than those treated with a pharmaceutical carrier alone without a Calpain Inhibitor.

EXAMPLE 10

A human diagnosed as having suffered a mild heart attack is prescribed an oral formulation of a Dipeptide α-Ketoamide (Subclass A) Calpain Inhibitor providing 2 mg/kg of the inhibitor. The formulation is taken once per day to protect the individual in the event he or she suffers a further heart attack.

EXAMPLE 11

A human who has been picked up by an emergency team within several minutes of suffering a heart attack is administered an injectable composition providing 10 mg/kg of a Dipeptide α-Ketoamide (Subclass B) Calpain Inhibitor. In this way permanent tissue damage to the myocardial tissue is avoided.

2. Treatment of Skeletal Muscle Tissue Damage

Damage to muscle tissues can also be prevented or reduced through the administration of Calpain Inhibitors. Research has shown that Calpain is involved in the degeneration of muscle tissues. During the autolysis of muscle fibers, for example, Calpain degrades the Z-disc of skeletal muscle myofibrils. Calpain also degrades myosin, a muscle protein, over a wide range of protease concentrations. Since Calpain is activated by elevated intracellular levels of calcium, any rise in such levels can lead to damage to a muscle cell. Any condition in which intracellular calcium levels become elevated, therefore, can be treated with a Calpain Inhibitor in order to prevent or limit the damage to skeletal muscle tissue due to the activity of Calpain.

Calpain Inhibitors can be used to treat skeletal muscle tissue damage caused by a variety of factors. A physical insult to skeletal muscle tissue that damages the muscle cell membrane and results in increased intracellular levels of calcium, for example, can be treated with a Calpain Inhibitor, which blocks the proteolysis of muscle cell

constituents. Muscular dystrophy, a condition characterized by elevated intracellular calcium levels, can also be treated with Calpain Inhibitors.

Identifying an individual with skeletal muscle tissue damage is normally done by a physician. A physician, for example, can identify an individual with the clinical symptoms of muscular dystrophy. Other damage to skeletal muscle tissue can likewise be diagnosed.

As with the treatment of myocardial tissue, the route of administration of Calpain Inhibitors to skeletal muscle tissue will vary, depending on the site of the damaged tissue. An injury to the bicep is most directly treated through an intramuscular injection of a Calpain Inhibitor into the bicep muscle of the arm, the site of injury. A more systemic condition, such as muscular dystrophy, however, is better treated through a systemic injection of a Calpain Inhibitor. Such an injection can be intramuscular, intraperitoneal, or intravenous. Alternatively, the oral administration of Calpain Inhibitors can effect systemic administration of such Inhibitors.

EXAMPLE 12

An individual is diagnosed with Duchenne's muscular dystrophy by a physician. To treat that condition, between approximately 0.1 mg/kg and 10 mg/kg of a Calpain Inhibitor in a phosphate-buffered saline solution is injected intravenously into the individual once per day for the course of the treatment.

3. Treatment of Smooth Muscle Injury

Calpain is involved as well in the breakdown of smooth muscle cells. It has been reported that certain smooth muscle cell proteins, such as calponin, are degraded by Calpain. As indicated by studies of the proteolytic proclivity of Calpain in other tissues, Calpain poses a threat to smooth muscle cells whenever those cells experience elevated intracellular calcium levels. In particular, mammals which have experienced physical damage to their smooth muscle tissue or whose blood circulation to their smooth muscle tissue has been cut off would benefit from the administration of Calpain Inhibitors. Prophylactic administration of such Inhibitors reduces the damage done by such conditions. Administration of Calpain Inhibitors after smooth muscle tissue has been damaged, however, is also beneficial since such Inhibitors can slow or stop the progress of the proteolytic activity of Calpain in such tissues.

The identification of an individual with smooth muscle tissue damage, such as an intestinal ulcer, is normally done by a physician. After diagnosing an individual as having damage to her smooth muscle tissue, the physician can give a Calpain Inhibitor to that individual to slow further damage to such smooth muscle tissue.

The administration of a Calpain Inhibitor to smooth muscle tissue can be accomplished by any method known to the art. While intramuscular injection is a possible route of delivery, in most cases intravenous, intraperitoneal, or oral delivery will be preferred, depending on the location of the damaged tissue. For example, someone with an ulcer is more effectively treated with an oral delivery of a Calpain Inhibitor, since the Inhibitor can then coat the affected area directly in the course of passing through the digestive system. Intravenous delivery can also be used, however, and would be preferable if the Inhibitor is itself upsetting to the digestive system of the individual or if the low pH of the stomach interferes with the effectiveness of the Inhibitor. Between approximately 0.001 mg/kg and 100 mg/kg, and preferably between 0.01 mg/kg and 10 mg/kg is used to treat smooth muscle tissue damage.

EXAMPLE 13

An individual is diagnosed as having an ulcer by her physician, and is prescribed a Calpain Inhibitor along with other ulcer medications. Approximately 2 mg/kg of the Calpain Inhibitor is administered orally in tablet form to the individual with every meal as long as the individual is experiencing the ulcer.

4. Treatment of Smooth Muscle Contraction

The tonic contraction of smooth muscle in appropriate circumstances is a normal process. However, in inappropriate circumstances, tonic contraction can lead to serious pathological conditions. For example, contraction of the bronchial smooth muscle leads to the shortness of breath and other symptoms of asthma. Contraction of the coronary arteries can lead to angina, partial coronary hypoxia and subsequent loss of coronary function. Contraction of the smooth muscle in cerebral arteries can lead to cerebral vasospasm and hypoxia of the brain tissue, a serious condition that can leave patients mentally disabled and permanently brain damaged.

Angina generally results from both an occlusive and a spastic component. In some patients, angina is largely a result of either the occlusive or spastic component.

Advantageously, the Calpain Inhibitors of the present invention can be used to treat both components of angina.

Vasospasm is a condition which affects smooth muscles, particularly blood vessels. Vasospasm is a sustained spastic or tonic contraction of the vascular tissue. Such contractions are associated with a rise in intracellular calcium levels. Calpain activity has been linked to these contractions, and contracted blood vessels can be dilated with Calpain Inhibitors.

An individual experiencing vasospasm is normally identified by a physician. A physician might, for example, detect vasospasm during surgery, or might detect vasospasm indirectly through the observation of external symptoms. Vasospasm is also often detected by angiogram.

Vasospasms frequently occur as a result of subarachnoid hemorrhage, which causes blood clots. Such blood clots are believed to provide factors that promote vasospasm. Such hemorrhaging is therefore an indication that an individual is at risk for vasospasm.

It is one of the surprising discoveries of the present invention that Calpain Inhibitors can be used to block the establishment of the tonic state, or to relax tonically contracted smooth muscle. We have found Calpain Inhibitors of several classes to be particularly useful in this regard, including the Substituted Heterocyclic Compounds, Peptide Keto-Compounds, Peptide Aldehydes, Halo-Ketone Peptides. Various subclasses of compounds within these broad classes that we have found to be useful in the inhibition of tonic smooth muscle contraction include the peptide ketoamides, chloromethyl ketone peptides, epoxysuccinates, diazomethane peptides and peptide aldehydes.

The Calpain Inhibitors of the present invention provide a number of advantages in the treatment of smooth muscle contraction. These inhibitors provide unexpectedly high efficacy in the treatment of smooth muscle contraction, such as in the treatment of vasospasm disorders of many types. Furthermore, we have shown that Calpain Inhibitors of the present invention are additionally beneficial because they have relatively little effect on resting tension in smooth muscle. Thus, we consider these Calpain Inhibitors to be particularly useful in the treatment of human subjects without adverse side effects.

The administration of Calpain Inhibitors to prevent smooth muscle tonic contraction or to relax tonically contracted smooth muscle can be by any of a number of methods known to those of skill in the art. Such methods include the systemic delivery of Calpain Inhibitors through intravenous, intraperitoneal, or intramuscular injection, or through oral delivery, as described hereinbelow. Administration of the Calpain Inhibitor for this purpose can also be through use of a catheter system such as will be readily known to those having skill in the art. A solution of Calpain Inhibitor can be injected directly into the cerebrospinal fluid. Aerosolization of solutions containing a Calpain Inhibitor is a preferred mode of administration. Finely dispersed dry powders can also be used successfully. Other known methods of delivery are also acceptable. Thus, formulations including Calpain Inhibitors can be of many forms, including tablets, troches, solutions, powders and the like, as described herein.

In order to induce dilation of a spastic or tonically contracted blood vessel and thereby reverse vasospasm, a Calpain Inhibitor can be administered by direct topical application to the blood vessel. This method, of course, necessitates the exposure of the blood vessel so that it can be physically manipulated, and thus requires surgery. A Calpain Inhibitor at a concentration of approximately 1 - 500 μM can be topically applied to achieve vasodilation.

However, when it is desired to treat vasospasm without surgery, a Calpain Inhibitor can be administered intravenously. In this event, between approximately 0.001 mg/kg and 100 mg/kg, and preferably between 0.01 mg/kg and 10 mg/kg, of a Calpain Inhibitor suspended or dissolved in a pharmaceutically acceptable carrier is administered once to an individual. The oral administration of a Calpain Inhibitor in like amounts is also possible, although this route of administration would not be as fast-acting as intravenous administration.

For inhibition of other tonic smooth muscle contractions, between approximately 0.001 mg/kg body weight and 100 mg/kg body weight of a Calpain Inhibitor can be administered daily, divided into one to eight doses, or via continuous infusion intravenously. More preferably, the daily dosage to an individual to prevent or relax tonic smooth muscle contraction would provide between 0.01 mg/kg to 10 mg/kg body weight. Optimum dosages can be determined for each particular Calpain Inhibitor using techniques known to those having ordinary skill in the art.

Higher concentrations of Calpain Inhibitors can be administered through the direct application of such Inhibitors to the smooth muscle tissue. For example, the Calpain Inhibitor can be loaded into a microsphere and the microsphere driven into the smooth muscle to effect direct application. For the relaxation of spastic arteries, the artery can be surgically exposed and a solution of Calpain Inhibitor applied directly to the artery. Calpain Inhibitors can also be delivered through the use of a slow-release compound, such as a gel or ointment, applied directly to the smooth muscle tissue during surgery. Local administration strategies include inhalation formulations for use in treating bronchospasm.

In the following examples, certain specific Calpain Inhibitor compounds were tested in order to verify the results of the present invention. These drugs are specifically identified hereinabove in the Brief Description of the Figures, upon reference to the figures referred to in the Examples. Drugs A, B, F (the same drug as B), G, H, J and CX are all Peptide Keto-Compounds that are inhibitors of Calpain. Drug H is another inhibitor of Calpain. Drugs C, E and I are not inhibitors. Drug D is a relatively poor inhibitor that is a compound of the Halo-Ketone Peptide class.

EXAMPLE 14

Isolated arteries *in vitro* were treated with 10^{-8}M endothelin (ET) to induce contraction of the smooth muscle. The arteries were then treated with Calpain Inhibitor at a concentration of between 10^{-7}M and 10^{-4}M. The results of this procedure are shown in Figures 12 and 13 for Calpain Inhibitors A-J, as described hereinabove in the Brief Description of the Figures. These Figures show that administration of a Calpain Inhibitor can effectively reduce endothelin-induced contraction of isolated arteries *in vitro*.

EXAMPLE 15

Isolated arteries *in vitro* were treated with 10^{-7}M phorbol dibutyrate (PDB) to induce contraction of the smooth muscle. The arteries were then treated with compounds E-J (as described hereinabove in the Brief Description of the Figures) at a concentration of between 10^{-6}M and 10^{-4}M. The results of this procedure are shown in Figure 14. This Example demonstrates that administration of a Calpain Inhibitor in accordance with the present invention can effectively reduce PDB-induced contraction of isolated arteries *in vitro*.

EXAMPLE 16

Isolated arteries *in vitro* were treated with Calpain Inhibitors E-J (as described hereinabove in the Brief Description of the Figures) at concentrations of $3x10^{-6}M$, $10^{-5}M$, and $3x10^{-5}M$. The tension in the arteries, in mg, was then measured. The results are shown in Figure 15. This example verifies that Calpain Inhibitors of the present invention have relatively little effect on resting tension in smooth muscle.

EXAMPLE 17

The protective effect of Z-Leu-Phe-$CONH(CH_2)_3$ on the acute phase of vasospasm was evaluated in live rabbits. Arterial contraction measured for approximately 10 minutes in order to establish a baseline. Z-Leu-Phe-$CONH(CH_2)_3$ at concentrations ranging from 100 μM to 300 μM, or vehicle was then administered to the animals. At a time approximately 60 minutes after establishment of a baseline, oxyhemoglobin was administered in order to induce constriction. Figure 16 shows that higher concentrations of Calpain Inhibitor resulted in less constriction, with virtually no constriction occurring in the animal receiving the Calpain Inhibitor at 300 μM. Constriction was reversed by administration with aCSF.

EXAMPLE 18

Figure 17 provides an example of the effect of a Calpain Inhibitor of the present invention on an artery in a live animal that was constricted by subarachnoid hemorrhage (SAH). The "normal" resting size of the vessel, as estimated from an age-matched control animal, was approximately 750 μm. The vessel was constricted to approximately 400 μm following hemorrhage. Perfusion of aCSF alone for 90 minutes had no effect. Z-Leu-Phe-$CONH(CH_2)_3$ at 100 μM reversed the SAH-induced constriction by approximmately 60% to about 600 μm.

EXAMPLE 19

Three rabbits were subjected to SAH resulting in constriction of cerebral arteries. Z-Leu-Phe-$CONH(CH_2)_3$ was then administered to produce a concentration of 100μM and the amount of relaxation measured. Figure 18 shows the summary of data from all three animals. In all three, Z-Leu-Phe-$CONH(CH_2)_3$ reversed constrictions induced by SAH. The constricted diameter is taken as the "100% value" in this graph (unlike in Figure 16) and the relaxation is expressed as a percentage of

the 100 % value. The data were analyzed in this fashion because the absolute amount of consstriction following SAH varies from animal to animal.

EXAMPLE 20

A human diagnosed as suffering from tonic smooth muscle contraction is intramuscularly administered approximately 10 mg of a Calpain Inhibitor, such as Z-Leu-Phe-CONH$(CH_2)_2$Ph, in a phosphate buffered saline solution by intravenous injection at least once per day for approximately one week or until it is determined that the risk of tonic contraction has subsided.

EXAMPLE 21

A human diagnosed as suffering from angina associated with coronary artery vasospasm is orally administered approximately 100 mg of Z-Leu-Phe-CONHEt. The Calpain Inhibitor is delivered by surgically exposing the artery and applying a solution of Calpain Inhibitor in a phosphate buffered saline solution directly to the artery.

EXAMPLE 22

A human diagnosed as suffering from asthma associated with bronchospasm is administered between approximately 100 mg of $(Ph)_2$CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph by inhalation. The Calpain Inhibitor is delivered by inhalation of a formulation containing the Calpain Inhibitor directly into the patient's lungs.

EXAMPLE 23

A human diagnosed a suffering from cerebral vasospasm is administered approximately 100 mg of Z-Leu-Phe-CONHEt into the CSF. The Calpain Inhibitor is delivered by injecting said Calpain Inhibitor in a phosphate buffered saline solution directly into the patient's cerebrospinal fluid.

EXAMPLE 24

During surgery, a blood vessel is discovered to be experiencing tonic smooth muscle contraction. A solution containing a 100μM solution of a Calpain Inhibitor is topically applied to the contracted blood vessel. If one application fails to produce full dilation of the blood vessel, further topical applications are performed.

EXAMPLE 25

Cerebral vasospasm in an individual is detected, and a solution containing approximately 2 mg/kg of a Calpain Inhibitor is dissolved in phosphate buffered saline and then injected intravenously into the individual.

EXAMPLE 26

We tested the ability of several Calpain Inhibitors to dilate *ex vivo* blood vessels treated to induce vasospasm. We found that Calpain Inhibitor-1 provided low activity in inducing vasodilation, while at least three Peptide Ketoamide Calpain Inhibitors within the scope of the present invention provided appreciably greater activity. We found that highly lipophilic compounds were particularly effective. Thus, we found that each of Z-Leu-Phe-CONH$(CH_2)_2C_6H_5$, Z-Leu-Phe-CONHEt, and Z-Leu-Abu-CONHEt were more effective than Calpain Inhibitor-1; however these compounds are listed in decreasing order of effectiveness.

5. Treatment of Hypertension-Related Injury

The activity of calpastatin, a natural inhibitor of Calpain, is significantly reduced in the erythrocytes of individuals who have hypertension. The activity of Calpain in such cells concomitantly increased, and both functional and structural lesions have been observed in the erythrocytes of hypertensive mammals. Calpain Inhibitors can therefore be administered to a hypertensive mammal in order to counteract the harmful effects of hypertension on such a mammal's erythrocytes caused by the increased proteolytic activity of Calpain in such cells.

An individual with hypertension is diagnosed by a physician, and in conjunction with other therapies to lower that individual's blood pressure a Calpain Inhibitor is administered to that individual. Approximately 1 - 10 mg/kg of a Calpain Inhibitor is administered to the individual daily, preferably in an oral formulation for ease of administration.

EXAMPLE 27

An individual is diagnosed as having hypertension. Approximately 2 mg/kg of a Calpain Inhibitor is administered daily in tablet form to that individual until the individual's blood pressure returns to a normal range.

6. Treatment of Cataracts

Calpain has been implicated in the causation of cataracts. In a murine model of cataractogenesis, Calpain was found to have a very high activity in the lenses of mice just before visible cataracts appeared. The activity of Calpain then decreased as cataracts formed on the lenses of such mice. Other indicators of Calpain activity include the fact that the concentration of calcium increases markedly in the lenses of

such mice as cataracts begin to develop. Proteins similar to the *in vitro* reaction products of the degradation of the lens protein crystallin by Calpain are also found in the lenses of such mice.

The formation of cataracts is linked to a number of external and internal factors, including age, diabetes, hereditary diseases, UV radiation, drugs such as steroids, and toxic chemicals. Calpain Inhibitors can protect people exposed to agents known to cause cataracts, or who show signs of developing cataracts, through the administration of a Calpain Inhibitor to such people before the development of a cataract.

Calpain Inhibitors can also be administered to people who have not developed fully mature cataracts, in order to halt or slow the progress of cataract development. Such treatments can potentially save millions of dollars in medical care costs. In one study, it was estimated that if cataract development in individuals could be slowed for 10 years or more, over $600 million (in 1973 dollars) could be saved in annual medical care costs in the U.S. alone.

A person having a cataract who would benefit from treatment with a Calpain Inhibitor is easily identified by a physician trained in diagnosing cataracts. Such a person is then treated through the administration of Calpain Inhibitors to slow or halt the progress of cataract formation.

Individuals who would benefit from protection from cataract formation using Calpain Inhibitors can also be identified by a physician. For example, patients whose families have a history of developing cataracts are candidates for treatment with Calpain Inhibitors. Preventative therapy with Calpain Inhibitors is also indicated for people with diabetes or who are regularly exposed to agents, such as steroids, which can cause cataracts.

The administration of Calpain Inhibitors for the treatment or prevention of cataracts can be by any of a number of methods. Cataracts can be treated by the systemic delivery of Calpain Inhibitors through intravenous, intraperitoneal, or intramuscular injection or through oral delivery. Between approximately 0.001 mg/kg and 100 mg/kg of a Calpain Inhibitor is administered daily to an individual to prevent or slow cataract development.

