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(54) Title: LACTONES AND THEIR PHARMACEUTICAL APPLICATIONS

(57) Abstract

Novel lactones useful as antiviral, antifungal, antiproliferative, immunosuppressive and detoxifying agents as well as pharmaceutical compositions and methods based thereon are disclosed.

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"LACTONES AND THEIR PHARMACEUTICAL APPLICATIONS"

BACKGROUND

Brefeldin A (decumbin, "BFA") was first isolated in 1958 as a fungal metabolite from Penicillium decumbens (Singleton, V. L., et al., Nature 181:1072-1073 (1958)). BFA has a molecular weight of 280.37 ($C_{16}H_{24}O_4$) and reportedly has a wide range of biological activities, including antifungal, antiviral and antitumor effects. See Betina, Folia Microbiol. 37(1):3-11 (1992) for a recent review. At the cellular level, BFA has dramatic effects on the secretory pathway and protein trafficking in mammalian cells. (Pelham, H. R. B., Cell, 67:449-451 (1991); (Klausner, R. D., et al., J. Cell Biol., 116:1071-1080 (1992)). BFA has been shown to also inhibit protein transport in fungi, such as Candida albicans (Arioka, M., et al., J. Gen. Microbiol., 137:1253-1262 (1991) and inhibit the presentation of endogenous and exogenous protein antigens by MHC class II-restricted T-cells (Adorini, L., et al., Nature, 246:63-66 (July 1990)). BFA has also been shown to have selective cytotoxic activity against human tumor cell lines (Ishii, S., et al., J. Antibiot., XLII:1877-1878 (1989)).

BFA also inhibits virus replication by interfering with the intracellular transport and maturation of viral proteins. Inhibition, as defined herein, means a significant reduction in virus particle replication, as well as complete abrogation of virus particle replication. Enveloped viruses, such as herpes viruses (including Herpes Simplex) and Human Immunodeficiency Virus (HIV), require the host cell secretory apparatus for transport and processing of envelope (membrane) glycoproteins during the course of virus assembly and maturation. BFA has also been shown to inhibit infectious viral particle formation by preventing the transport of envelope glycoprotein to the cell surface as required for assembly of mature, infectious viral particles. (Cheung, P., et al., J. Virol., 65:1893-1904 (1991); Pal, R., et al., Aids Res. Human Retroviruses, 7:707-712 (1991); see also Takatsuki et al, Agric. Biol. Chem. 49(3):899-902 (1985)).

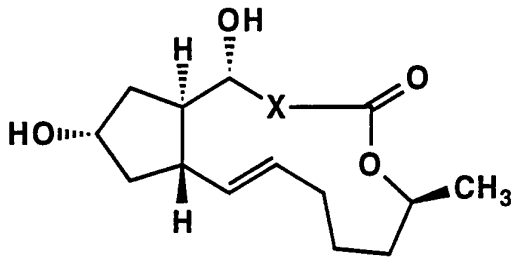
BFA has a short biological half-life. It is rapidly deactivated in vivo via conjugation with glutathione by glutathione S-transferase and subsequently transported out of the cell (Bruning, A., et al., J. Biol. Chem.,

267:7726-7732 (1992)). Compounds having some or all of the biological activities of BFA combined with an extended useful biological half-life and/or improved overall therapeutic profiles would be valuable for the treatment of viral, bacterial, fungal and other diseases, as immunosuppresive agents and as detoxifying agents.

DESCRIPTION OF THE INVENTION

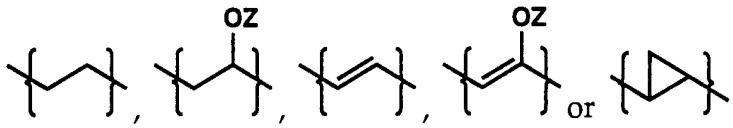
This invention concerns novel lactones related structurally to Brefeldin A; methods of synthesizing these lactones; use thereof as antiviral, antifungal, detoxification and antiproliferative agents (e.g., antitumor agents); pharmaceutical compositions which contain these lactones as active components; and pharmaceutical methods involving administration of these compounds to mammals, preferably human patients, in need thereof. These lactones block, or inhibit, the transport of proteins from the endoplasmic reticulum (ER) and through the Golgi apparatus in a cell and are also useful as experimental research reagents.