Higher concentrations of Calpain Inhibitors can be delivered, however, through the direct application of such Inhibitors to the eyes. Direct application can be done either through the injection of Calpain Inhibitors into the eye, or through the topical application of Calpain Inhibitors which have been suspended in or mixed with eye drops, ointments, or solutions which are then placed on the eye. Calpain Inhibitor solutions can also be soaked into contact lenses which then deliver a Calpain Inhibitor to the eye slowly over time. Between approximately 0.00001 mg/kg and 1 mg/kg, and preferably between 0.0001 mg/kg and 0.1 mg/kg, are administered daily in such direct applications.

EXAMPLE 28

An elderly individual with partially developed cataracts is treated with eye drops containing Calpain Inhibitors. One drop in each eye is administered twice daily. The eye drop solution is formulated so that each drop contains between 0.001 mg/ml and 1 mg/ ml of a Calpain Inhibitor.

EXAMPLE 29

An individual whose family has a history of cataract development is administered a Calpain Inhibitor in tablet form to be taken once daily. Each tablet contains approximately 2 mg of a Calpain Inhibitor approximately per kilogram of bodyweight.

7. Treatment of Restenosis

When blood vessels become blocked by arterial plaques and fatty deposits, therapeutic angioplasty can be used to open such stenotic regions. One of the most commonly used angioplasty procedures, coronary balloon angioplasty, makes use of a catheter which has an inflatable balloon at its distal end. In this procedure, the catheter is inserted into the arterial lumen, and the distal end of the catheter is guided to the stenotic region. Once positioned within the stenotic area, the balloon is expanded in order to flatten the arterial plaque against the wall of the vessel.

Other types of mechanical procedures for opening stenoses within the vasculature have also been used. These include the use of lasers or atherectomy devices to remove occlusions. Similar procedures for mechanically opening stenoses are also performed in heart valves (valvuloplasty) and peripheral vessels (peripheral angioplasty).

Smooth muscle cell proliferation of the vascular wall is a normal response to various physiological stimuli, including those associated with procedures for mechanically opening stenoses. Such stimuli can trigger the proliferation of smooth muscle cells and the migration of these cells to the vascular subintima, where they continue to proliferate. Smooth muscle cell proliferation is normally a desirable process, one in fact that is often necessary for healing, but which is not necessary for recovery from angioplasty.

However, following therapeutic angioplasty for the opening of obstructed arteries, the proliferation of smooth muscle cells can result in restenosis and blockage of the opened artery. As many as 50% of the patients who undergo successful coronary angioplasty can develop recurrent coronary artery obstructions following the procedure due to such restenosis. It would therefore be of great medical value to be able to inhibit smooth muscle cell proliferation in order to prevent restenosis following angioplasty.

It is one of the surprising discoveries of the present invention that Calpain Inhibitors can be used to inhibit smooth muscle cell proliferation and thereby prevent restenosis following angioplasty. A class of Calpain Inhibitors which are particularly useful in this application are the Peptide Keto-Compounds. These inhibitors are potent inhibitors of calpain. For example, Z-Leu-Phe-CONHEt inhibits the proliferation of cultured bovine smooth muscle cells with an IC50 of around 150 μM. Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$ has been found to inhibit the proliferation of cultured bovine smooth muscle cells with an IC50 of around 60 μM. Other Calpain Inhibitors which are effective in preventing restenosis can be determined by routine experimentation, such as through thymidine incorporation into cultured bovine aortic smooth muscle cells. Additional Calpain Inhibitors believed to be particularly effective in this regard are the Halo-Ketone Peptides, and the Substituted heterocyclic Compounds.

The administration of Calpain Inhibitors to inhibit smooth muscle cell proliferation and to prevent restenosis can be by any of a number of methods known to those of skill in the art. Such methods include the systemic delivery of Calpain Inhibitors through intravenous, intraperitoneal, or intramuscular injection, or through oral delivery. Formulations used for injection may also contain elements such as

ethanol, ethoxylated castor oil, and N,N'-dimethyl acetamide, which serve to solubilize the hydrophobic Calpain Inhibitors. Other art-known methods of delivery are also, of course, possible. Between approximately 0.001 mg/kg and 100 mg/kg of a Calpain Inhibitor can be administered daily to an individual to inhibit smooth muscle cell proliferation and to prevent restenosis.

Higher concentrations of Calpain Inhibitors can also be administered through the direct application of such Inhibitors to the vascular tissue. For example, during a coronary balloon angioplasty procedure, a balloon catheter can be inserted into the desired blood vessel and Calpain Inhibitors can be delivered through the catheter. One such method of application involves passing a balloon through the lumen of a vessel to the site of a vascular lesion or stenosis, after which the balloon is inflated in order to flatten plaque against the wall of the vessel. In this method, a Calpain Inhibitor is directly applied to the blood vessel through the angioplasty balloon. In another method, the angioplasty balloon or another tool used during the angioplasty procedure is coated with the Calpain Inhibitor so that the Calpain Inhibitor is applied directly to the site of stenosis. Alternatively, the Calpain Inhibitor can be loaded into a microsphere and the microsphere can be driven into the injured tissue to effect direct application. A Calpain Inhibitor can also be delivered through the use of a slow-release compound, such as a gel or ointment, applied directly to the injured tissue during surgery.

EXAMPLE 30

Two cultures of bovine aortic smooth muscle cells at low density were serum starved. One cell culture was treated with about 10-100 μM Z-Leu-Phe-CONHEt, and the other culture was treated with about 10-100 μM Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$. Treatment with Calpain Inhibitor began 6 hours prior to stimulation with serum. The cells were then stimulated to divide by changing the old, serum free media to the same media containing 10% fetal bovine serum. The results of this procedure for two Calpain Inhibitor compounds are shown in Figure 19. This Figure shows that the peptide keto compounds Z-Leu-Phe-CONHEt and Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$ effectively inhibit the proliferation of cultured bovine smooth muscle cells with an IC50 of around 150 μM and 60 μM, respectively. Results of this same testing using several different Calpain Inhibitors are shown in Table 30.

TABLE 30

Inhibition of BASMC Proliferation
Structure-Activity Relationship

structure	IC_{50}, μM
$(Ph)_2CHCO$-Leu-Phe-CONH-CH_2-2-Py	30
Z-Leu-Nva-CONH-CH_2-2-Py	30
Z-Leu-Phe-CONH-$CH_2CH(OH)Ph$	40
$(Ph)_2CHCO$-Leu-Abu-CONH-$CH_2CH(OH)Ph$	60
Z-Leu-Phe-$CONH_2$	75
Z-Leu-Abu-CONH-$CH_2CH(OH)Ph$	130
Z-Leu-Phe-CONHEt	150
Z-Leu-Abu-CONHEt	>200
$(Ph)_2CHCO$-Leu-Abu-COOEt	no inhib

EXAMPLE 31

To ensure that the inhibition of cell proliferation in Example 30 was not due to a toxic effect on the cells, the bovine aortic smooth muscle cells treated according to the procedure of Example 30 were further treated for one week with about 10-100 μM Ph_2CHCO-Leu-Abu-CONH-$CH_2CH(OH)Ph$. Figure 20 shows that treatment with Calpain Inhibitor did not cause significant cell death despite a complete inhibition of proliferation. In addition, no increase in trypan blue permeability was seen after treatment for 1 week with the aforementioned Calpain Inhibitor, thus indicating the continued viability of the cells. Furthermore, the antiproliferative effect of the Calpain Inhibitor $(Ph)_2CHCO$-Leu-Abu-CONH-$CH_2CH(OH)Ph$ was found to be rapidly reversed upon washout of the drug, another indication of the continued viability of the smooth muscle cells.

EXAMPLE 32

A human diagnosed as undergoing restenosis is administered between approximately between 0.01 mg/kg and 100 mg/kg of a Calpain Inhibitor in a phosphate buffered saline solution by intravenous injection at least once per day for approximately one week or until it is determined that the risk of restenosis has subsided.

EXAMPLE 33

A human who is determined to be at risk for developing restenosis, such as someone who has undergone angioplasty, is administered between about 0.01 mg/kg and 100 mg/kg of a Calpain Inhibitor in phosphate buffered saline by intravenous injection once per day for approximately one week or until it is determined that the risk of restenosis has passed.

EXAMPLE 34

A human undergoing coronary balloon angioplasty is administered a Calpain Inhibitor to prevent restenosis. The Calpain Inhibitor is delivered by means of an ointment containing between about 0.2 - 10% (2 g/kg to 100 g/kg) of a Calpain Inhibitor. The ointment is coated on the surface of the balloon used in the angioplasty procedure. The Calpain Inhibitor is thus delivered directly to the injured tissue when the balloon is inflated during the procedure.

8. Synchronization of the Cell Cycle

The ubiquitous distribution of calpain in mammalian cells, taken together with the fact that the cleavage of substrate proteins by calpain does not appear to be part of protein catabolism or general protein turnover, points to a regulatory role for calpain in cells. While not wishing to be bound by any particular theory, we believe that a cell's progression through the cell cycle is dependent upon the calpain mediated cleavage of regulatory proteins. This theory has been deduced from our discovery that the inhibition of calpain in a cell with the Calpain Inhibitors of the present invention prevents the progression of the cell's reproductive cycle from the G_1 phase to the S phase. Thus, we have found that Calpain Inhibitors can be used to synchronize the reproductive cycles of cells.

An experiment which was conducted to show that Calpain Inhibitors block the passage of cells from G_1 to S phase is shown in Figure 21. By serum starving the cells used in this experiment, these cells were all blocked at the G_1 phase due to lack of sufficient nutrients to allow passage into the S phase. In this experiment, serum was added to the cells in order to allow them to pass into S phase, and the DNA content of the cells was analyzed with a flow cytometer. The cells were divided into several groups, and the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph was added to each of these groups at the various points in time following serum addition

shown above the top row of squares (at 0, 12, 18, 21, 24,, 30, or 48 hours after the addition of serum to the cells). The peaks shown in each of the individual squares depict the amount of DNA in the cells of the analyzed group at the designated point in time, where the leftmost peak in each of these squares represents the amount of DNA in cells which are in the G_1 phase. The peak to the right of this G_1 peak represents the amount of DNA which has accumulated in cells which have passed through the S phase.

The numbers shown along the diagonal axis of the graph represent the time after the addition of serum that the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph was added to each of the samples of the cells. Thus, the leftmost square in each row represents the amount of DNA in cells which have not been exposed to a Calpain Inhibitor. As can be seen from the first row of squares, when Calpain Inhibitor was added at the time of serum addition, the cells thus treated remained in G_1 phase and did not progress through to the S phase (as shown by the absence of a second, right-shifted peak). However, if Calpain Inhibitor is added after some cells have already begun passing into S phase, such as at 18 or 21 hours after serum addition, then these cells will continue on in the cell cycle. When such cells again reach G_1 phase, however, they will be prevented from progressing, as shown in the squares representing cells sampled at 48 hours, in which only a very small right-shifted peak is visible in all of the cell cultures.

The discovery that Calpain Inhibitors can block the cell cycle has been utilized to devise a treatment for cancer. This treatment involves the synchronization of the cell cycles of cancer cells, followed by a course of chemotherapy. According to this embodiment of the present invention, a patient is first treated with a Calpain Inhibitor, which blocks the patient's actively dividing cells, including cancer cells, from passing from the G_1 phase (the "gap" between mitosis and the beginning of DNA synthesis) to the S phase. After the patient has been treated with the Calpain Inhibitor for the length of one cell cycle, all of the patient's cancerous cells will be in the G_1 phase. Treatment with the Calpain Inhibitor is then stopped, thereby allowing the actively dividing cells to enter the S phase. All of the cancer cells which have been exposed to the Calpain Inhibitor will then progress synchronously into the S phase. At this point, a chemotherapeutic agent which interferes with proper DNA replication is

administered to the patient. Since all of the cancerous cells exposed to the calpain inhibitor will be progressing synchronously into the S phase when the chemotherapeutic agent is administered, all of these cells will be affected by the agent. This course of treatment can be repeated in order to treat cells which did not get previously exposed to the calpain inhibitor.

Figure 22 shows that cancer cells in particular are susceptible to having their cell cycles blocked by Calpain Inhibitors, and that such blockage can be reversed by removing the Calpain Inhibitor. In the experiment illustrated by Figure 22, the cell cycles of cells from AT-2 and HeLa cell lines were synchronized through the use of a the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph. However, we have also found that P388 leukemia cells, L1210 leukemia cells, and human myeloma cells can also be similarly synchronized through the use of a Calpain Inhibitor. Thus, it is believed that all cancer cells can be treated according to the methods of the present invention

As in Figure 21, the squares in Figure 22 depict the results of subjecting cells to flow cytometry analysis, in which the amount of DNA in cells is quantitated. The cells used in this experiment were first exposed to the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph for 48 hours, after which the cell medium (which contained this Calpain Inhibitor) in which the cells were suspended was changed. At time 0 in this figure, when the cell medium was changed, most of the AT-2 (top row) and HeLa (bottom row) cells were in G_1 phase, as shown by the presence of a large left-shifted peak and only a very small right-shifted peak. After washing out the medium containing the Calpain Inhibitor, the cells began to progress through S phase, until after 30 hours quite a number of cells had progressed through the cell cycle (as shown by the significantly larger right-shifted peak). Thus, Calpain Inhibitors can be used to synchronize the cell cycles of cancer cells and allow them to synchronously pass into S phase so that they can be effectively treated with a chemotherapeutic agent.

One of the benefits of using Calpain Inhibitors in conjunction with a chemotherapeutic agent is that the use of the chemotherapeutic agent can be discontinued after the length of the S phase of a patient's cancer cells, rather than requiring the agent to be administered for a full cell cycle in order to affect all treatable cancer cells. This results in a shorter duration of treatment and therefore a

lessening of the discomfort and side effects of the chemotherapy. The efficacy of the chemotherapeutic agent is also increased.

In order to determine the length of a cancerous cell's cell cycle or the length of such a cell's S phase, the type of cancer cell should first be determined. This can be done, for example, by taking a biopsy of the tissue which is or is believed to be cancerous. Once the type of cancer cell is determined, the length of that cell's cell cycle and of the S phase of that cell's cycle can be approximately determined by reference to information known to those of skill in the art. Alternatively, cancerous tissue extracted during the biopsy can be observed in order to determine the length of the cell cycle of the cells in such cancerous tissue and the length of the S phase of the cell cycle of such cells. Since the S phase of a cell's reproductive cycle typically makes up a relatively short period in the cell's reproductive cycle, it is anticipated that standard chemotherapy regimens which target the DNA synthesis of actively dividing cancer cells can be considerably shortened through the use of Calpain Inhibitors according to this aspect of the present invention.

The administration of Calpain Inhibitors to synchronize the cell cycle can be by any of a number of methods known to those of skill in the art. Such methods include the systemic delivery of a Calpain Inhibitor through intravenous, intraperitoneal, or intramuscular injection, or through oral delivery. Administration of a Calpain Inhibitor can also be accomplished through the use of a catheter. Other methods of delivery known to those of skill in the art are as well possible.

A pharmacologically effective does of a calpain inhibitor for blocking the cell cycle of cells from progressing from G phase to S phase of between approximately 0.001 mg/kg and 100 mg/kg Calpain Inhibitor can be administered daily to an individual to cause synchronization of the cell cycle. Preferably, between 1 and 50 mg/kg of a Calpain Inhibitor are administered to such an individual. In one possible course of treatment, a patient can be administered 1 mg/kg of a Calpain Inhibitor. Approximately 24-48 hours after the patient has received this dose of the Calpain Inhibitor, the patient is administered between about 60-75 mg/m^2 of adriamycin, a chemotherapeutic drug sold by Adria Laboratories, Dublin, Ohio. This course of treatment can then be repeated approximately every 21 days or otherwise as needed until the cancer is eradicated or in remission.

Higher concentrations of Calpain Inhibitors than those discussed above can also be administered through the direct application of such Inhibitors to living cells. For example, the Calpain Inhibitor can be loaded into a microsphere, and the microsphere can then be driven into tissue to effect direct application to cancer cells. A Calpain Inhibitor can also be delivered through the use of a slow-release compound, such as a gel or ointment, which is applied directly to cells. Other strategies for the local administration of a Calpain Inhibitor include the injection of a solution containing a Calpain Inhibitor directly into a malignant tumor to effect synchronization of the cell cycles of the cancerous cells of the tumor.

The Calpain Inhibitors of the present invention can also be used in assessing the effectiveness of a chemotherapeutic agent. By synchronizing the cell cycles of cells grown *in vitro*, chemotherapeutic agents that interfere with DNA synthesis can be assayed most effectively. Thus, to growing cancerous cells, an amount of a Calpain Inhibitor is administered to synchronize the cells at the end of G_1 phase. The Calpain Inhibitor can then be rinsed or washed out in a manner well known to those having ordinary skill in the art. Thereafter, the cells are allowed to enter S phase, and a potential chemotherapeutic agent is administered to the cells in an amount believed to kill cancerous cells. The number of killed cells can be determined using any of a number of techniques known in the art, such as by measuring the cells' ability to convert MTT into its blue product. The more effective chemotherapeutic agents will be more effective at killing cells at low dosages.

EXAMPLE 35

Determining the Lengths of the Phases of a Cell Cycle

A biopsy is performed on a patient who has been determined to have cancer, and cancerous cells are thereby removed from the patient. These cells are given a brief pulse of ^{3}H-thymidine and are then washed. Samples of these cells are taken at various times over the course of approximately 24 hours. Autoradiographs are then prepared from these samples. Initially, the cells that are in the S phase are radiolabeled, while cells in the G_2, M and G_1 phases are not labeled. After a length of time equal to the length of the G_2 phase, the labeled cells will enter the M phase. By monitoring when labeled cells pass into the M phase, and then eventually re-enter the M phase, one can

determine the average durations of the G_2, M, S and G_1 phases of the cell cycle, as well as the length of the entire cell cycle.

EXAMPLE 36

Inhibition of the Passage of Cells into S Phase with a Calpain Inhibitor

Bovine aortic smooth muscle cells (BASMC) were grown in tissue culture medium for at least 48 hours. These cells were then serum starved for an additional 48 hours, resulting in a population of cells arrested in G_1. The cells were then exposed to media containing about 10% fetal bovine serum to stimulate cell cycle progression and division. At various times following the addition of the serum, the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph was added to the culture medium to a final concentration of 70mM. Cells from these cultures were removed and stained with DNA dye and analyzed for DNA content using fluorescence activated cell counting.

The results of this experiment are shown in Figure 21. This experiment demonstrates that cells exposed to a Calpain Inhibitor at the time of serum addition do not increase their DNA content over the course of the experiment, which means that they do not progress into the S phase of the cell cycle. The addition of serum to serum-starved cells normally allows cells to progress synchronously into the S phase. The addition of a Calpain Inhibitor 18 or more hours after the addition of serum, however, does not inhibit the increase in DNA content and the subsequent division of the cells. Thus, Calpain Inhibitors act to block the progression of the cell cycle into the S phase.

EXAMPLE 37

Removal of a Calpain Inhibitor from a Cell Culture Arrested in G1 Phase Will Allowthe Culture to Progress to S Phase

Two cell cultures, one of HeLa cells and one of AT-2 cells, were each grown in the presence of serum and the Calpain Inhibitor Ph_2-CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph at a final concentration of 70mM for 48 hours. The culture media was then replaced with media lacking this Calpain Inhibitor. This allowed the cells to progress through the cell cycle. At various times after the removal of the Calpain Inhibitor the cells were stained with DNA dye and analyzed using fluorescence activated cell counting.

The results of this experiment are illustrated in Figure 21. Both cell types were predominantly in the G_1 phase after 48 hours of treatment with Calpain Inhibitor, as

shown by their normal DNA content. After washout of the Calpain Inhibitor, both cell types progressed into the S phase, as shown by the increase in their DNA content. Thus, it was shown that cells can be made to synchronously progress into the S phase of the cell cycle after being treated with a Calpain Inhibitor after removal of the Calpain Inhibitor.

EXAMPLE 38

Use of Substituted Isocoumarins In Chemotherapy

A human diagnosed as having a cancerous tumor is administered a Substituted Isocoumarin. Such administration is performed by injecting directly into the tumor a solution containing approximately 1 mg/kg of a Substituted Isocoumarin in phosphate buffered saline. Beginning 24-48 hours after administration of the Substituted Isocoumarin, 70 mg/m^2 of adriamycin (Adria Laboratories, Dublin, OH) is administered to the patient. This treatment is repeated at 21 day intervals until the tumor is eradicated or in remission.

EXAMPLE 39

Use of Peptide Ketoamides in Chemotherapy

A human diagnosed as having a cancerous tumor is administered a Peptide Ketoamide. Such administration is performed by injecting directly into the tumor a solution containing approximately 1 mg/kg of a Peptide Ketoamide in phosphate buffered saline. Beginning 24-48 hours after administration of the Peptide Ketoamide, 70 mg/m^2 of adriamycin (Adria Laboratories, Dublin, OH) is administered to the patient. This treatment is repeated at 21 day intervals until the tumor is eradicated or in remission.

EXAMPLE 40

Use of Peptide Ketoacids in Chemotherapy

A human diagnosed as having a cancerous tumor is administered a Peptide Ketoacid. Such administration is performed by injecting directly into the tumor a solution containing approximately 1 mg/kg of a Peptide Ketoacid in phosphate buffered saline. Beginning 24-48 hours after administration of the Peptide Ketoacid, 70 mg/m^2 of adriamycin (Adria Laboratories, Dublin, OH) is administered to the patient. This treatment is repeated at 21 day intervals until the tumor is eradicated or in remission.

EXAMPLE 41

Use of Peptide Ketoesters in Chemotherapy

A human diagnosed as having a cancerous tumor is administered a Peptide Ketoester. Such administration is performed by injecting directly into the tumor a solution containing approximately 1 mg/kg of a Peptide Ketoester in phosphate buffered saline. Beginning 24-48 hours after administration of the Peptide Ketoester, 70 mg/m^2 of adriamycin (Adria Laboratories, Dublin, OH) is administered to the patient. This treatment is repeated at 21 day intervals until the tumor is eradicated or in remission.

*. **Increasing the Efficiency of Cell Transformation**

With the discovery that Calpain Inhibitors can prevent a cell from entering the S phase of the cell cycle, we have found that such Inhibitors can be used to increase the efficiency with which cells are transformed with DNA. When foreign DNA is introduced (transformed) into a cell, such DNA can be incorporated into the genome of that cell. Whether such incorporation takes place depends upon the presence of DNA splicing and replication enzymes which are most active during the S phase of the cell cycle. Thus, the efficiency of the incorporation of foreign DNA can be increased by introducing the foreign DNA into a population of cells which have been synchronized in the S phase using a Calpain Inhibitor.

In this aspect of the present invention, cells *in vitro*, such as mammalian cells in culture, can first be synchronized as described above by administering a Calpain Inhibitor to such cells. A dose of a Calpain Inhibitor which is pharmacologically effective to block the cell cycle of cells form progressing from G_1 phase to S phase is between approximately 10 μM and 500 mM. Such a dose can be administered to the cells by adding the Calpain Inhibitor in solution to the media in which the cells are suspended. Following the addition of the Calpain Inhibitor, the cells will pass into the G_1 phase and remain in that state until the Calpain Inhibitor is washed out of the cell media or until it is used up by the cells.

Once the cells are thus synchronized in the G_1 phase, the Calpain Inhibitor can be removed from the cell media by removing the cell media and adding fresh medium. After allowing sufficient time to allow the cells to pass into the S phase, the cells can be transformed by methods known to those of skill in the art. For example, the methods disclosed in *Molecular Cloning* (Sambrook, Jr., Fritsch, E.F., and Maniatis, T., *Molecular*

Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)) or in *Short Protocols in Molecular Biology*, (Ausbel, et al. eds., *Short Protocols in Molecular Biology*, John Wiley & Sons (1989)), which are hereby incorporated by reference, can be used to transform the cells. In a preferred embodiment, exogenous oligonucleotides are included in vector sequences for transformation of the cells. In this embodiment, the exogenous oligonucleotides preferably code for protein are operatively linked to a promoter sequence for transcription and later translation. Following transformation, the cells can be used in a variety of ways known to those having skill in the art.

In one embodiment, this method can be used to treat a mammal which has a disease caused by a genetic mutation that results in a protein deficiency in a particular tissue. In this embodiment, cells of the affected tissue can be removed from a patient synchronized in the G, phase as described above. After allowing the cells to pass into the S phase, these cells are transformed by methods known to the art, for example by introduction of a viral vector carrying exogenous nucleotide sequences. The cells transformed with oligonucleotides coding for a normal gene can be retransplanted into the patient from whom the cells were taken, where they will then be able to function normally due to the incorporation of the normal gene.

EXAMPLE 42

Use of Calpain Inhibitors to Increase Efficiency of Transformation in Gene Therapy

A human is diagnosed with sickle cell anemia, which is caused by a genetic mutation that results in a deficiency of normal hemoglobin in red blood cells. Hematopoietic bone marrow cells are removed from the patient and put into culture *in vitro* in the presence of 100 mM of a Calpain Inhibitor, which causes the cells to synchronize at the G_1 phase. After synchronization, the Calpain Inhibitor is washed out, allowing the cells to proceed to the S phase. The cells are then transformed with foreign DNA which includes the normal gene coding for hemoglobin. After transformation with such foreign DNA, the cells are reintroduced into the patient, where they will repopulate the bone marrow and produce normal hemoglobin protein in red blood cells.

10. Other Non-Neurological Uses

It is known that a large number of medical conditions and diseases are associated with an increase in the activity of Calpain and other calcium-activated proteases. We therefore believe that the compositions of the present invention are beneficial in treating a large number of these other conditions, and the treatment of these other conditions can properly be considered within the scope of the present invention.

H. DRUG DELIVERY

The ability of the various Calpain Inhibitors to penetrate plasma membranes is a significant advantage of these compounds from a pharmaceutical perspective. We believe that this ability, advantageously, allows the Calpain Inhibitors to provide excellent permeation of the blood-brain barrier. This is in contrast to many pharmaceuticals, especially peptides, which often exhibit poor permeation of the blood-brain barrier. Thus, we believe that the Calpain Inhibitors will exhibit excellent results as pharmaceutically neuroprotective agents.

For treatment of neurodegeneration and other medical conditions, the Calpain Inhibitors can be administered orally, topically, intraperitoneally or parenterally. The term "parenteral" as used herein includes all non-oral delivery techniques including transdermal administration, subcutaneous injection, intravenous, intramuscular or intrasternal injection, intrathecal injection (directly into the CNS) or infusion techniques.

The dosage depends primarily on the specific formulation and on the object of the therapy or prophylaxis. The amount of the individual doses as well as the administration is best determined by individually assessing the particular case. However, in preferred compositions, the dosages of Calpain Inhibitors per day are preferably in the range of 1 μg/kg total body mass to 100 mg/kg total body mass, more preferably in the range of 10 μg/kg total body mass to 10 mg/kg total body mass.

The pharmaceutical compositions containing the active ingredient may be in a form suitable for oral use, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules or syrups or elixirs. Dosage levels of the order to 0.2 mg to 140 mg per kilogram of body weight per day are useful in the treatment of above-indicated conditions (10 mg to 7 gms per patient per day). The amount of active ingredient that may be combined with carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode

of administration. However, typically, a single dose will contain sufficient Calpain Inhibitor to provide a complete day's dosage in a single orally acceptable form.

For injection, the therapeutic amount of the Calpain Inhibitors or their pharmaceutically acceptable salts will normally be made by subcutaneous injection, intravenous, intramuscular, intraperitoneal or intrasternal injection, or by intrathecal injection (directly into the brain). For injection, the therapeutic amount of the peptide a-ketoamides or their pharmaceutically acceptable salts will normally be in the dosage range from 0.2 to 140 mg/kg of body weight. In order to provide a single day's dose with a single injection, the pharmaceutical compositions for parenteral administration will contain, in a single dosage form, from about 70 μg to about 7 g of Calpain Inhibitor per dose of from about 0.5 ml to about 1 liter of carrier solution. In addition to the active ingredient, these pharmaceutical compositions will usually contain a buffer, e.g. a phosphate buffer that keeps the pH in the range from 3.5 to 7 and also sodium chloride, and can also contain mannitol or sorbitol for adjusting the isotonic pressure. In a preferred form of these compositions, DMSO or other organic solvent is added in order to assist the introduction of the Calpain Inhibitor across membranes.

Additionally, lipids can be introduced into the pharmaceutical compositions in order to facilitate entry of the Calpain inhibitor compounds into tissue of the CNS. These compositions are prepared in accordance with methods known to those of skill in the art. Briefly, a lipid such as, phosphatidyl choline, cholesterol, other well-known lipid carrier or mixtures thereof, is mixed with the active compound along with a solvent, the solvent is dried off and the material reconstituted in saline. The compositions can also include other ingredients known to those of ordinary skill in the art, such as detergents, surfactants or emulsifying agents.

A composition for topical application or infusion can be formulated as an aqueous solution, lotion, jelly or an oily solution or suspension. A composition in the form of an aqueous solution is obtained by dissolving the Calpain Inhibitor in aqueous buffer solution of pH 4 to 6.5 and, if desired, adding a polymeric binder. An oily formulation for topical application is obtained by suspending the Calpain Inhibitor in an oil, optionally with the addition of a swelling agent such as aluminium stearate and/or a surfactant. The addition of DMSO to these topical compositions is believed to allow at least partial penetration of

the active Calpain Inhibitor into the blood stream after application of the composition to the skin of a patient to allow for transdermal administration.

For treatment of neurodegeneration resulting from excitotoxicity, HIV-induced neuropathy, ischemia following denervation or injury, subarachnoid hemorrhage, stroke, multiple infarction dementia, Alzheimer's Disease (AD), Huntington's Disease, surgery-related brain damage, Parkinson's Disease, and other pathological conditions, the Calpain Inhibitors or pharmaceutically acceptable salts thereof may be administered orally or parenterally. The dosage depends primarily on the specific formulation and on the object of the therapy or prophylaxis. The amount of the individual doses as well as the administration is best determined by individually assessing the particular case.

In many acute neurodegenerative conditions and events, such as stroke and head injury, it is important to deliver the Calpain Inhibitor as soon after injury as is practicable. Thus, it is preferable to identify those individuals who have suffered stroke, head injury or other injury in which neurodegeneration is associated or is likely to occur, and to begin administration of a Calapin Inhibitor within 1 minute to 2 hours after the event, in order to prevent as much neurodegeneration as possible.

A particular application of the Calpain Inhibitors within the scope of the present invention is the application of these compounds during surgery to prevent neurodegeneration associated therewith. For example, for surgeries performed under general anesthesia, hypoxic conditions can occur through inadequate perfusion of the CNS while under anesthesia. Additionally, many major surgeries of the cardiovascular system require that a patient's heart be stopped and that perfusion be maintained through artificial means. In such surgeries, there is an increased danger of hypoxia occurring within the CNS, which can also result in neurodegeneration. Moreover, during neurosurgeries, there is an inherent risk of neurodegeneration resulting from inflammation, bleeding, hemorrhaging and the like. Such neurodegeneration can be inhibited by infusion with a solution containing Calpain Inhibitor. However, neurodegeneration resulting from neurosurgery can also be reduced prophylactically by administration of a Calpain Inhibitor through any of the foregoing administration techniques. Such administration is also believed to inhibit or prevent neurodegeneration associated with the use of anesthesia or with the use of artifical means of perfusion during

major surgeries. A surgical patient can also have Calpain Inhibitor administered throughout surgery through intravenous drip.

The following examples are intended to illustrate certain neuroprotective uses of the Calpain Inhibitors within the scope of the present invention. As such, they are not meant to limit the invention in any way.

EXAMPLE 35

A Neuroprotective Composition for Intravenous Injection

500 µg	CH_3CONH-CiTPrOIC from Example SHC2
4 ml	Propylene Glycol
1 ml	DMSO

EXAMPLE 36

A Neuroprotective Composition for Intravenous Drip

250 mg	Z-Leu-Phe-CONH-Et from Example PKC 48
1000 ml	Phosphate Buffered Saline (pH 6.0)
10 ml	DMSO

EXAMPLE 37

A Neuroprotective Composition for Transdermal Application

25 mg	Z-Leu-DL-Abu-COOEt from Example PKC19
3 ml	Phosphate Buffered Saline (pH 6.0)
2 ml	DMSO

EXAMPLE 38

Neuroprotection after Head Injury

A first group of patients who are victims of head trauma is given 2 ml of the injectable composition of Example 30 intravenously within ten minutes of the time of injury. A second group of similarly matched patients does not receive the composition. The first group of patients exhibits markedly fewer and less severe outward symptoms of neurodegeneration, such as dementia, memory loss and paralysis.

EXAMPLE 39

Neuroprotection During Surgery

A patient about to undergo a triple bypass heart surgery is administered 500 ml of the composition of Example 31 per hour using an intravenous drip system. During surgery, the patient's heart is stopped and perfusion continued through artificial means. Although complications develop while restarting the heart and disconnecting the patient from the artificial means of perfusion, the patient becomes conscious within several hours of surgery. Within a few days, the patient's mental status is normal with no indications of neurodegeneration.

For the inhibition of smooth muscle cell proliferation in the treatment or prevention of restenosis, Calpain Inhibitor can also be administered directly to the site of injured smooth muscle tissue. Such administration can be accomplished, for example, by means of an ointment or gel applied to the surface of a balloon or other surgical tool used in an angioplasty procedure. In this way, if damage is done during angioplasty that would otherwise result in restenosis, restenosis can be prevented.

The direct administration of a Calpain Inhibitor to the site of injured tissue can also be accomplished by loading the Calpain Inhibitor into microspheres and imbedding the microspheres into the injured tissue. This can be accomplished by applying the microspheres to the surface of the balloon used in the angioplasty procedure. When the balloon is inflated inside the artery, the force of the expansion drives the microspheres into the arterial wall, where they become lodged. The microspheres then release the Calpain Inhibitor slowly over time and provide local application to the injured tissue.

For the treatment of numerous medical conditions, Calpain Inhibitors can be injected in solutions either intravenously, intraocularly, intramuscularly, intraperitoneally, or intrasternally. These solutions will contain a Calpain Inhibitor in the range of from about 70 μg to about 7 g per dose in about 0.5 ml to 1 liter of a pharmaceutically acceptable carrier solution. The solution preferably contains a buffer, such as a phosphate buffer, that keeps the pH in the range of about 3.5 to 7. The solution also preferably contains approximately 9000 mg/l of sodium chloride (0.9% saline), as well as mannitol or sorbitol for adjusting the isotonic pressure. DMSO at 0.01 to 10 ml/liter can also be used in injectable solutions of the present invention in order to potentiate the Calpain

Inhibitor, or help it to penetrate membranes. Other additives such as ethanol or ethoxylated oils can also be used.

When using Calpain Inhibitors to protect cardiac, skeletal, or smooth muscle tissue from damage, intravenous drip infusion is preferred to the periodic injection of such Inhibitors. A solution suitable for intravenous infusion can be prepared by suspending approximately 250 mg of a Calpain Inhibitor in 1000 ml of an aqueous solution of phosphate buffered saline containing 10 ml DMSO. However, in the treatment of muscular dystrophy, or when treating a condition for a long period of time with Calpain Inhibitors, an oral formulation of a Calpain Inhibitor is preferred. Such an oral formulation can be in the form of a tablet, in which a powdered form of a Calpain Inhibitor is mixed with a pharmaceutically acceptable filler material capable of being formed into a tablet.

In the treatment of cataracts, the injectable solutions referred to above can be administered by soaking them into a contact lens, which is then worn for a period of time long enough to allow the solution to diffuse into the eye from the lens. Other methods of delivering Calpain Inhibitors to an eye with the injectable solutions described above include the administration of eye drops comprising the solution.

Calpain Inhibitors can also be formulated in an ointment for administration to the eye. This can be accomplished by dissolving a Calpain Inhibitor in an aqueous solution and then adding a pharmaceutically acceptable polymeric binder. A Calpain Inhibitor can also be directly dissolved or suspended in such a polymeric binder.

For the treatment or prevention of tonic smooth muscle contraction, Calpain Inhibitor can also be administered directly to the smooth muscle, including application to coronary tissue. Such administration can be accomplished by means of an ointment, gel or solution applied directly to the smooth muscle during surgery. Direct administration can also be accomplished by loading the Calpain Inhibitor into a microsphere and imbedding the microsphere into the smooth muscle tissue. The microsphere then releases the Calpain Inhibitor slowly over time and provides local appliction to the tissue.

For the treatment of cerebral vasospasm, a solution of a Calpain Inhibitor can be injected directly into the cerebrospinal fluid of the patient. For the treatment of bronchospasm, such as that which occurs in asthmatic patients, a solution containing a Calpain Inhibitor can be inhaled directly into the patient's lungs.

The following additional examples are provided to further illustrate certain embodiments of pharmaceutical compositions within the scope of the present invention.

EXAMPLE 40

An Injectable Composition for Non-Neurological Uses

1 mg	Z-Leu-Abu-CONH-Bzl from Example PKC58
4 ml	Propylene Glycol
1 ml	DMSO

EXAMPLE 41

An Ophthalmic Solution for Treating Cataracts

500 µg	Z-Leu-Abu-CONH-iBu from Example PKC57
5 ml	sterile phosphate buffered saline

EXAMPLE 42

A Solution for Topical Application to a Tonic, Contracted Blood Vessel

2 mg	Z-Leu-Abu-CONH-$(CH_2)_3$-N$(CHCH_2)_2$O from Example PKC60
1 ml	DMSO
10 ml	sterile phosphate buffered saline

It will be appreciated that certain variations may suggest themselves to those skilled in the art. The foregoing detailed description is to be clearly understood as given by way of illustration, the spirit and scope of this invention being interpreted through reference to the appended claims.

WHAT IS CLAIMED IS:

1. A method of synchronizing the reproductive cycle in actively dividing cells, comprising administering to said cells an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cells from G_1 phase into S phase, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

2. The method of Claim 1, wherein the administering step comprises administering a Calpain Inhibitor to cells located *in vivo* in a mammal.

3. The method of Claim 1, wherein the administering step comprises administering a Calpain Inhibitor to cells *in vitro*.

4. The method of Claim 1, wherein the administering step comprises administering a Peptide Keto-Compound.

5. The method of Claim 1, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to said cells.

6. A method of blocking the progression of the cell cycle from G_1 phase into S phase in actively dividing cells in a mammal, comprising administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cell cycles of actively dividing cells in said mammal from G_1 phase into S phase, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

7. The method of Claim 6, wherein the administering step comprises administering a Peptide Keto-Compound.

8. The method of Claim 6, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to said living cells.

9. The method of Claim 6, wherein the administering step comprises direct application.

10. The method of Claim 9, wherein the direct application comprises applying a gel to an area of living cells.

11. The method of Claim 9, wherein the direct application comprises driving microspheres loaded with said Calpain Inhibitor into tissue comprising said living cells.

12. The method of Claim 9, the direct application comprises injecting a solution containing said Calpain Inhibitor directly into tissue comprising said living cells.

13. A method of enhancing the efficacy of chemotherapy in the treatment of cancer in a human patient having cancer cells, comprising:

administering to the cancer cells an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cell cycles of said cancerous cells from G_1 phase into S phase; and thereafter

administering to said cells a chemotherapeutic agent.