The lactones of this invention have the general formula:



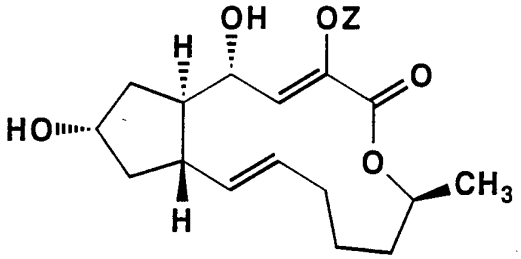
wherein (X) is a cyclopropyl moiety or saturated or unsaturated 2-carbon unit which may be optionally substituted (preferably at the alpha position relative to the carbonyl moiety) with a hydroxy or lower alkoxy moiety.

"X" may thus comprise one of the following functional groups:



wherein Z is -H or substituted or unsubstituted alkyl, preferably lower alkyl.

Compounds of this invention in which X is a hydroxy- or alkoxy-substituted unsaturated 2-carbon unit are depicted as follows:



where Z may be substituted or unsubstituted.

These lactones can be used as biological reagents to inhibit the intracellular transport of proteins from the ER of a cell through the compartments of the Golgi apparatus, and, ultimately, to the cell surface. For example, the lactones of this invention can be used to inhibit maturation of viral membrane glycoproteins in a cell infected with a virus by inhibiting the transport of viral membrane glycoproteins from the ER of the cell through the compartments of the Golgi apparatus, and, ultimately, to the cell surface, as required for the assembly of mature, infectious virus particles. These lactones can likewise be used to inhibit the transport, and thus the otherwise concomitant processing and presentation, of antigens by antigen presenting cells.

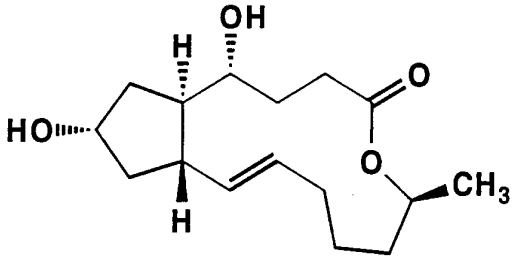
These lactones can also be used in pharmaceutical applications as antiviral, antifungal, immunosuppressive and antiproliferative agents (e.g., antitumor agents) and as detoxification agents. Accordingly, this invention further relates to pharmaceutical compositions which contain the compounds described herein as active components and which can be administered to an individual in need thereof. For example, a lactone of this invention can be combined with a physiologically compatible carrier for administration to an individual infected with a fungus or virus, or to an individual harboring a tumor. Without wishing to be bound by a particular theory, we do note that the compounds of this invention may act, at least in part, by inhibiting the transport of proteins critical, for example, to the maturation, intracellular replication and/or infectivity of virus, to the growth and/or proliferation of tumor cells, or to fungal growth.

SYNTHESIS

The compounds of this present invention can be produced synthetically from BFA or other starting materials as illustrated in the Examples which follow. BFA can be prepared by fermentation followed by

product recovery from the culture medium as described in detail in Harri, E., et al., Helv. Chim. Acta, 46:1235 (1963). Alternately, BFA can be synthesized using standard laboratory methods. (Baudouy, R., et al., Tetrahedron Letters, 34:2973-2976 (1977); LeDrian, C., et al., J. Am. Chem. Soc.,104:5473-5483 (1982); Kitahara, T. and Mori, K., Tetrahedron, 40:2935-2944 (1984)).

For example, Compound (I),

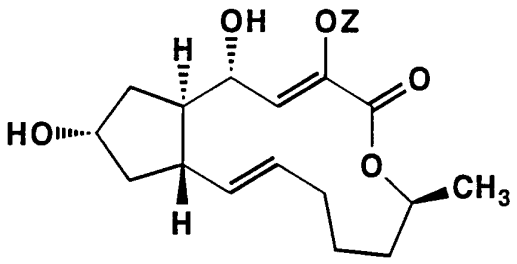


(I)

can be synthesized by reacting BFA with a thiol and a base to form a thiol addition product which is then reacted with a Raney Nickel catalyst. An example of a thiol and base combination which can be used is thiophenol and pyrrolidine. This method is illustrated in Example 1.

Alternatively, Compound I can be synthesized by reacting BFA with magnesium and hydrochloric acid. The preferred reaction sequence of this method is detailed in Example 1.