14. The method of Claim 13, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

15. The method of Claim 13, wherein the administering step comprises administering a Peptide Keto-Compound.

16. The method of Claim 13, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to said cancerous cells.

17. The method of Claim 13, wherein the chemotherapeutic agent is administered beginning 24-48 hours after the administration of said Calpain Inhibitor.

18. A method of determining the effectiveness of a chemotherapeutic agent, comprising:

growing cancerous cells *in vitro*;

administering to said cells an amount of a Calpain Inhibitor which is effective to block the progression of said cells from G_1 phase into S phase;

administering to said cells said chemotherapeutic agent in an amount sufficient to kill said cells;

determining the amount of cell death that occurs, wherein the amount of cell death is indicative of the effectiveness chemotherapeutic agent.

19. A method of increasing the efficiency of cell transformation and the integration of foreign DNA into living cells, comprising:

administering to a population of cells comprising actively dividing cells an amount of a Calpain Inhibitor which is pharmacologically effective to block the progression of the cell cycles of said cells from G_1 phase into S phase, wherein said

Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds;

discontinuing administration of said Calpain Inhibitor; and thereafter

introducing foreign DNA into said population of cells.

20. The method of Claim 19, wherein the administration of said Calpain Inhibitor continues for the length of one cell cycle in said population of living cells.

21. The method of Claim 19, wherein the administering step comprises administering said Calpain Inhibitor to a population of cells located in a mammal.

22. The method of Claim 19, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to said population of cells in said mammal.

23. The method of Claim 19, wherein the administering step comprises administering said Calpain Inhibitor to a population of cells *in vitro*.

24. The method of Claim 19, wherein the administering step comprises administering a Peptide Keto-Compound.

25. A method of medical treatment for a medical condition in a mammal, comprising administering a pharmaceutical composition containing a Calpain inhibitor compound to said mammal, said composition being administered in an amount that is pharmacologically effective to treat said condition, wherein said Calpain inhibitor is selected from the group consisting of Peptide Keto-Compounds and Substituted Heterocyclic Compounds, said condition being one which is associated with increased proteolytic activity of Calpain.

26. The method of Claim 25, wherein said inhibitor is administered intravenously, intraperitoneally, intramuscularly, or orally.

27. The method of Claim 25, wherein said inhibitor is administered in an ointment.

28. The method of Claim 25, wherein said inhibitor is administered in eye drops or by being released into the eye from a contact lens.

29. The method of Claim 25, wherein said inhibitor is administered with a catheter.

30. A method of treating a mammal with cardiac muscle tissue damage in order to slow or prevent further damage, comprising:

identifying a mammal with cardiac muscle tissue damage;

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for treating cardiac muscle damage in said mammal.

31. The method of Claim 30, wherein said inhibitor is administered intravenously, intraperitoneally, intramuscularly, or orally.

32. The method of Claim 30, wherein said inhibitor is administered with a catheter.

33. The method of Claim 30, wherein said inhibitor is administered directly to heart tissue during surgery or is injected into the pericardial space.

34. A method of protecting a mammal at risk for developing cardiac muscle tissue damage, comprising:

identifying a mammal which is at risk for developing cardiac muscle tissue damage; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for protecting said mammal from developing cardiac muscle tissue damage.

35. The method of Claim 34, wherein said inhibitor is administered intravenously.

36. The method of Claim 34, wherein said inhibitor is administered intraperitoneally, intramuscularly, orally, or with a catheter.

37. A method of treating a mammal with a cataract, comprising:

identifying a mammal having a developing cataract; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for treating said cataract in said mammal.

38. The method of Claim 37, wherein said inhibitor is administered intravenously, intraperitoneally, intramuscularly or orally.

39. The method of Claim 37, wherein said inhibitor is injected directly into the eye (intraocularly).

40. The method of Claim 37, wherein said inhibitor is administered in an ophthalmic ointment, in eye drops or by being released into the eye from a contact lens.

41. A method of slowing or preventing cataract formation in the eyes of a mammal, comprising:

identifying a mammal which is at risk for developing a cataract; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective to slow the development of a cataract.

42. The method of Claim 41, wherein said inhibitor is administered intravenously, intraperitoneally, intramuscularly or orally.

43. The method of Claim 41, wherein said inhibitor is injected directly into the eye (intraocularly).

44. The method of Claim 41, wherein said inhibitor is administered in an ophthalmic ointment, in eye drops or by being released into the eye from a contact lens.

45. A method of treating a mammal with skeletal muscle damage, comprising:

identifying a mammal with skeletal muscle damage; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for healing damaged skeletal muscle.

46. The method of Claim 45, wherein said inhibitor is administered intravenously, intraperitoneally, intramuscularly or orally.

47. A method of treating a mammal with smooth muscle damage, comprising:

identifying a mammal with damage to its smooth muscle tissue; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted

Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for healing said smooth muscle tissue.

48. The method of Claim 47, wherein said inhibitor is administered intravenously, intraperitoneally, intramuscularly or orally.

49. A method of reversing vasospasm in a mammal, comprising:

identifying a mammal experiencing vasospasm; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for reversing vasospasm.

50. The method of Claim 49, wherein said inhibitor is administered intravenously, topically or orally.

51. A method of treating a mammal with damage to that mammal's erythrocytes due to hypertension, comprising:

identifying a mammal with hypertension; and

administering a Calpain inhibitor selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds to said mammal in an amount which is pharmacologically effective for treating damaged erythrocytes.

52. The method of Claim 51, wherein said inhibitor is administered orally.

53. The method of any one of Claims 25, 30, 34, 37, 41, 45, 47, 49 or 51 wherein said Calpain Inhibitor comprises a Peptide Ketoamide.

54. The method of Claim 53, wherein said Peptide Ketoamide comprises a compound selected from the group consisting of: Z-Leu-Abu-CONH-Et, Z-Leu-Phe-CONH-Et and Z-Leu-Phe-CONH$(CH_2)_2C_6H_5$.

55. A method of halting or inhibiting the proliferation of smooth muscle cells, comprising:

identifying smooth muscle cells that are proliferating; and

administering to said cells an amount of a Calpain Inhibitor which is pharmacologically effective to halt or inhibit smooth muscle cell proliferation, wherein said Calpain Inhibitor is selected from the group consisting of Peptide

Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

56. The method of Claim 55, wherein the step of identifying smooth muscle cells comprises identifying smooth muscle cells located in a living organism.

57. The method of Claim 55, wherein the step of identifying smooth muscle cells comprises identifying smooth muscle cells in vitro.

58. The method of Claim 55, wherein the administering step comprises administering a Peptide Keto-Compound.

59. The method of Claim 58, wherein said Peptide Keto-Compound is selected from the group consisting of:

$(Ph)_2CHCO$-Leu-Phe-CONH-CH_2-2-Py;

Z-Leu-Nva-CONH-CH_2-2-Py;

Z-Leu-Phe-CONH-$CH_2CH(OH)Ph$;

$(Ph)_2CHCO$-Leu-Abu-CONH-$CH_2CH(OH)Ph$;

Z-Leu-Phe-$CONH_2$;

Z-Leu-Abu-CONH-$CH_2CH(OH)Ph$; and

Z-Leu-Phe-CONHEt.

60. The method of Claim 55, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to said smooth muscle cells.

61. A method of treating a mammal to prevent restenosis of a blood vessel following angioplasty, comprising:

identifying a mammal which has undergone angioplasty; and thereafter

administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to prevent restenosis following angioplasty, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

62. The method of Claim 61, wherein the administering step comprises administering a Peptide Keto-Compound.

63. The method of Claim 62, wherein said Peptide Keto-Compound is selected from the group consisting of:

$(Ph)_2CHCO$-Leu-Phe-CONH-CH_2-2-Py;

Z-Leu-Nva-CONH-CH_2-2-Py;

Z-Leu-Phe-CONH-CH_2CH(OH)Ph;

$(Ph)_2$CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph;

Z-Leu-Phe-$CONH_2$;

Z-Leu-Abu-CONH-CH_2CH(OH)Ph; and

Z-Leu-Phe-CONHEt.

64. The method of Claim 61, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to said blood vessel.

65. The method of Claim 64, wherein said direct application comprises applying a gel to an area of vascular tissue which has been treated by angioplasty.

66. The method of Claim 65, wherein said gel is coated onto the balloon of a balloon catheter or onto the end of a surgical instrument that is inserted into said blood vessel.

67. The method of Claim 61, wherein said direct application comprises driving microspheres loaded with said Calpain Inhibitor into said blood vessel.

68. A method of treating a mammal to prevent restenosis of a blood vessel following angioplasty, comprising:

performing angioplasty on a mammal; and

administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to prevent restenosis following the angioplasty procedure, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

69. The method of Claim 68, wherein the administering step comprises administering a Peptide Keto-Compound.

70. The method of Claim 69, wherein said Peptide Keto-Compound is selected from the group consisting of:

$(Ph)_2$CHCO-Leu-Phe-CONH-CH_2-2-Py;

Z-Leu-Nva-CONH-CH_2-2-Py;

Z-Leu-Phe-CONH-CH_2CH(OH)Ph;

$(Ph)_2$CHCO-Leu-Abu-CONH-CH_2CH(OH)Ph;

Z-Leu-Phe-$CONH_2$;

Z-Leu-Abu-$CONH\text{-}CH_2CH(OH)Ph$; and

Z-Leu-Phe-CONHEt.

71. The method of Claim 68, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, or by direct application to the blood vessel.

72. The method of Claim 70, wherein the direct application comprises applying a gel to an area of vascular tissue which has been treated by angioplasty.

73. The method of Claim 71, wherein said angioplasty is balloon catheter angioplasty.

74. The method of Claim 72, wherein the administering step comprises coating a balloon of a balloon catheter with a Calpain Inhibitor, so that said Calpain Inhibitor is applied directly to the vascular tissue on which the angioplasty is performed.

75. The method of Claim 68, wherein the administering step comprises loading a Calpain Inhibitor into a microsphere and delivering said microsphere directly to the vascular tissue on which the angioplasty is performed.

76. A method of preventing the restenosis of a blood vessel, comprising the steps of:

identifying a mammal at risk for developing restenosis; and

administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to prevent restenosis following the angioplasty procedure, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides, and Substituted Heterocyclic Compounds.

77. The method of Claim 76, wherein said Calpain Inhibitor is a Peptide Keto Compound.

78. The method of Claim 77, wherein said Peptide Keto-Compound is selected from the group consisting of:

$(Ph)_2CHCO$-Leu-Phe-$CONH\text{-}CH_2$-2-Py;

Z-Leu-Nva-$CONH\text{-}CH_2$-2-Py;

Z-Leu-Phe-$CONH\text{-}CH_2CH(OH)Ph$;

$(Ph)_2CHCO$-Leu-Abu-$CONH\text{-}CH_2CH(OH)Ph$;

Z-Leu-Phe-$CONH_2$;

Z-Leu-Abu-CONH-$CH_2CH(OH)Ph$; and

Z-Leu-Phe-CONHEt.

79. A method of inhibiting tonic smooth muscle contraction in a mammal susceptible to inappropriate contraction in a smooth muscle thereof, comprising:

administering to said smooth muscle an amount of a Calpain Inhibitor that is pharmacologically effective to suppress the contraction thereof, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides and Substituted Heterocylic Compounds.

80. The method of Claim 79, wherein the administering step comprises administering a Peptide Keto-Compound.

81. The method of Claim 79, wherein the administering step comprises administering said Calpain Inhibitor intravenously, intramuscularly, intraperitoneally, topically, orally, by injection into cerebrospinal fluid, by inhalation, or by direct application to said smooth muscle.

82. The method of Claim 81, wherein said direct application comprises applying to an area of smooth muscle.

83. The method of Claim 81, wherein said direct application comprises driving microspheres loaded with said Calpain Inhibitor into said smooth muscle.

84. The method of Claim 79, wherein relaxation of said smooth muscle is induced.

85. A method of treating coronary vasospasm in a mammal, comprising:

administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to stop vasospasm of coronary tissue in said mammal, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides and Substituted Heterocylic Compounds.

86. The method of Claim 85, wherein the administering step comprises administering a Peptide Keto-Compound.

87. The method of Claim 85, wherein the administering step comprises surgically exposing said coronary tissue and applying a solution of Calpain Inhibitor directly to said tissue.

88. The method of Claim 87, wherein said coronary tissue comprises a coronary artery.

89. The method of Claim 85, wherein said mammal is suffering from angina and said method comprises a treatment for said angina.

90. A method of treating bronchial vasospasm in a mammal, comprising:

administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to stop vasospasm of bronchial tissue in said mammal, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides and Substituted Heterocylic Compounds.

91. The method of Claim 90, wherein the administering step comprises administering a Peptide Keto-Compound.

92. The method of Claim 90, wherein the administering step comprises surgically exposing said bronchial tissue and applying a solution of Calpain Inhibitor directly to said tissue.

93. The method of Claim 90, wherein said mammal is suffering from asthma and said method comprises a treatment for said asthma.

94. A method of treating cerebral vasospasm in a mammal, comprising:

administering to said mammal an amount of a Calpain Inhibitor which is pharmacologically effective to stop vasospasm of cerebral tissue in said mammal, wherein said Calpain Inhibitor is selected from the group consisting of Peptide Keto-Compounds, Halo-Ketone Peptides and Substituted Heterocylic Compounds.

95. The method of Claim 94, wherein the administering step comprises administering a Peptide Keto-Compound.

96. The method of Claim 94, wherein the administering step comprises surgically exposing said cerebral tissue and applying a solution of Calpain Inhibitor directly to said tissue.

97. The method of Claim 94, wherein the administering step comprises injecting said Calpain Inhibitor into the mammal's cerebrospinal fluid.

98. A method of medical treatment for a medical condition in a mammal, comprising administering a pharmaceutical composition containing a morpholine Peptide Keto-Compound to said mammal, said composition being administered in an amount that

is pharmacologically effective to treat said condition, said condition being one which is associated with increased proteolytic activity of Calpain.

99. The method of Claim 98, wherein said morpholine Peptide Keto-Compound is a C-terminal morpholine Peptide Keto-Compound.

100. The method of Claim 98, wherein said morpholine Peptide Keto-Compound is an N-terminal morpholine Peptide Keto-Compound.

101. A method of medical treatment for a medical condition in a mammal, comprising administering a pharmaceutical composition containing a Peptide Ketoamide, Subclass C to said mammal, said composition being administered in an amount that is pharmacologically effective to treat said condition, said condition being one which is associated with increased proteolytic activity of Calpain.

102. The method of either Claim 98 or Claim 101, wherein said medical condition is selected from the group consisting of: cardiac muscle tissue damage, cataracts, skeletal muscle damage, vasospasm and restenosis following cardiac angioplasty.

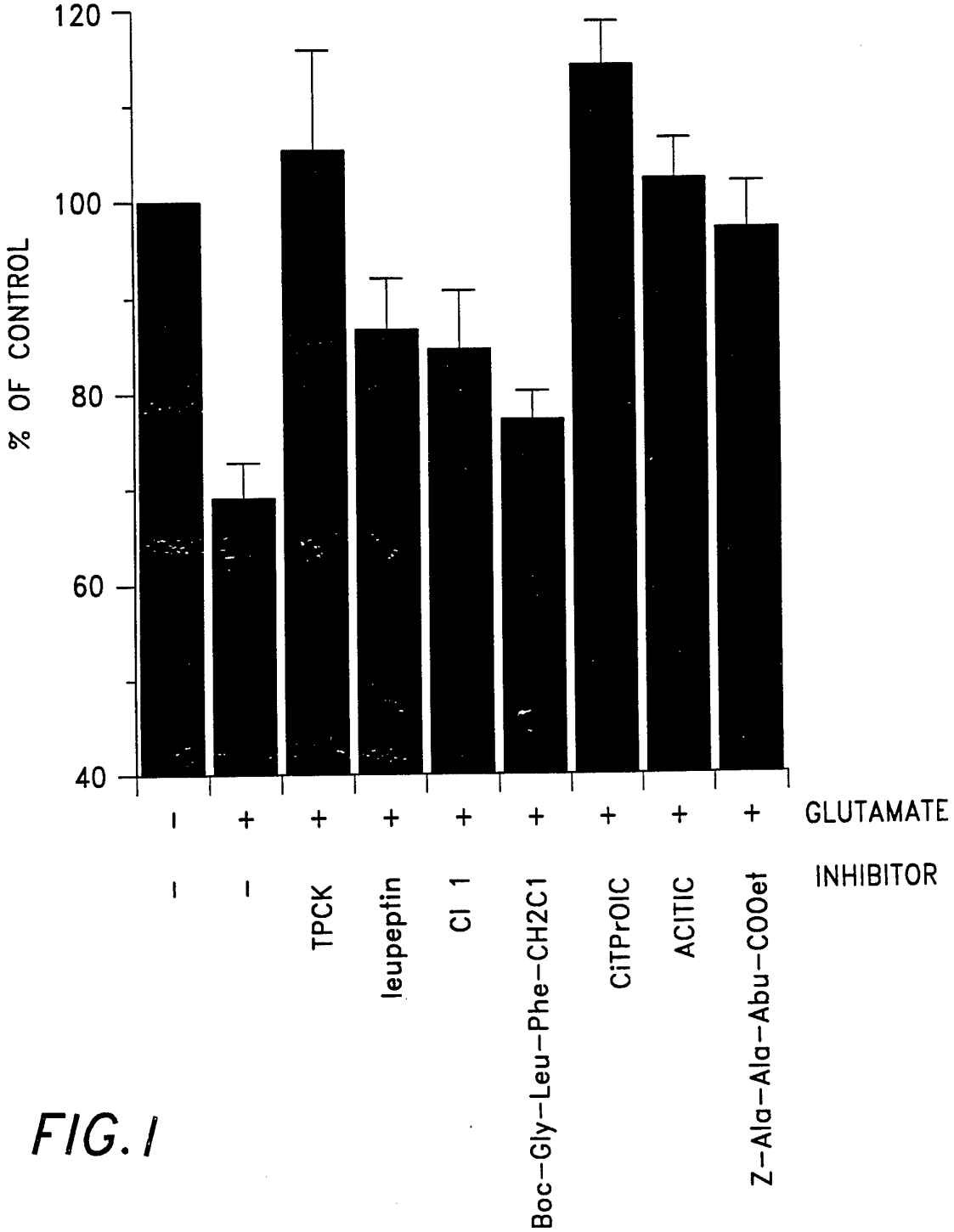

120
100
80
60
40
% OF CONTROL
- + + + + + + + + GLUTAMATE
- - TPCK leupeptin CI 1 Boc-Gly-Leu-Phe-CH2C1 CiTPrOIC ACITIC Z-Ala-Ala-Abu-COOet INHIBITOR

FIG. 1

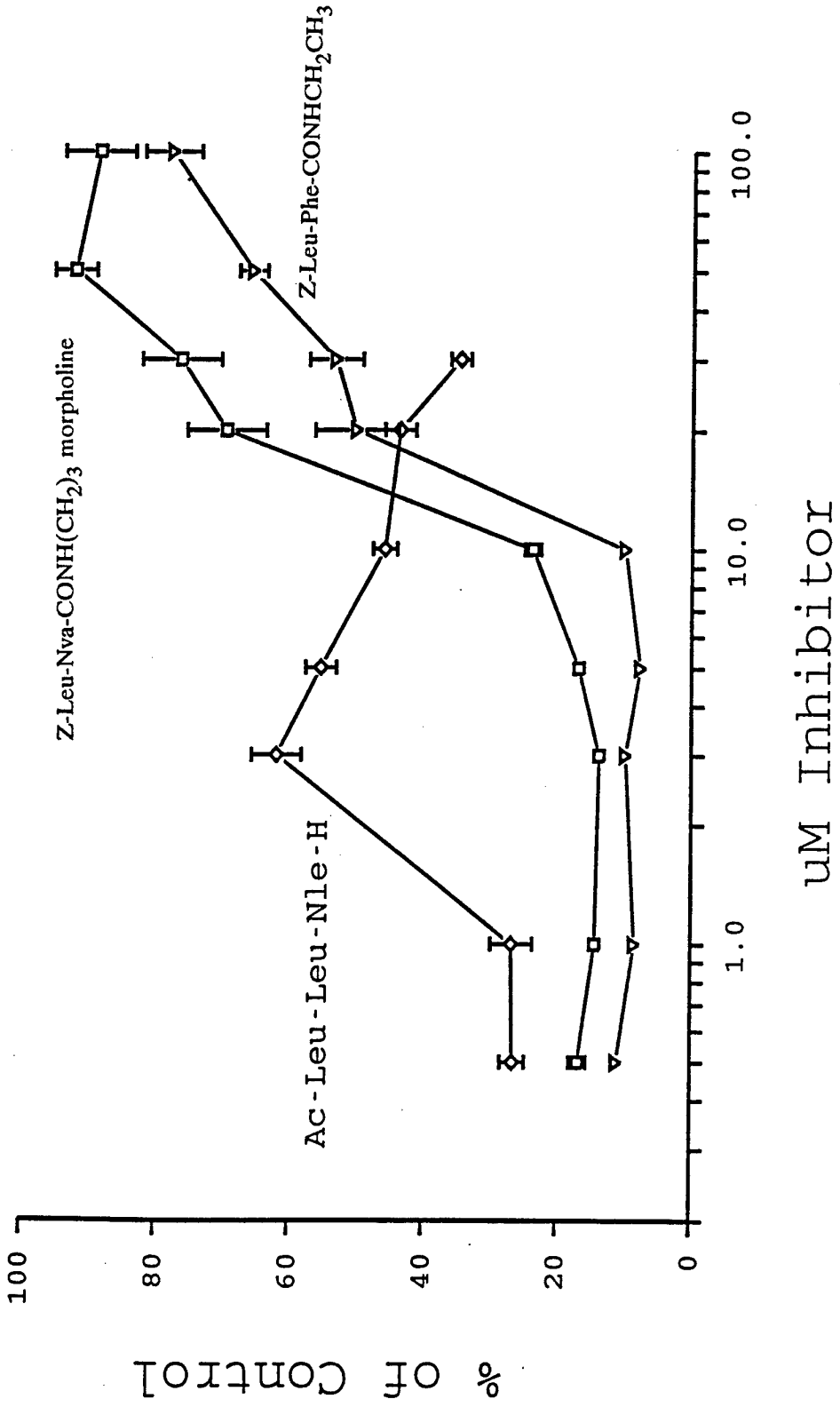

% of Control
100
80
60
40
20
0
1.0
10.0
100.0
uM Inhibitor
Z-Leu-Nva-CONH(CH2)3 morpholine
Z-Leu-Phe-CONHCH2CH3
Ac-Leu-Leu-Nle-H

FIGURE 2

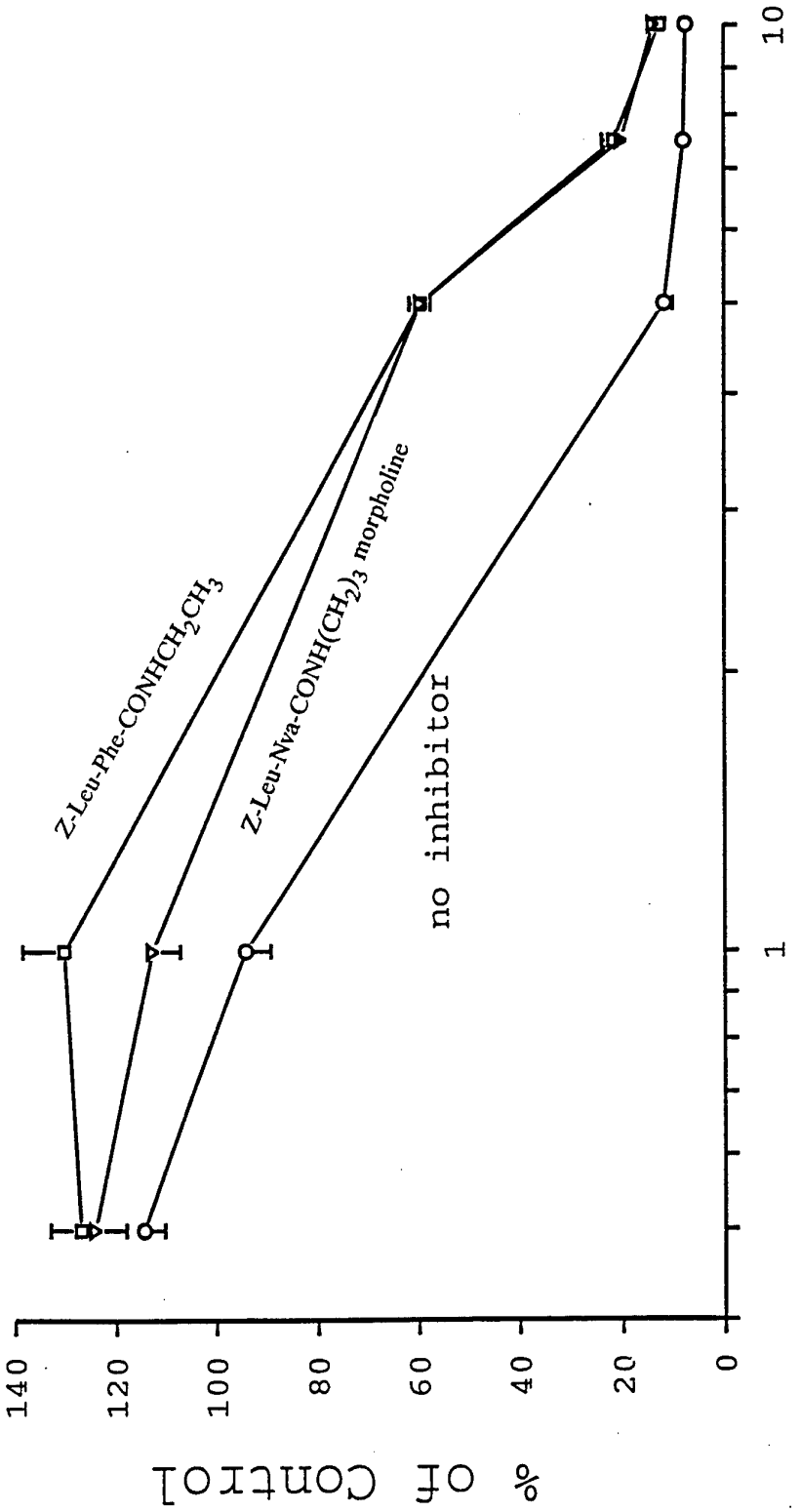

% of Control
140
120
100
80
60
40
20
0
1
10
mM Glutamate
Z-Leu-Phe-CONHCH2CH3
Z-Leu-Nva-CONH(CH2)3 morpholine
no inhibitor

FIGURE 3

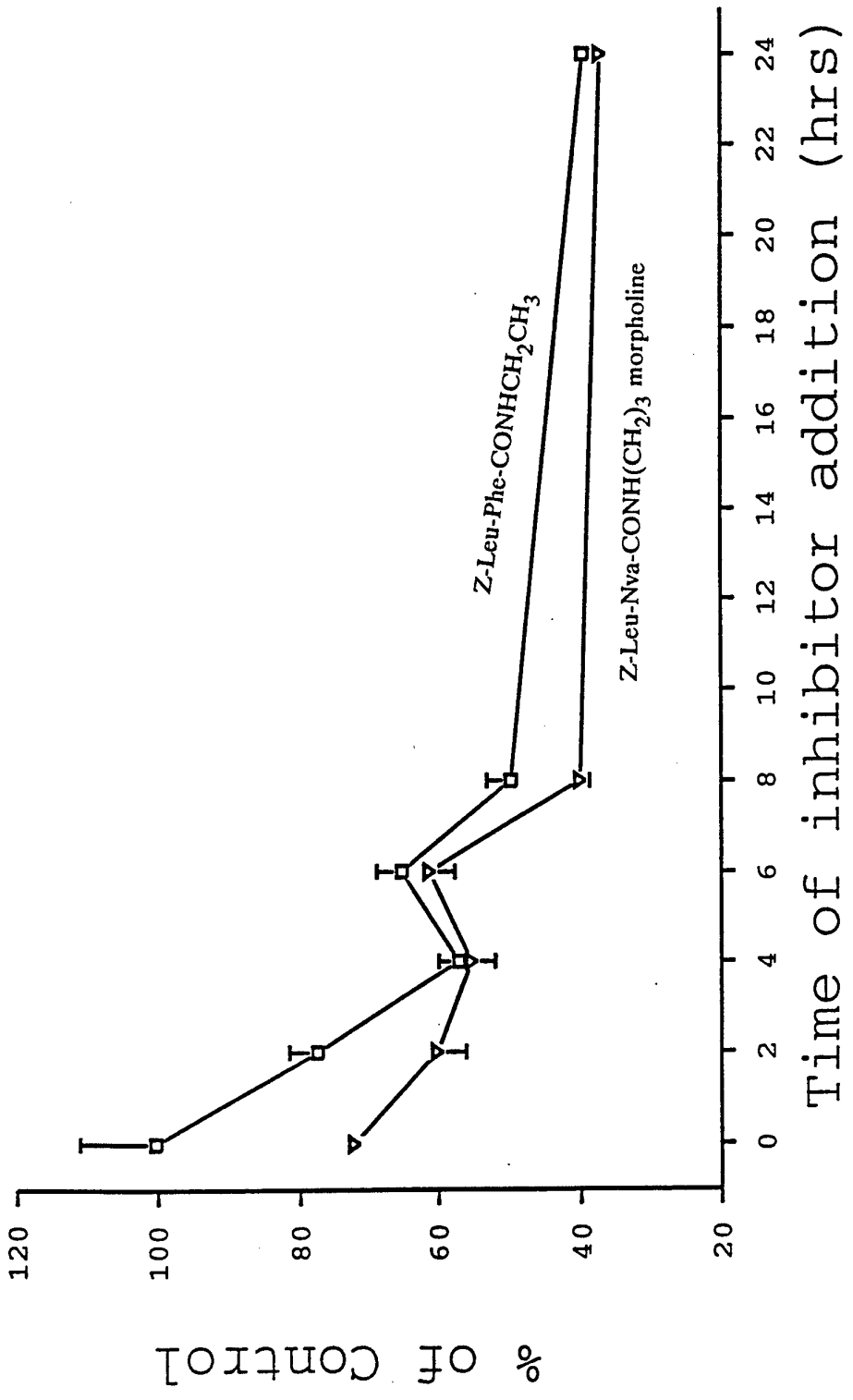

% of Control
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
Time of inhibitor addition (hrs)
Z-Leu-Phe-CONHCH2CH3
Z-Leu-Nva-CONH(CH2)3 morpholine

FIGURE 4

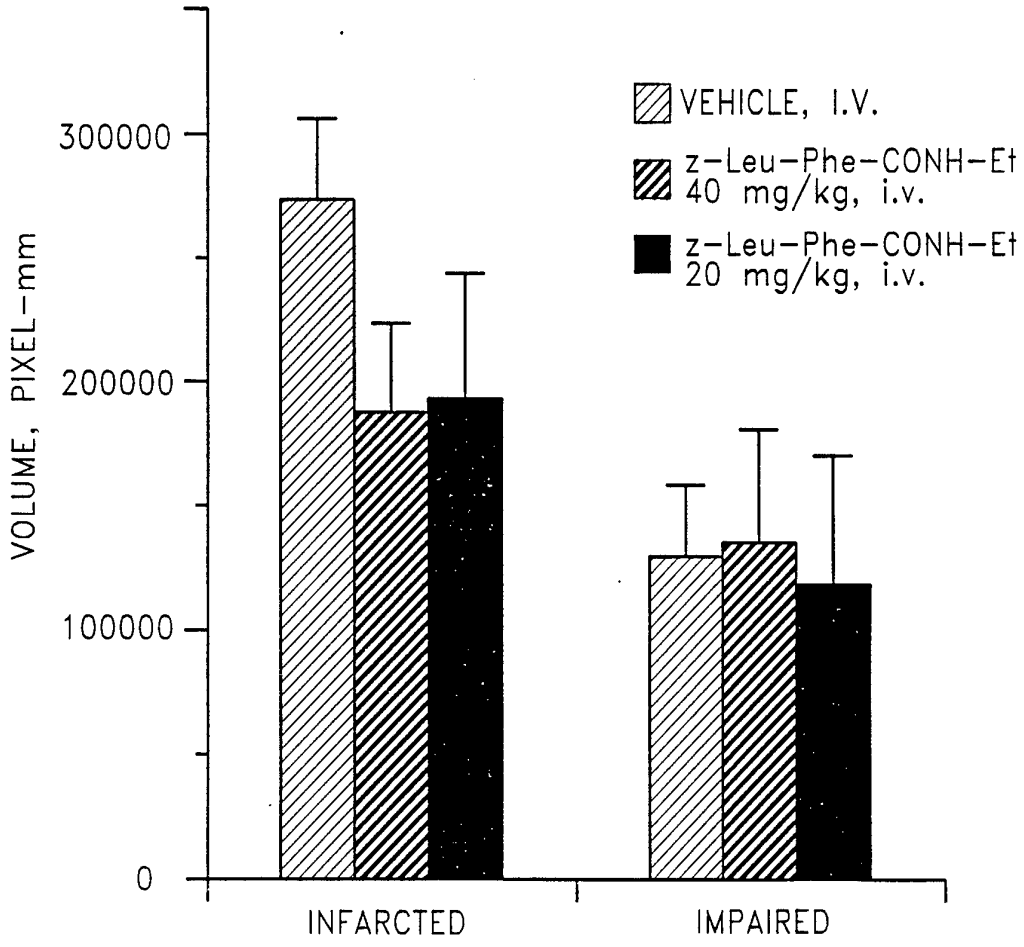

VOLUME, PIXEL-mm
300000
200000
100000
0
INFARCTED
IMPAIRED
VEHICLE, I.V.
z-Leu-Phe-CONH-Et
40 mg/kg, i.v.
z-Leu-Phe-CONH-Et
20 mg/kg, i.v.

FIG.5

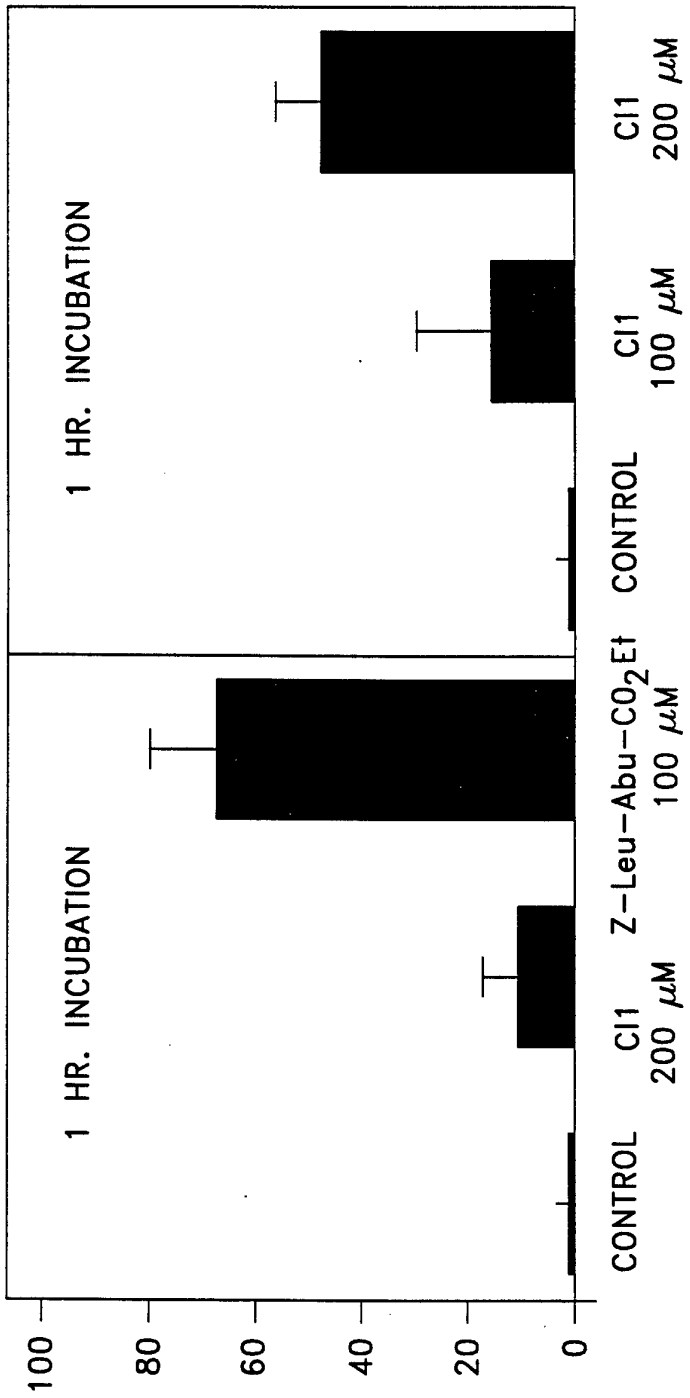

100
80
60
40
20
0
1 HR. INCUBATION
1 HR. INCUBATION
CONTROL
CI1 200 μM
Z-Leu-Abu-CO2Et 100 μM
CONTROL
CI1 100 μM
CI1 200 μM

FIG. 6

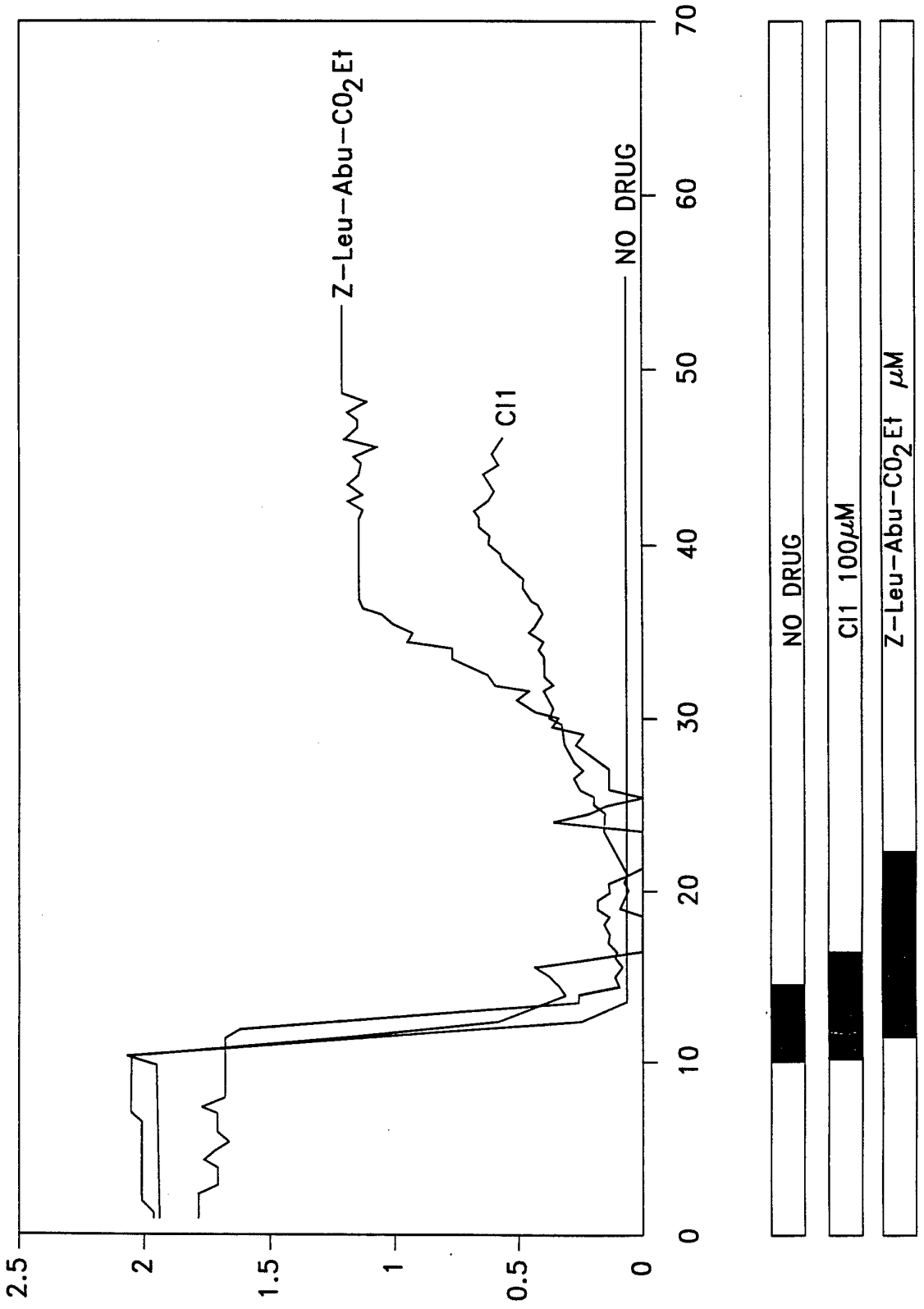

Z-Leu-Abu-CO2Et
NO DRUG
CI1
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
NO DRUG
CI1 100μM
Z-Leu-Abu-CO2Et μM