The 2-hydroxy and 2-alkoxy compounds:

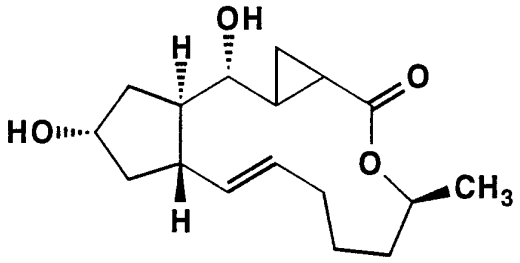


(II)

can be synthesized by reacting BFA with hydrogen peroxide and sodium hydroxide to form an intermediate product which is reacted with diazabicyclo-octane to form an alpha-keta ester of BFA. The alpha-keta ester is further reacted with acetic acid, and then with an alkyl orthoformate and toluenesulfonic acid-monohydrate to yield Compound II. An example of an alkyl orthoformate which can be used is triethyl orthoformate. An illustrative example is detailed in Example 2. Z can be hydrogen, methyl or other unbranched or branched lower alkyl (having 2-

5 carbon atoms). When Z is hydrogen, Compound II can exist as two tautomers where the hydrogen can reside either on the oxygen at C2 or on the carbon, C3, in which case the oxygen becomes double bonded to C2, i.e., it is a carbonyl group.

The cyclopropyl compounds **(III)**:



can be synthesized by reacting BFA with a slurry of trimethylsulfoxonium iodide, dimethyl sulfoxide and pentane washed sodium hydride. An illustrative example is provided in Example 3. The method of synthesis described herein, results in a mixture of two diastereomers, both of which possess the desired characteristics of an antiviral compound. Under the conditions described in Example 3, Compound IIIA (the 2R, 3R-diastereomer) is the major product (also referred to herein as BFAP). The two diastereomers can be separated by standard laboratory methods. In addition, the apex of the cyclopropyl ring can be further modified to include one or two, halogens (e.g., fluoride), or, alternately, one, or two, unbranched alkyl groups.

EVALUATION OF IN VITRO BIOLOGICAL ACTIVITY

The biological activity of these compounds can be evaluated and compared using conventional *in vitro* assays for inhibition of protein trafficking between the endoplasmic reticulum (ER) and the Golgi apparatus and specifically for antiviral, antitumor, immunosuppressive and antifungal activity as discussed in further detail below.

(a) Inhibition of Protein Transport

The inhibitory activity of the lactones with respect to protein transport can be evaluated in a cell-free system as described in Orci, L., et al., Cell, 64:1183-1195 (1991). Generally, secretory proteins, such as membrane glycoproteins, are transported from the endoplasmic reticulum to the Golgi apparatus, and subsequently to the cell surface, via transport

vesicles. To evaluate the ability of the compounds described herein to prevent the formation of transport vesicles, Golgi apparatus membranes can be isolated and incubated with cytosol, ATP, an ATP regenerating system and the compounds to be tested as described in Orci, L., et al., Cell, 64:1183-1195 (1991).

The activity of our lactones in inhibiting protein transport may also be evaluated using a Guanine Nucleotide Exchange Factor (GEF) assay as described in detail in Example 4. The GEF assay is based on assays described in Donaldson, J. G., et al., Nature, 360:350-352 (1992), and Helms, J. B., et al., Nature, 360:352-354 (1992). A number of cytosolic proteins are specifically associated with the Golgi apparatus. One such protein, ß-COP, is rapidly released from the Golgi upon treatment with BFA. This release occurs within 20 seconds of BFA treatment and is complete in 1-2 minutes. Upon removal of BFA, ß-COP rapidly reassociates with the Golgi apparatus. (Klausner, R.D., et al., J. Cell Biol. 116:1071-1080 (1992)). The binding of ß-COP to Golgi membranes has been shown to be dependent on the interaction of another protein, ADP-ribosylation factor (ARF) with the Golgi membrane. ARF association with the Golgi is, in turn, dependent on binding the guanine nucleotide, GTP. A component of Golgi membranes specifically catalyzes the exchange of GTP onto ARF. BFA prevents the assembly of ß-COP onto the Golgi membrane by inhibiting the GTP-dependent interaction of ARF with the Golgi membrane. (Donaldson, J. G., et al., Nature, 360:350-352 (1992); Helms, J. B., et al., Nature, 360:352-354 (1992)).

The activity of our lactones in preventing Golgi membranes from catalyzing the exchange of GTP onto ARF may be evaluated as described in Example 4.

(b) Anti-viral Activity

BFA has been shown to have dramatic effects on membrane protein glycosylation and processing, key steps which affect the egress of enveloped viruses from infected cells. (Whealy, M. E., et al., J. Virol., 65:1066-1081 (1991)). The envelopment of a virus, during the maturation process in an infected host cell, appears to be a multistep pathway. The viral capsid acquires a membrane by budding of the capsid through the nuclear membrane such that an immature enveloped virion is formed. This immature virion is transported through the endoplasmic reticulum

(ER) and undergoes subsequent de-envelopment, with release of the immature virus particle in proximity to the Golgi apparatus. Subsequent maturation of the immature virion occurs at the Golgi apparatus, which involves a second envelopment of these immature capsids by membrane proteins derived from the Golgi apparatus, containing fully processed viral glycoproteins. The resulting mature, infectious enveloped virus particle is released from the cell by fusion of the outer membrane of the virion envelope with the plasma membrane of the host cell, or, alternately, can be transported via transport vesicles to the cell surface, where membrane fusion results in presentation of viral glycoproteins on the cell surface. BFA does not affect protein synthesis at the translational level, but blocks the post-translational processing and export of viral glycoproteins to the Golgi apparatus, thus, inhibiting viral replication by preventing the formation and/or release of mature, infectious virus particles.