FIG. 7

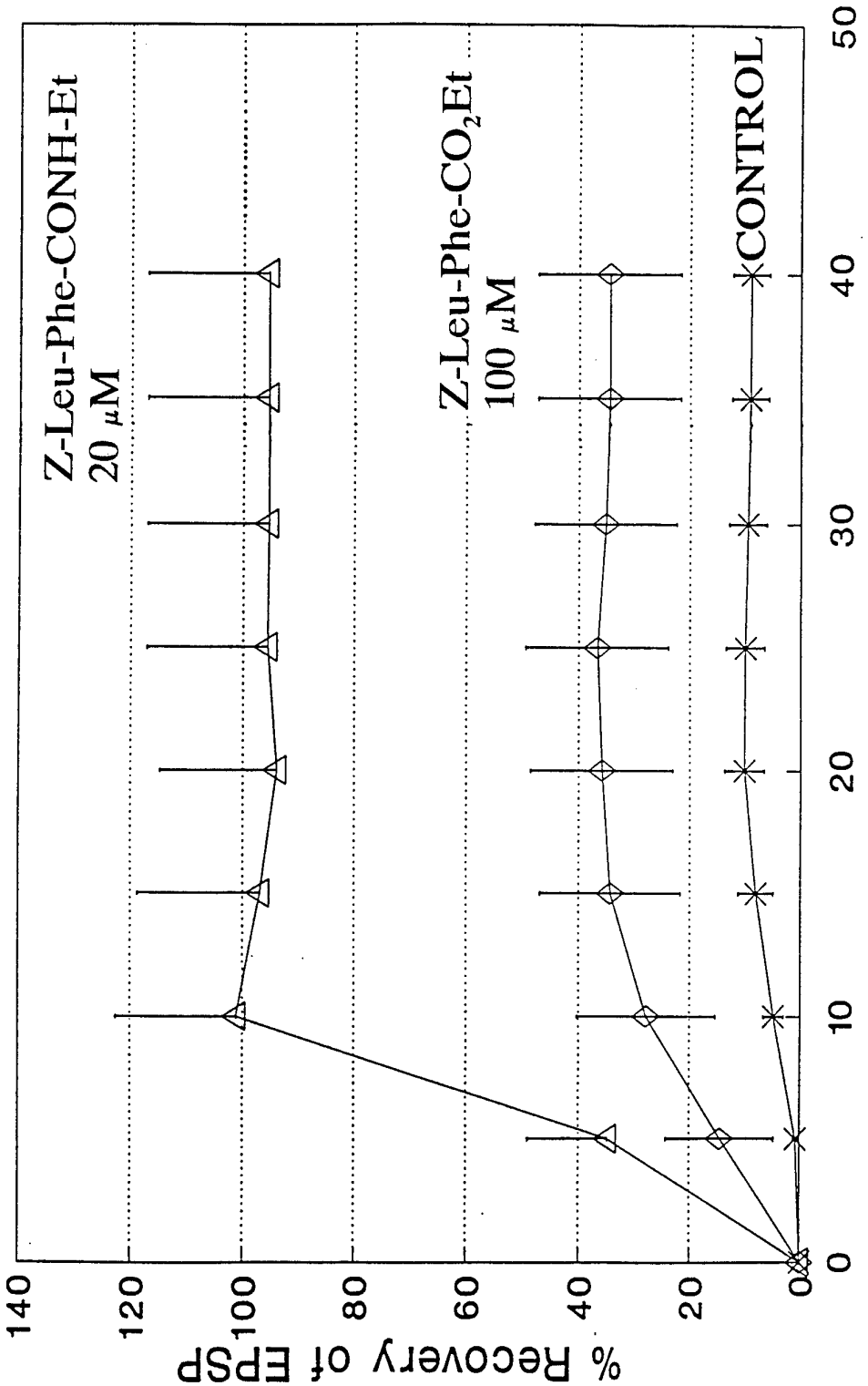

Z-Leu-Phe-CONH-Et
20 μM
Z-Leu-Phe-CO2Et
100 μM
CONTROL
% Recovery of EPSP
Time(min)
0
20
40
60
80
100
120
140
0
10
20
30
40
50

FIGURE 8

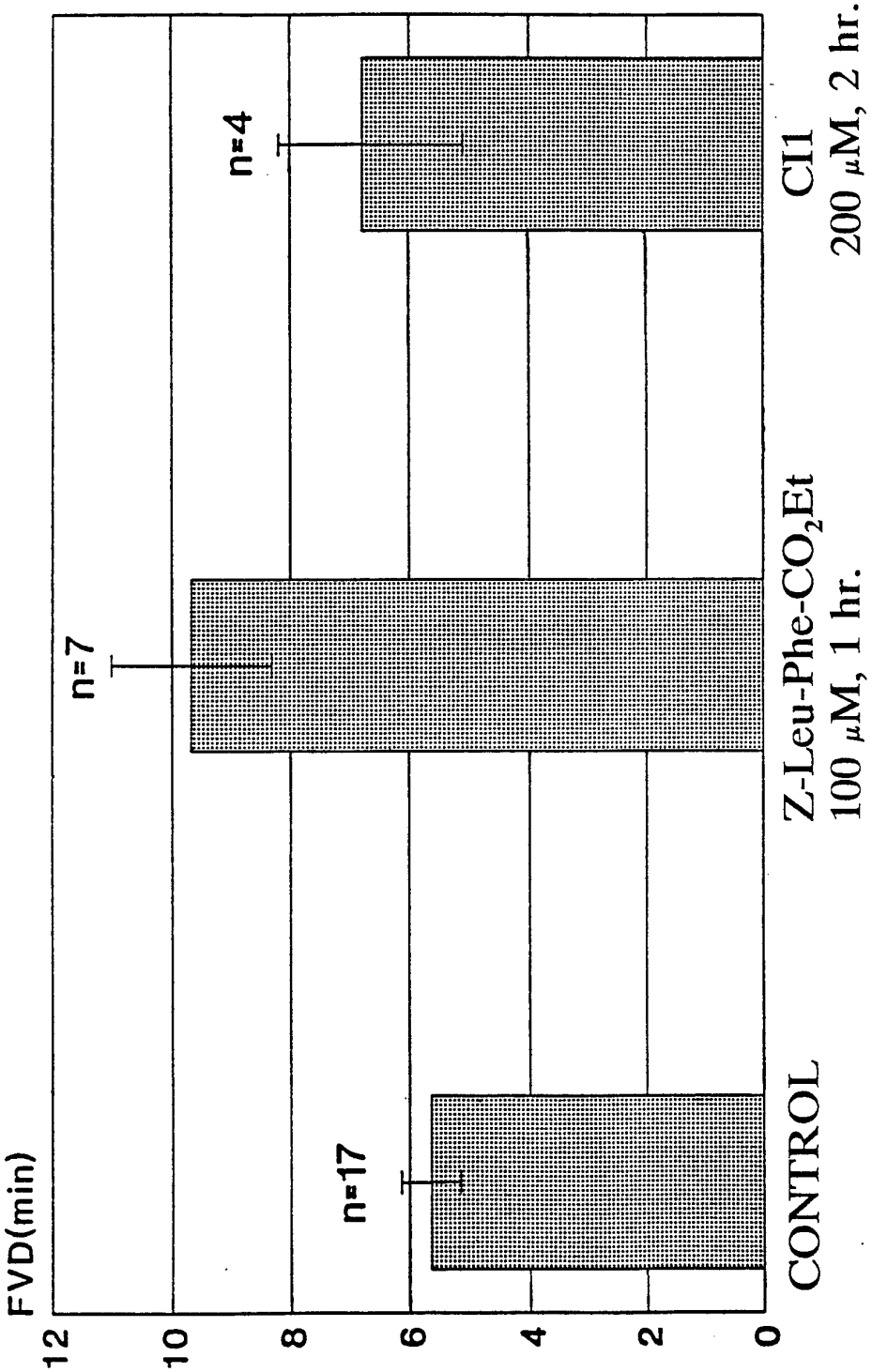

FVD(min)
12
10
8
6
4
2
0
n=17
n=7
n=4
CONTROL
Z-Leu-Phe-CO2Et
100 μM, 1 hr.
CI1
200 μM, 2 hr.

FIGURE 9

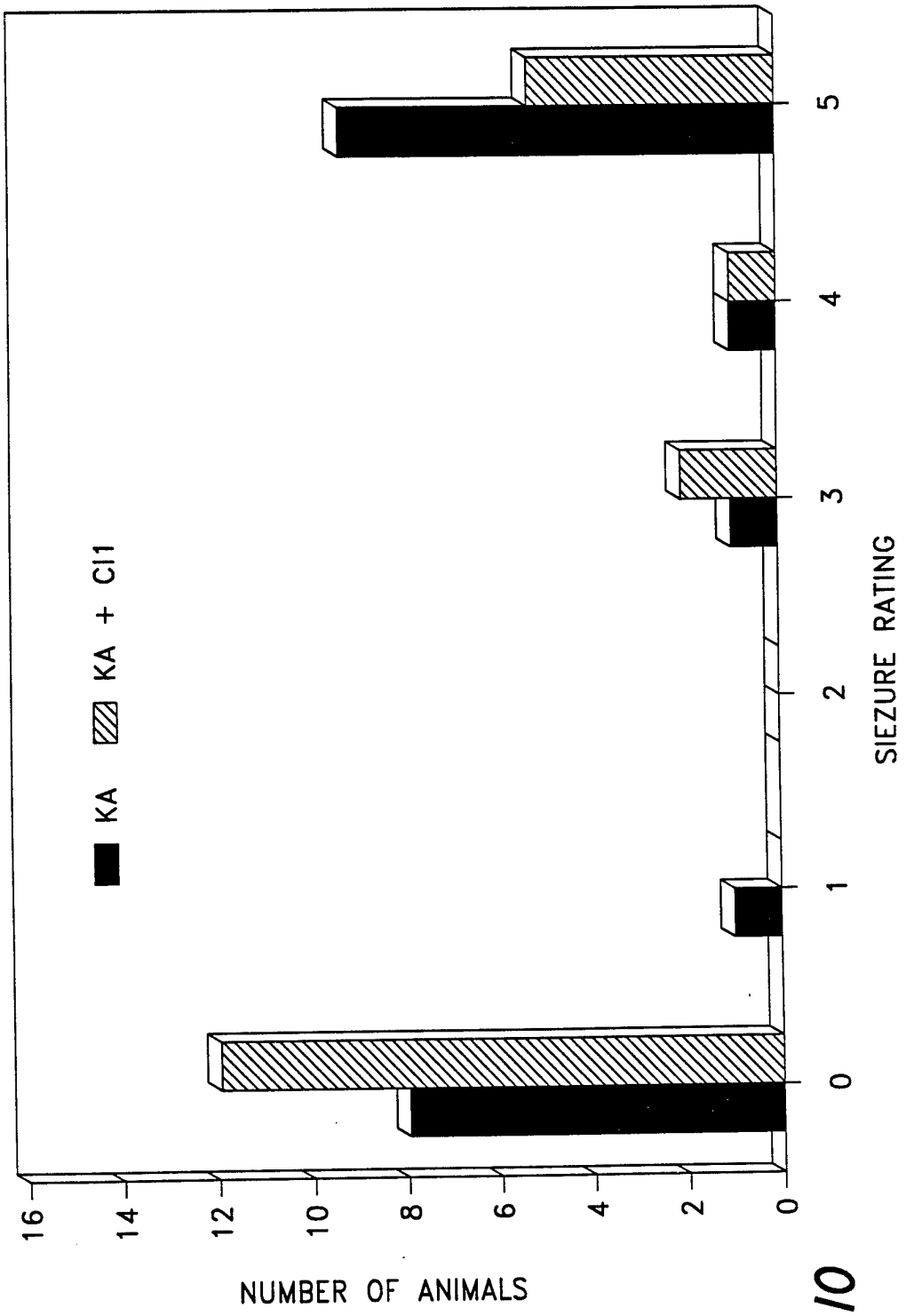

KA
KA + CI1
0
1
2
3
4
5
SIEZURE RATING
0
2
4
6
8
10
12
14
16
NUMBER OF ANIMALS

FIG. 10

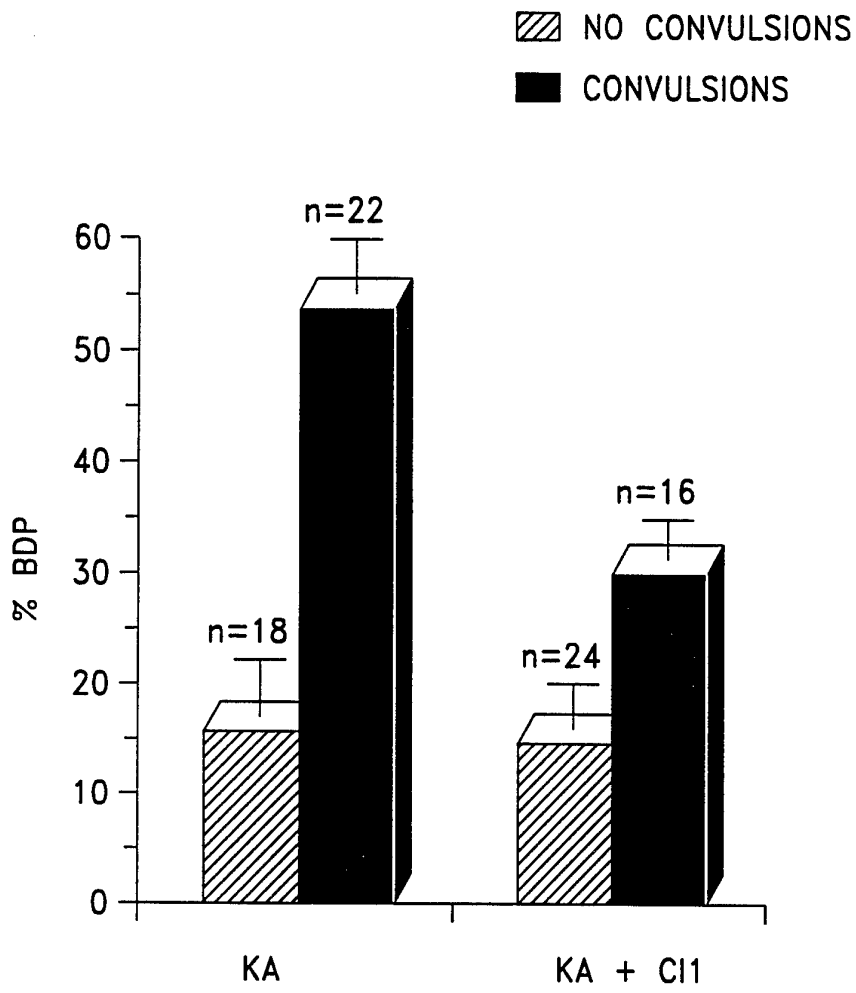

NO CONVULSIONS
CONVULSIONS
% BDP
60
50
40
30
20
10
0
n=22
n=18
n=16
n=24
KA
KA + CI1

FIG. 11

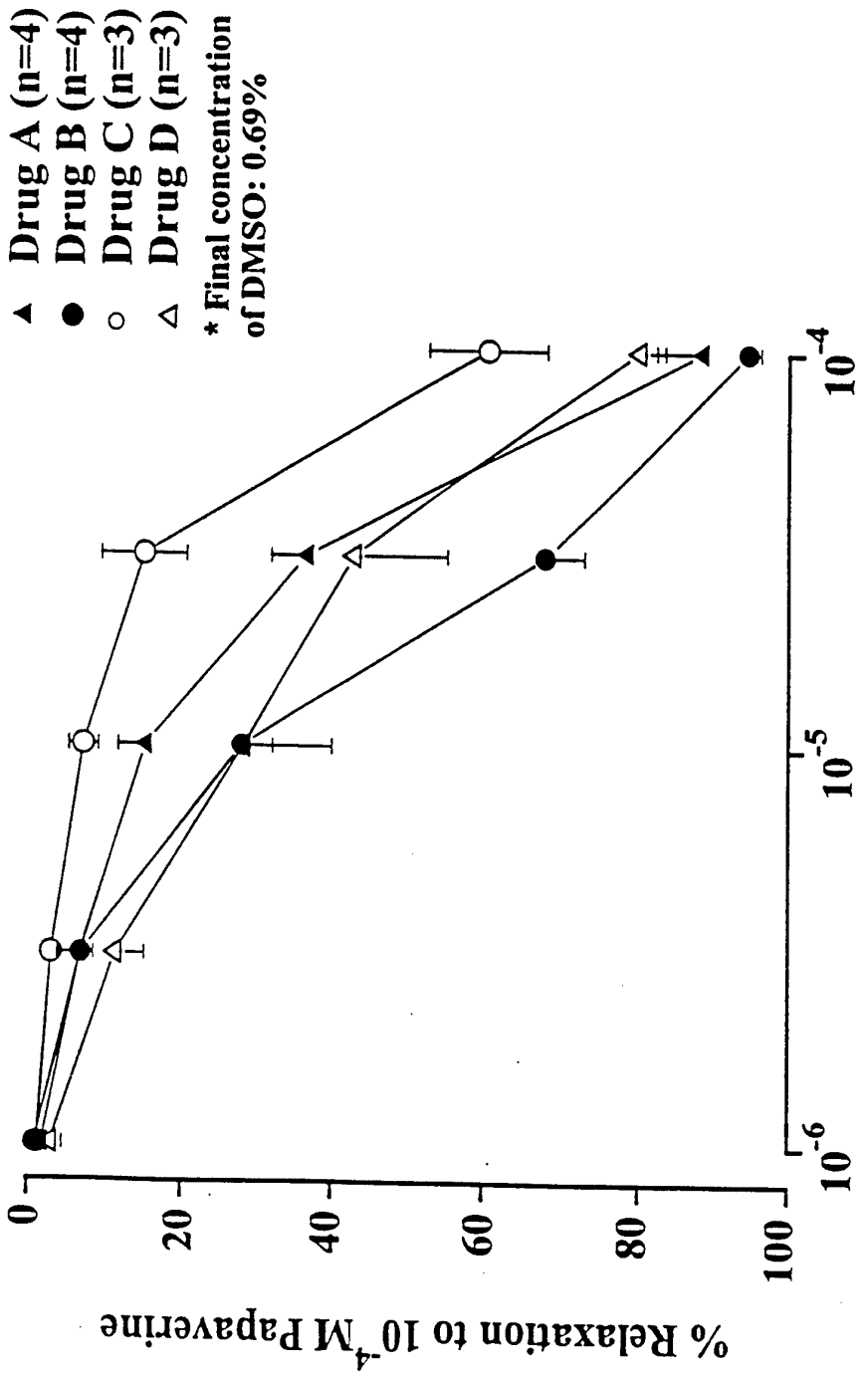

Drug A (n=4)
Drug B (n=4)
Drug C (n=3)
Drug D (n=3)
* Final concentration of DMSO: 0.69%
% Relaxation to 10^{-4}M Papaverine
0
20
40
60
80
100
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