The lactones of this invention can be tested for specific antiviral activity as described in Example 5 as well as by other conventional antiviral assay methods. See e.g. Whealey et al, supra; Johnson et al, J Virol 43(3):1102-1112 (1982); Sidwell et al, Nucleotides and Nucleosides 8:833-836 (1989) and Chen et al, J Virol 65(3):1427-1439 (1991). As described in detail in Example 5, the antiviral activity and non-specific cytotoxic effects of these lactones can be readily evaluated using Hep2 cells infected with Herpes Simplex Virus type 1 (HSV-1). BFA can be used as a control, as can clinically relevant or other known positives, such as IUDR (iodouracyl deoxyribocyte) which can be used as an antiviral, positive control.

(c) Evaluation of Other in vitro Activities

Compounds can be evaluated with respect to specific antifungal, anticancer, immunosuppressive or other pharmaceutically relevant activities using conventional materials and methods. See e.g. Arioka, J. Gen. Microbiol., 137:1253-1262 (1991)(evaluation of antifungal activity); Ishii et al., J. Antibiot. XLII:1877-1878 (1989)(evaluation of cytotoxic/antitumor activity); Sun et al, US Patent 5,206,249 (issued 27 April 1993)(evaluation of in vitro growth inhibitory activity on cultured leukemia cells); and Yoshida et al., Experimental Cell Research 192:389-395 (1991)(evaluation of anti-toxin activity).

EVALUATION OF IN VIVO BIOLOGICAL ACTIVITY

Bioactivity of compounds of this invention can be further evaluated in conventional animal model systems including anti-viral, anti-fungal, antitumor, immunosuppression and detoxification assays involving experimental animal models, e.g. using rats, mice, rabbits, guinea pigs, sheep or non-human primates. Numerous animal models for such studies, as well as animal models for determining biological half-life, pharmacokinetics and toxicology, are well known in the art. The in vivo toxicity of these lactones can be readily evaluated with conventional toxicity assays as well as by the method described in Example 6.

(a) in vivo antiviral activity

The effectiveness of the lactones of this invention in controlling viral infection can be evaluated in any of the conventional assay systems. See e.g. Stanberry, "Pathogenesis of Herpes Simplex Virus Infection and Animal Models for its Study" and Renegar, Laboratory Animal Science 42(3):222. For instance, HSV infection can be evaluated using guinea pig and mouse model systems that are art-recognized models used in the study of genital herpes. The guinea pig model system is described in detail in Stanberry, L.R., et al., J. Infect. Diseases, 153:1055-1061 (1986), and Bourne, N., et al., Antimicrob. Agents and Chemo., 36:2020-2024 (1992). The effectiveness of antiviral agents against influenza virus can be evaluated in mice as described by Sidwell et al, in Antiviral Res. 6:343-353 (1985) and in Antimicrob. Ag. Chemother. 36:473-476 (1992).

(b) in vivo antitumor activity

The antitumor effectiveness of our lactones can be evaluated in vivo with conventional xenograft models using various human tumor cell lines xenografted into mice as described, for example, in Sun et al, supra, as well as in various transgenic animal models (again, see Sun et al, supra, col 21).

PHARMACEUTICAL APPLICATIONS

Compounds of this invention which prevent, inhibit or reduce the severity of viral infection (e.g. an infection by a virus such as a Herpes Simplex virus), fungal infection (e.g. an infection by a fungus

such as Candida albicans), tumors or tumor growth or the effect of toxic substances or which have an immunosuppressive effect may be used for treatment or prevention in a mammal in need thereof.

Mammals include rodents such as mice, rats and guinea pigs as well as dogs, cats, horses, cattle, sheep, non-human primates and humans.

The preferred method of such treatment or prevention is by administering to a mammal an effective amount of a compound of this invention to prevent, alleviate or cure said disease or disorder. An effective amount of a lactone of this invention is an amount of one or more lactones of this invention which inhibits one or more of protein transport from the endoplasmic reticulum, viral replication, fungal growth, tumor cell growth, and pathological effect(s) of a toxin, or which results in immunosuppression, as the case may be. Such effective amounts can be readily determined by evaluating the lactones of this invention in conventional assays well-known in the art, including assays described herein.

Therapeutic/Prophylactic Administration & Pharmaceutical Compositions

The invention provides methods of treating, preventing and/or alleviating the symptoms and/or severity of a disease or disorder referred to above by administration to a subject a compound of the invention in an amount effective therefor. The subject will be an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human.

Various delivery systems are known and can be used to administer a compound of this invention, e.g., encapsulation in liposomes, microparticles, microcapsules, etc. One mode of delivery of interest is via pulmonary administration, as detailed more fully infra. Other methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral routes. A compound of this inventions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents.

Administration can be systemic or local. For treatment or prophylaxis of nasal, bronchial or pulmonary infections or tumors, preferred routes of administration are oral, nasal or via a bronchial aerosol or nebulizer.

In specific embodiments, it may thus be desirable to administer a compound of this invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application (e.g., for viral or fungal infections or tumors of the skin), by injection, by means of a catheter, by means of a suppository, or by means of a skin patch or implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

This invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically (or prophylactically) effective amount of a compound of this invention, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The carrier and composition can be sterile. The formulation should suit the mode of administration.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

In a specific embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the side of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed

with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

Administration to an individual of an effective amount of one or more of the compounds described herein can also be accomplished topically by administering the compound(s) directly to the affected area of the skin of the individual. For this purpose, the compounds are administered or applied in a composition including a pharmacologically acceptable topical carrier, such as a gel, an ointment, a lotion, or a cream, which includes, without limitation, such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils.

Other topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolaurate (5%) in water, or sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In addition, in certain instances, it is expected that the compounds of this invention may be disposed within devices placed upon, in, or under the skin. Such devices include patches, implants, and injections which release the compound into the skin, by either passive or active release mechanisms.

In a specific application of this invention, we note that genital infection with HSV is characterized by herpetic lesions on the external genital skin. As a consequence of initial genital infection, latent infection is established. One possible mechanism for the maintenance of latency involves the migration of virus from recurrent lesions back to sensory ganglia, where a new set of neurons are infected and become a source of latent virus responsible for recurrent disease. (Stanberry, L.R., et al., J. Infect. Dis., 153:1055-1061 (1986)). Thus, administration of an antiviral agent which inhibits the formation of mature infectious virus particles would be useful to prevent migration of HSV and reasonably prevent establishment of a latent HSV infection. Topical administration of a lactone of this invention directly to the areas of the skin affected with the herpetic lesions would be an attractive method of administration. As an illustrative example of anti-viral application of a

pharmaceutical agent, see Whitley et al, "Acyclovir: A Decade Later", New England Journal of Medicine pp. 782-789 (10 September 1992).

Materials and methods for producing the various formulations are well known in the art [see e.g. US Patent Nos. 5,182,293 and 4,837,311 (tablets, capsules and other oral formulations as well as intravenous formulations)].

A compound of this invention can be formulated in neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2- ethylamino ethanol, histidine, procaine, etc.

The effective dose of compounds of this invention will typically be in the range of about 0.01 to about 50 mg/kgs, preferably about 0.1 to about 10 mg/kg of mammalian body weight, administered in single or multiple doses. Generally, the compounds of this invention may be administered to patients in need of such treatment in a daily dose range of about 1 to about 2000 mg per patient.

The amount of a compound of this invention which will be effective in the treatment or prevention of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, in vitro or in vivo assays may optionally be employed to help identify optimal dosage ranges. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. The precise dosage level of our compounds, as the active component(s), should be determined by the attending physician or other health care provider and will depend upon well known factors, including route of administration, biological activity of the particular compound, and the age, body weight, sex and general health of the individual; the nature, severity and clinical stage of the disease; and the use (or not) of concomitant therapies.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form

prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

Pulmonary Administration

In an embodiment of this invention of particular interest, a compound of this invention is administered by pulmonary administration, e.g. via aerosolization. This route of administration may be particularly useful for treatment or prophylaxis of bronchial or pulmonary infection or tumors.

Pulmonary administration can be accomplished, for example, using any of various delivery devices known in the art (see e.g., Newman, S.P., 1984, in Aerosols and the Lung , Clarke and Davia (eds.), Butterworths, London, England, pp. 197-224; PCT Publication No. WO 92/16192 dated October 1, 1992; PCT Publication No. WO 91/08760 dated June 27, 1991; NTIS Patent Application 7-504-047 filed April 3, 1990 by Roosdorp and Crystal), including but not limited to nebulizers, metered dose inhalers, and powder inhalers. Various delivery devices are commercially available and can be employed, e.g., Ultravent nebulizer (Mallinckrodt, Inc., St. Louis, Missouri); Acorn II nebulizer (Marquest Medical Products, Englewood, Colorado), Ventolin metered dose inhaler (Glaxo Inc., Research Triangle Park, North Carolina); Spinhaler powder inhaler (Fisons Corp., Bedford, Massachusetts); or Turbohaler (Astra, Inc.). Such devices typically entail the use of formulations suitable for dispensing from such a device, in which a propellant material may be present.