FIGURE 12

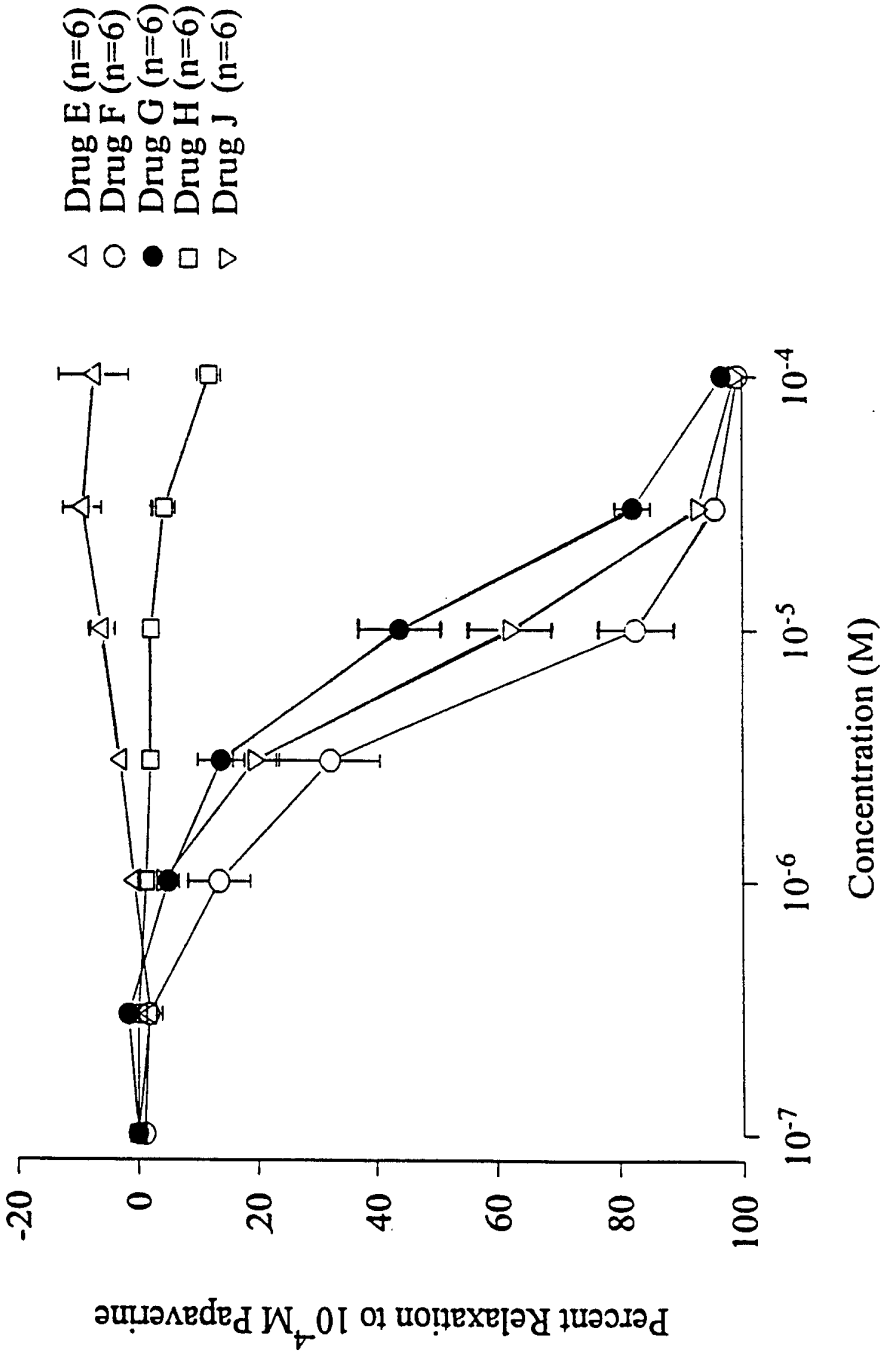

Drug E (n=6)
Drug F (n=6)
Drug G (n=6)
Drug H (n=6)
Drug J (n=6)
Percent Relaxation to 10^{-4}M Papaverine
-20
0
20
40
60
80
100
10^{-7}
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

FIGURE 13

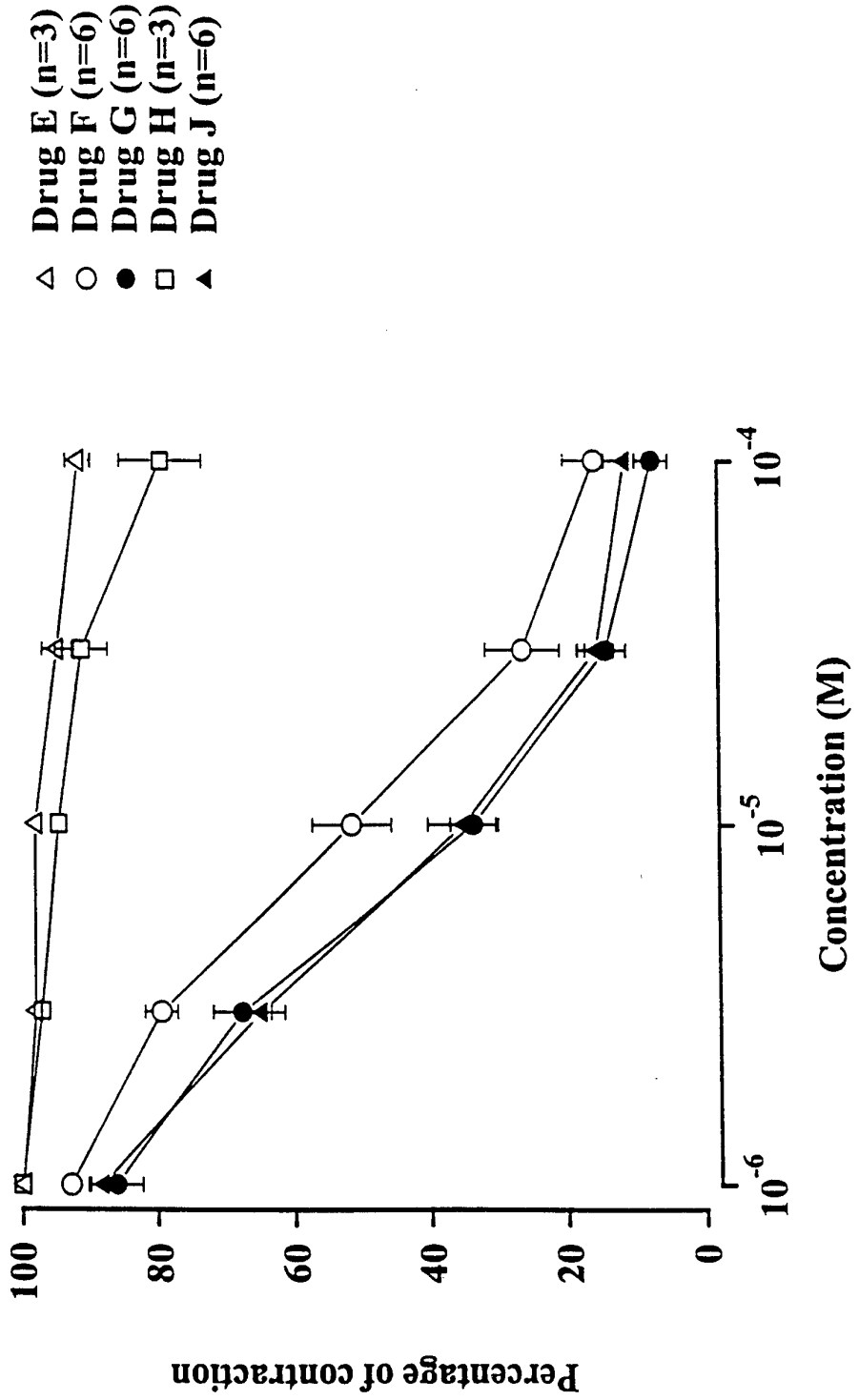

Drug E (n=3)
Drug F (n=6)
Drug G (n=6)
Drug H (n=3)
Drug J (n=6)
Percentage of contraction
100
80
60
40
20
0
10^{-6}
10^{-5}
10^{-4}
Concentration (M)

FIGURE 14

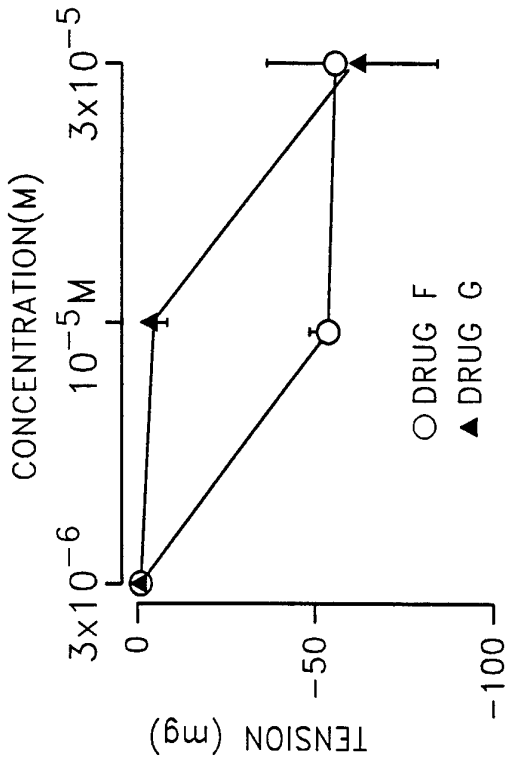


FIG. 15b

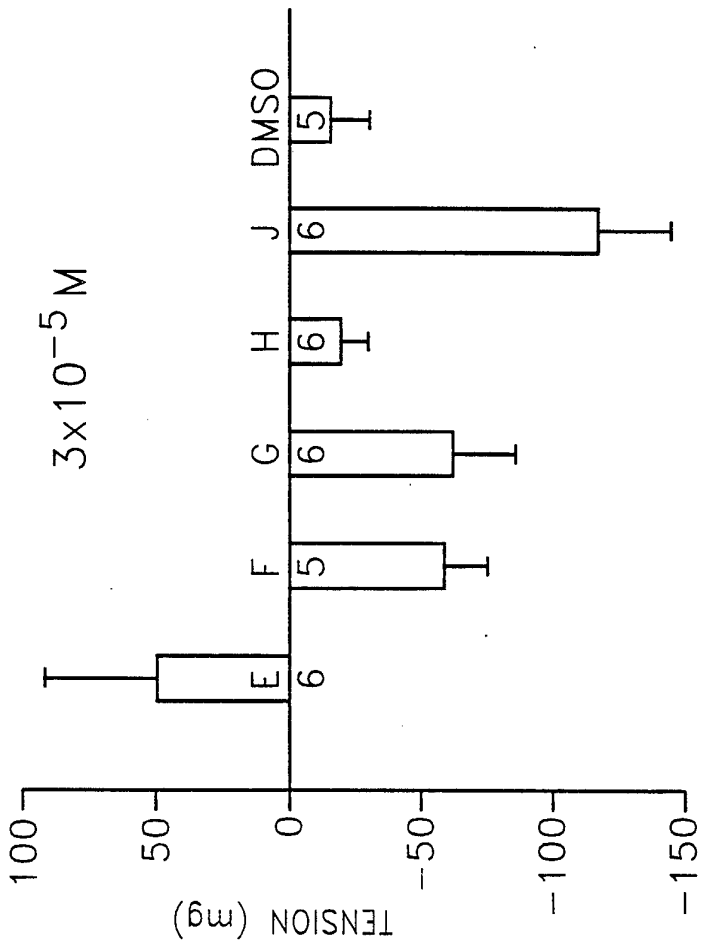


FIG. 15a

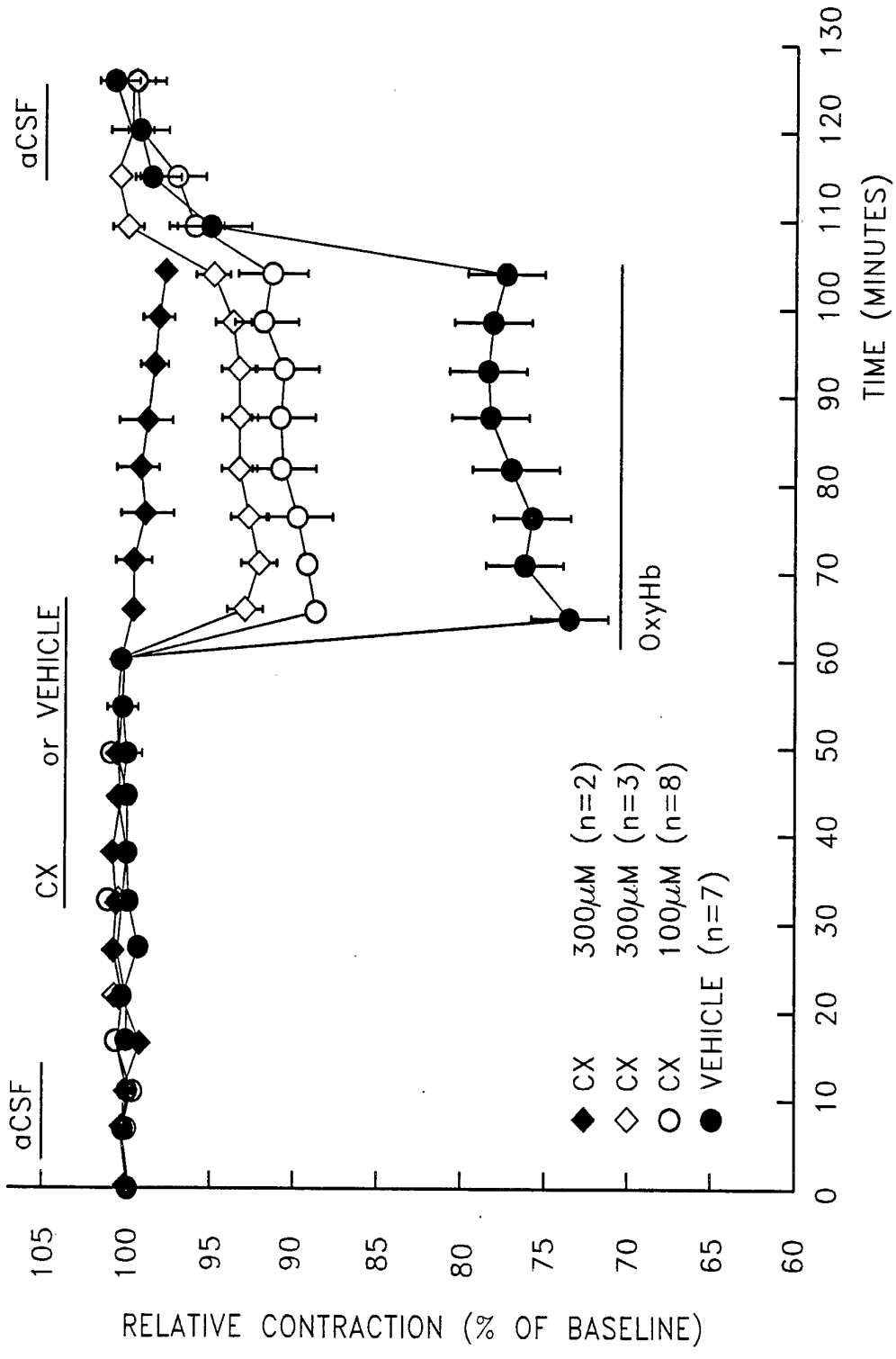

aCSF
CX or VEHICLE
aCSF
OxyHb
◆ CX 300μM (n=2)
◇ CX 300μM (n=3)
○ CX 100μM (n=8)
● VEHICLE (n=7)
RELATIVE CONTRACTION (% OF BASELINE)
105
100
95
90
85
80
75
70
65
60
0
10
20
30
40
50
60
70
80
90
100
110
120
130
TIME (MINUTES)