Ultrasonic nebulizers tend to be more efficient than jet nebulizers in producing an aerosol of respirable size from a liquid (Smith and Spino, "Pharmacokinetics of Drugs in Cystic Fibrosis," Consensus Conference, Clinical Outcomes for Evaluation of New CF Therapies, Rockville, Maryland, December 10-11, 1992, Cystic Fibrosis Foundation).

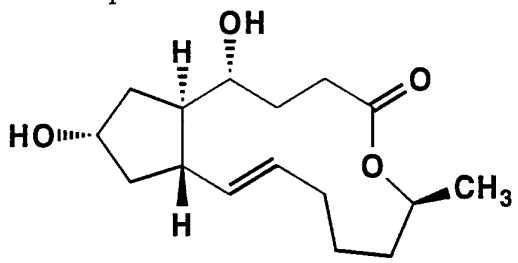
A nebulizer may be used to produce aerosol particles, or any of various physiologically acceptable inert gases may be used as an aerosolizing agent. Other components such as physiologically acceptable surfactants (e.g., glycerides), excipients (e.g., lactose), carriers, and diluents may also be included.

This invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

Examples

Example 1: Synthesis of Compound I



(I)

The 2,3-dihydro derivative **(I)** can be synthesized using either of the two following methods.

Method 1

BFA (0.5 gram) is added to ethanol (16 mL) and stirred under a nitrogen atmosphere at room temperature until the BFA is dissolved. Thiophenol (0.29 gram) is added to this solution of dissolved BFA, followed by the addition of pyrrolidine (0.13 gram). The solution is stirred for 25 hours, at room temperature. The reaction mixture is then diluted with methylene chloride and sequentially extracted with 0.05 N HCl, 0.05 N NaOH and brine. The organic layer is then dried over sodium sulfate, filtered and concentrated to an oil.

The above oil is taken into ethanol (20 mL) containing 5 grams of Raney Nickel catalyst (50% in water). The resulting slurry is stirred 4 hours at room temperature, and heated to reflux for an additional 4 hours. The catalyst is removed by filtration and the filtrate is diluted with methylene chloride and extracted sequentially with 0.5 N HCl, 0.5 N

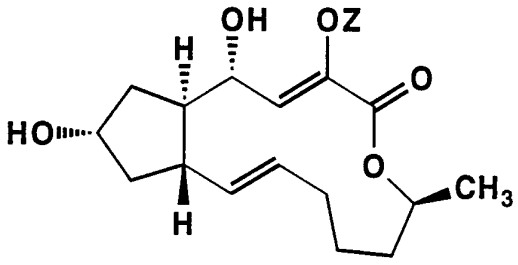
NaOH, and brine as described above. The organic layer is dried over sodium sulfate, filtered and concentrated to give the product as a solid.

Method 2

BFA (0.135 gram) is dissolved in methanol (4 mL) and stirred under a nitrogen atmosphere at room temperature until the BFA is dissolved. To this solution of dissolved BFA is added magnesium turnings (0.069 gram). The mixture is again stirred at room temperature until all of the magnesium metal is consumed (16 hours). The resultant turbid solution is poured into 1N hydrochloric acid (50 mL) and the solution is extracted three times with of ethyl acetate. The organic layers are combined and washed successively with water (50 mL) and brine (50 mL), dried over sodium sulfate, filtered and concentrated to a heavy clear oil.

The structure of **(I)**, made by either method 1 or method 2, was confirmed by NMR spectroscopy.

Example 2: Synthesis of alkoxylactones



(II)

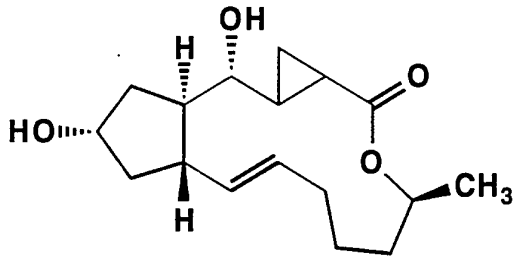
To a cooled, vigorously stirred solution of BFA (0.5 gram) and 30% hydrogen peroxide (0.6 mL) in ethanol (3 mL) is added 6 N NaOH (0.15 mL) over a 15 minute time period. The solution is stirred 3 hours at room temperature. Water (10 mL) is added. The solution is extracted with methylene chloride. The organic layer is first washed with water, then a 3% KI-water solution, dried over sodium sulfate, filtered and concentrated to an oil.

The above oil is taken into ethanol (5 mL) containing DABCO (diazabicyclo-octane, 0.24 gram) and stirred 24 hours at room temperature to obtain the alpha-keto ester of Brefeldin A. The mixture is diluted with water containing acetic acid, and extracted with methylene chloride. The

organic layer is dried over sodium sulfate, filtered and concentrated to give the alpha-keto ester of Brefeldin A as an oil.

The oil is taken into absolute ethanol (5 mL) and 2.7g of an alkyl orthoformate (triethyl orthoformate) is added. To this solution is added ethanol (1 mL) containing toluenesulfonic acid-monohydrate. The resulting mixture is heated to reflux for 30 minutes, cooled to room temperature and concentrated under vacuum. The residue is taken into toluene (5 mL) and heated to reflux for 24 additional hours. The toluene is removed under reduced pressure and the residue taken into methylene chloride and passed through a plug of silica gel. Evaporation of the solvent gives the desired product as an oil.

Example 3: Synthesis of Cyclopropyl Lactones **(III)**



Trimethylsulfoxonium iodide (0.258 gram) was added to a 10 mL flask containing a stir bar, nitrogen inlet, 2.5 mL dimethyl sulfoxide (DMSO) and pentane washed NaH. The resulting slurry was stirred at room temperature until gas evolution ceased (15 minutes). Brefeldin A (0.107 gram) was added at once and stirring was continued for an additional hour at ambient temperature. The reaction mixture was quenched with water (4 mL) and extracted with ethyl acetate (4x 50 mL). The combined organic extract was washed with water (2x 25 mL), brine (25 mL) and dried over sodium sulfate. The solvent was removed under vacuum and the residue chromatographed through a silica gel column, eluted with ethyl acetate, to separate isomers.

The major R, R-isomer gave an oil which crystallized upon trituration with acetonitrile. Recrystallization from acetonitrile gave crystals suitable for x-ray crystallography. The structure was consistent with the R, R-isomer as determined by x-ray crystallography and the following $^{1}H/^{13}C$ NMR data:

^{1}H NMR ($CDCl_3$)ppm 1.0 (d,2H, Me) 1.1-1.65(m, 8H) 1.7-2.25(m,10H) 3.5(d,1H,C(4)H-OH) 4.15(m,1H, C(7)H-OH) 4.9(m,1H, C(15)H-OR) 5.3 (m,2H, C(10)H=C(11)H)

^{13}C NMR ($CDCl_3$) ppm 173.9, 137.1, 129.4, 73.1, 72.6, 69.6, 50.4, 44.9, 44.1, 41.1, 34.2, 30.1, 26.2, 25.1, 20.6, 15.9, 11.1.

The minor S,S-isomer was isolated as an oil. The S,S-isomer structure was consistent with the following ^{1}H/^{13}C NMR data:

^{1}H NMR ($CDCl_3$) ppm 0.75(m,1H) 1.08(d, 2H, CH_3) 1.1-1.2(m,1H) 1.25-1.4(m, 5H) 1.55(m, 1H) 1.7(m, 1H) 1.8-2.2(m, 8H) 2.3(m, 1H) 3.7(m, 1H) 4.2(m, 1H) 4.8(d, 1H) 5.3(m, 2H)

^{13}C NMR ($CDCl_3$) ppm 12.5, 18.0, 22.0, 23.7, 25.7, 32.5, 35.7, 38.9, 42.9, 43.7, 49.2, 68.0, 71.8, 81.5, 131.4, 133.6, 176.8.

Example 4: Guanine Nucleotide Exchange Factor Assay

Recombinant myristoylated ADP-ribosylation factor (ARF) is purified from Escherichia coli co-expressing the human ARF-1 gene and N-myristoyltransferase as described in Weiss, O., et al., J. Biol. Chem., 264:21066-21072 (1989) and Duronio, R. J., et al., Proc. Natl. Acad. Sci. USA, 87:1506-1510 (1990).

Golgi membranes from rat livers are obtained by sucrose gradient centrifugation as described in Balch, W. E., et al., Cell, 39:525-536 (1984).

Incubations are carried out as described in Donaldson, J.G., et al., Nature 360:350-352 (1992) and Helms, J.B., and Rothman, J.E., Nature 360:352-354 (1992). Briefly, a 50.5 µl reaction mixture containing ARF, Golgi membranes, sucrose, ovalbumin, HEPES-KOH buffer containing KCI and Mg, 100 µM BFA analog and [^{35}S]GTP was incubated at 37°C for 15 minutes. The specific reactions are set up as described in Lanes 2 through 5 of Table 1.