FIG. 16

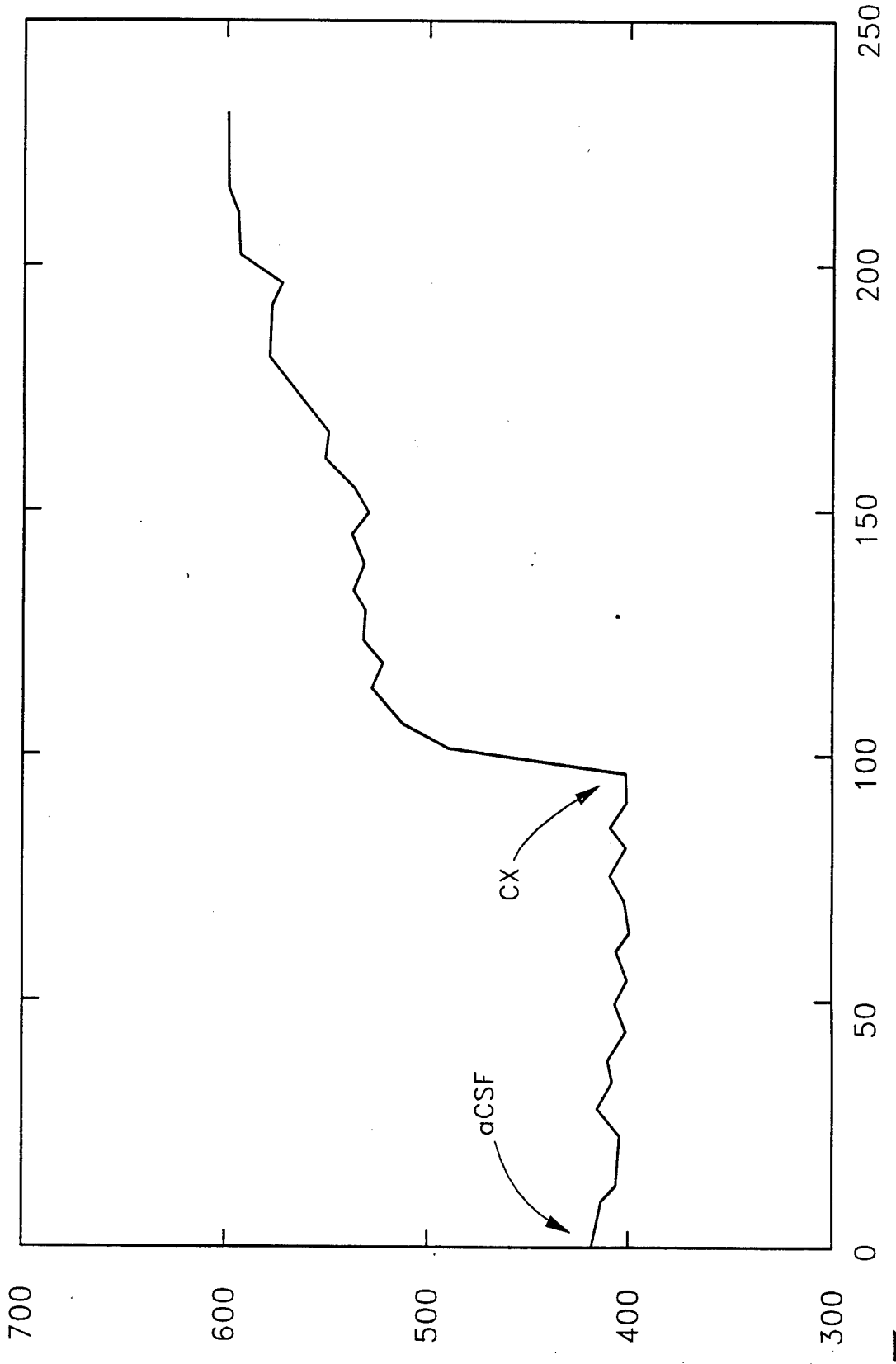


FIG. 17

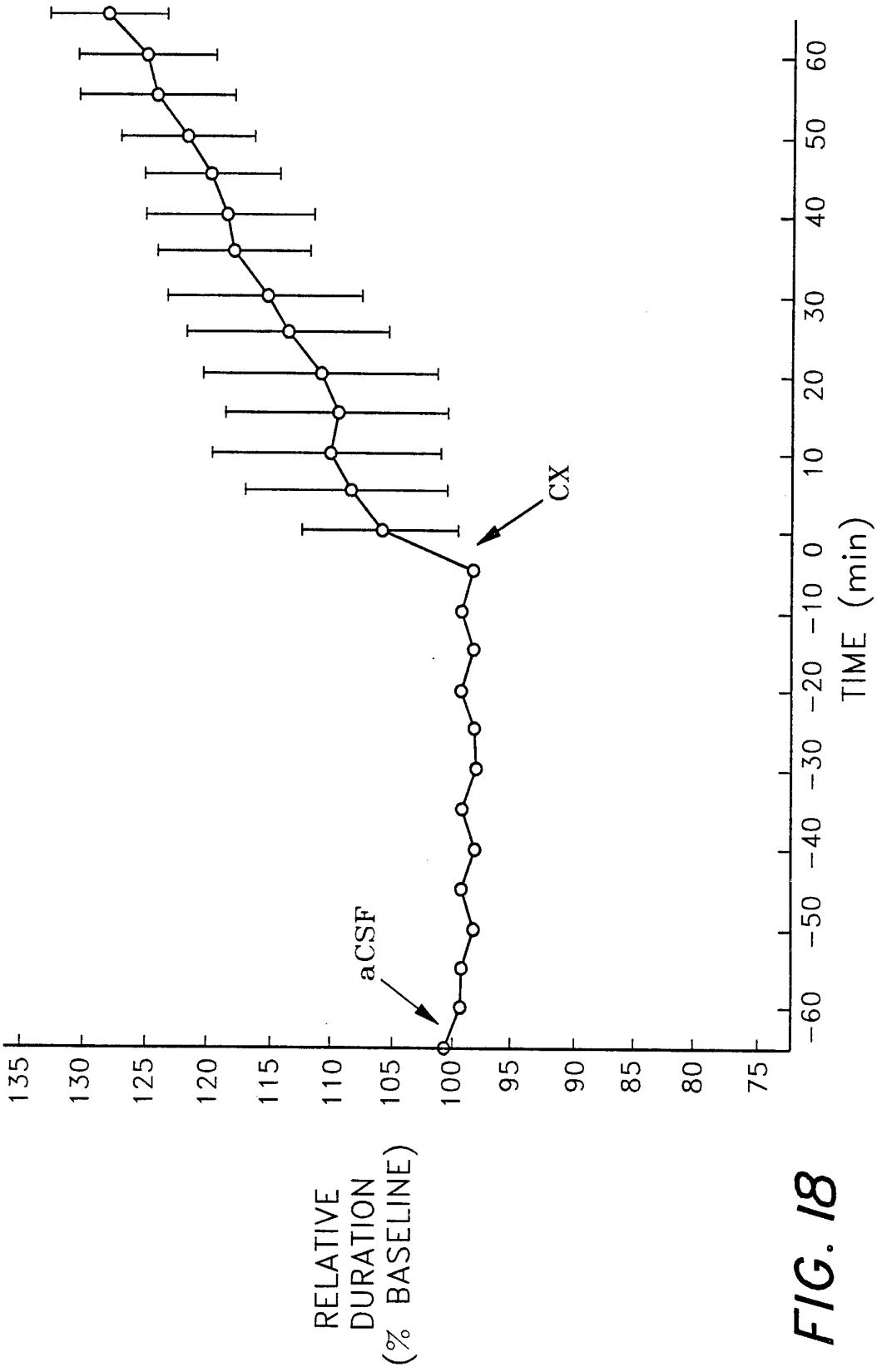

RELATIVE DURATION (% BASELINE)
135
130
125
120
115
110
105
100
95
90
85
80
75
aCSF
CX
TIME (min)
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
60

FIG. 18

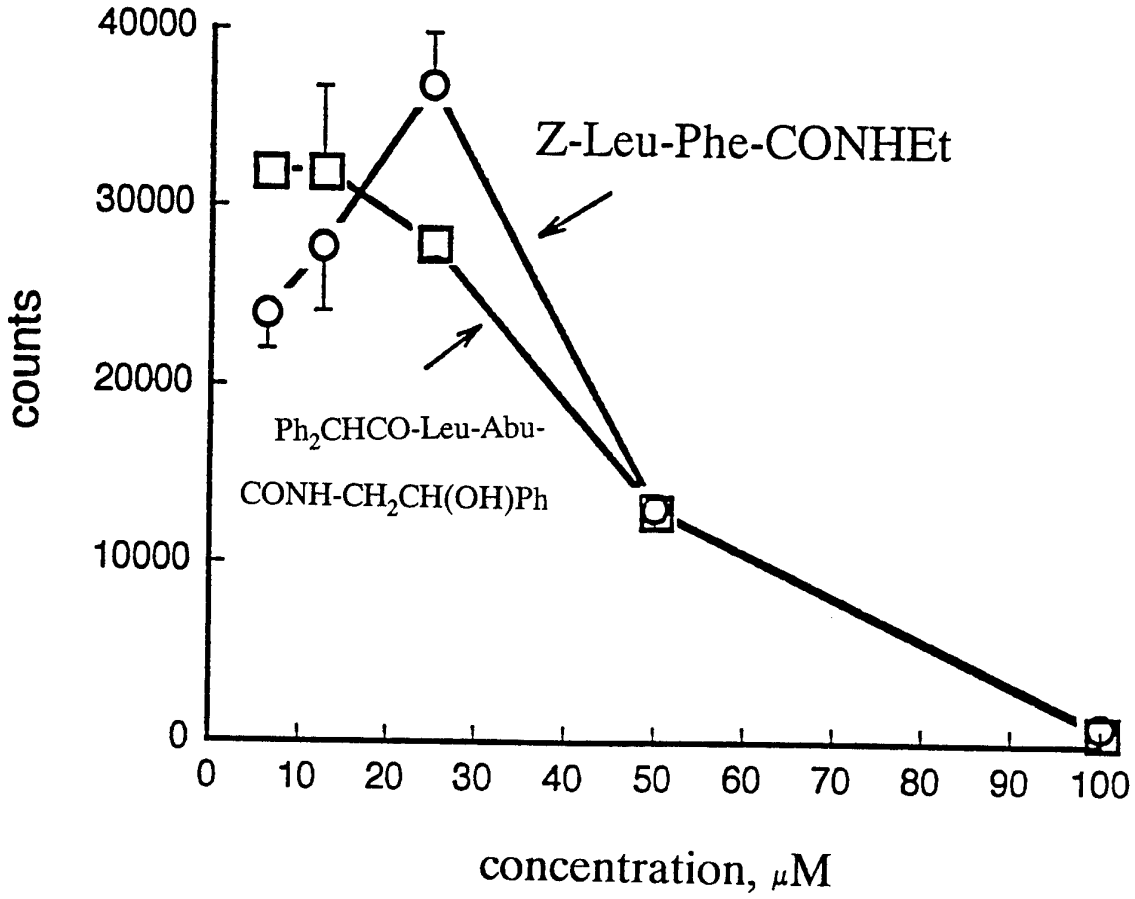

40000
30000
20000
10000
0
counts
Z-Leu-Phe-CONHEt
Ph2CHCO-Leu-Abu-
CONH-CH2CH(OH)Ph
0
10
20
30
40
50
60
70
80
90
100
concentration, μM

FIGURE 19

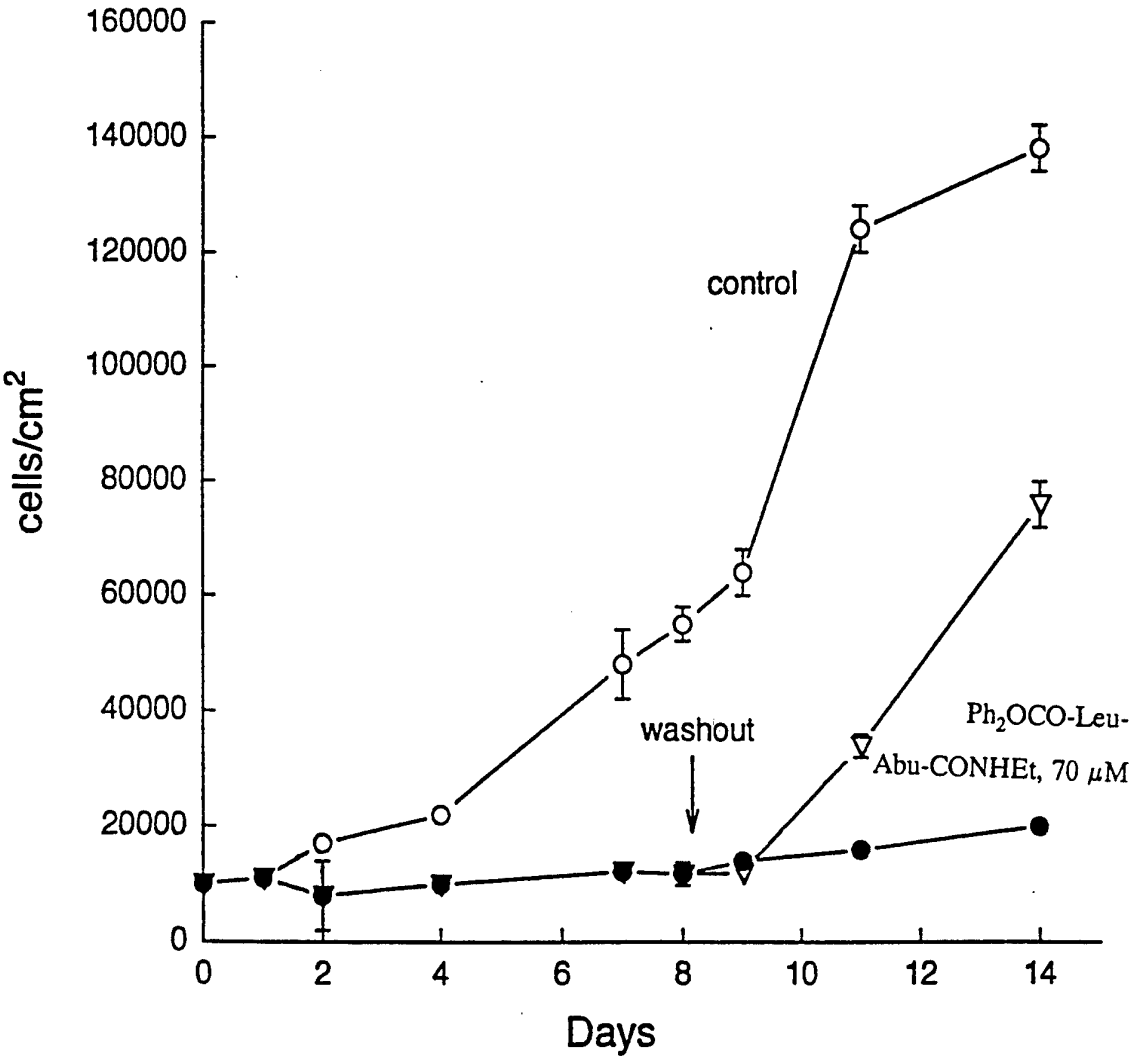

160000
140000
120000
100000
80000
60000
40000
20000
0
cells/cm2
0
2
4
6
8
10
12
14
Days
control
washout
Ph2OCO-Leu-
Abu-CONHEt, 70 μM

FIGURE 20

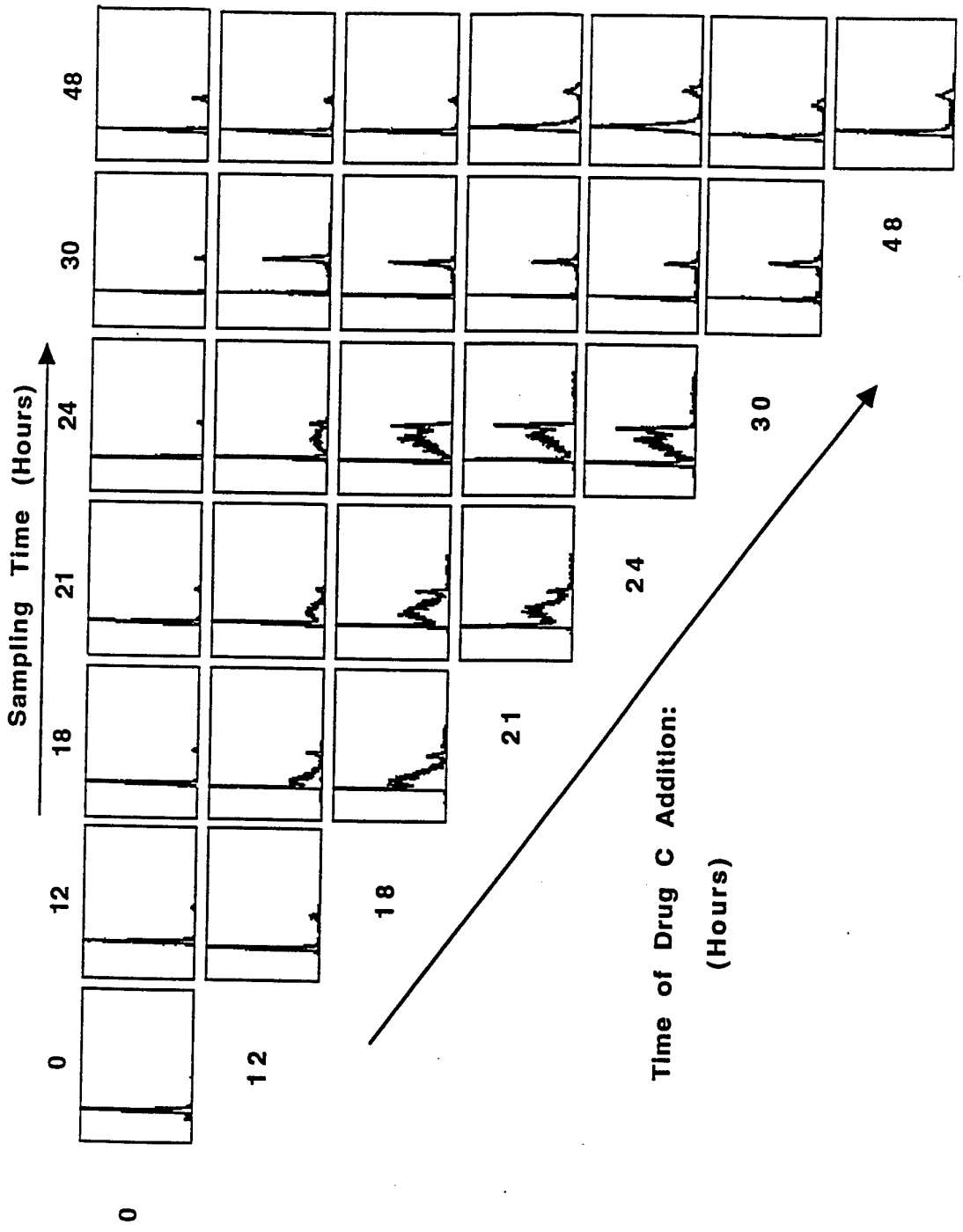

Sampling Time (Hours)
0
12
18
21
24
30
48
Time of Drug C Addition: (Hours)
0
12
18
21
24
30
48

FIGURE 21

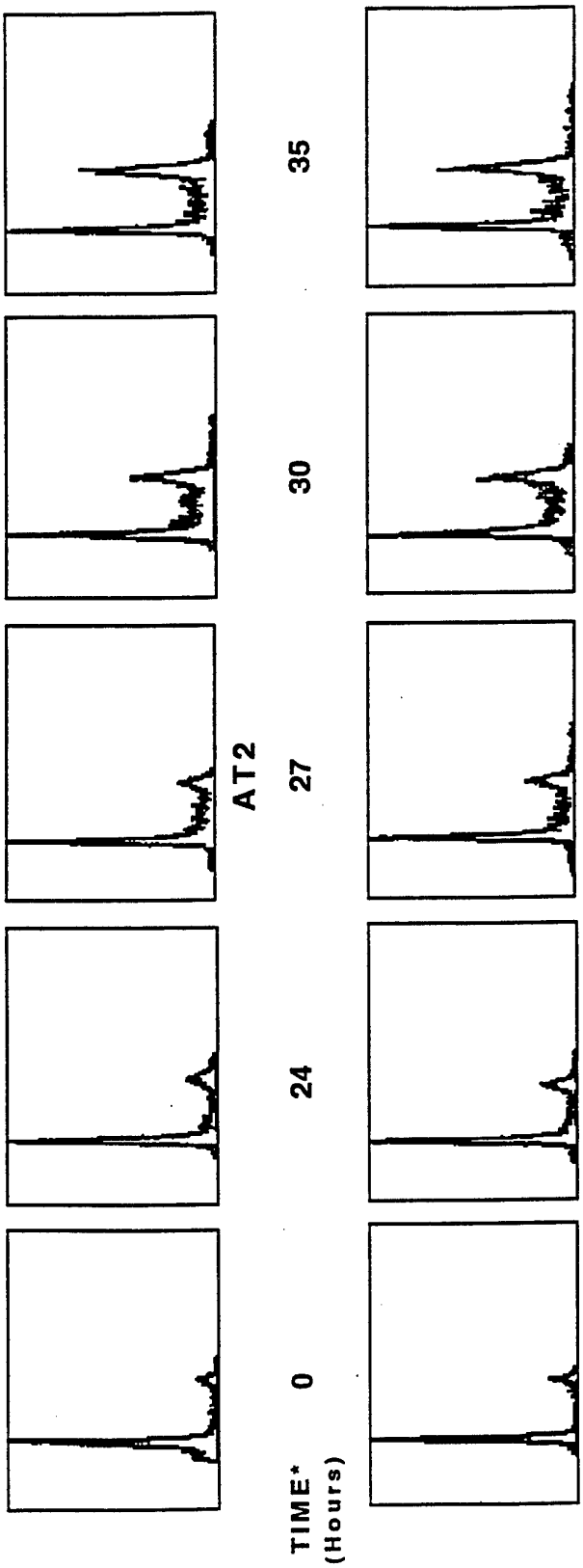

AT2
HeLa
TIME* (Hours)
0
24
27
30
35
*Note: 0 refers to washout after 48 hours of pre-incubation of cells in 70uM Drug C.

FIGURE 22