The amount of ARF-bound and -unbound [^{35}S]GTP is separated with 10 kD molecular weight cutoff cellulose filters. Nonspecific binding (Lane 0 of Table 1) is subtracted. Using Compound IIIA, for example, we have observed inhibition of the exchange of GTP onto ARF with this assay.

Alternatively, the ARF-bound [^{35}S]GTP can be separated by Sephadex G25 gel filtration. Again, Compound IIIA was shown to cause inhibition.

Example 5: Test for Antiviral Activity

The antiviral activity and cytotoxic microscopic effects of BFA, Compound I and Compound IIIA on Hep2 cells were determined in the following manner.

Hep2 cells in RPMI/1640 medium with 5% fetal calf serum were grown to provide a confluent sheet of cells, various concentrations of BFA, Compound I or Compound III, as indicated in Table 1, 2 and 3, were added. The cells were incubated at 37°C, in 5% carbon dioxide.

In a cytotoxicity screening assay, different concentrations of BFA, Compound I and Compound IIIA were added to the cell culture. Cytotoxicity was determined by microscopic examination on days 3 and 6. The results are shown in Table 2, and can be summarized as having demonstrated the cytotoxicity to have been highest in the BFA treated cells, reduced with Compound I, and further reduced in the Compound IIIA treated cells.

The antiviral and cytotoxic effects of BFA, Compound I and Compound IIIA on Hep2 cells infected with HSV-1 were determined as follows.

Hep2 cells in RPMI/1640 medium with 5% fetal calf serum were added to microtiter wells and incubated at 37°C in 5% carbon dioxide. To the Hep2 cells, various concentrations (4 wells/concentration compound) of BFA, Compound I, Compound IIIA or IUDR were added. HSV-1 virus was added 7 hours later. The cell culture specimens were examined for evidence of viral growth and Hep2 microscopic toxicity on days 1, 2, 3, 4, 5, 6, 7 and 10 post-infection. Virus controls at concentrations as shown in Table 3 were included. The results of this study are depicted in Table 3.

TABLE 1

Lane Number	0	1	2	3	4	5
2.3 M sucrose	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
0.5 mM BFA/analog*	-	-	10 µl	10 µl	10 µl	10 µl
Water	23 µl	10 µl	-	-	-	-
Buffer	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
16 mg/ml Ovalbumin	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
0.6 mg/ml golgi	-	5 µl	5 µl	5 µl	5 µl	5 µl
0.4 mg/ml Arf-1	-	8 µl	8 µl	8 µl	8 µl	8 µl
20 µM GTP (^{35}S)	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl	12.5 µl

Lane 0: background (CPM subtracted)
1: control
2: BFA
3: cyclopropyl-BFA
4: dihydro-BFA
5: BFC

TABLE 2 - Cytotoxicity Screen (Hep2 cells)

Drug	BFA		Compound I		Compound IIIA	
µg/ml	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
100	T	T	T	T	T	T
50	T	T	T	T	T	T
10	T	T	T	T	O	O
5	T	T	T	T	O	O
1.0	O	T	O	O	O	O
0.1	O	O	O	O	O	O
0.05	O	O	O	O	O	O
0.03	O	O	O	O	O	O
0.015	O	O	O	O	O	O
0.0075	O	O	O	O	O	O
Minimal Toxic Dose	1.0		5.0		50.0	

T = Toxic by microscopic observation; a cytotoxic effect

O = Cells normal

TABLE 3

HSV-1 10^{-12}; HEP2-cells PA #538 Viral Inoculum Dilution = 10^{-12} (about 5 to 7 $TCID_{50}$)
Microscopic Endpoints (lysis granulation, rounding, giant cells)

Drug	µg/ml	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 10
BFA	2	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	T T T T	T T T T
	1	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	T T T T	T T T T
	0.5	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
Compound I	4	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	T T T T	T T T T
	2	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	T T T T	T T T T
	1	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
Compound IIIA	25	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
	12.5	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
	6.25	O O O O	O O O O	O O O O	O O O +	O O O +	O O O +	O O O +	O O O +
IUDR	40	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
	30	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
	25	O O O O	O O O O	O O O O	O O O O	O + O O	O + O O	O + O O	O + O O
Virus Control	Dilution								
	10^{-10}	O O O O	O O + +	O + + +	O + + +	O + + +	O + + +	O + + +	+ + + +
	10^{-11}	O O O O	O O O +	O O O +	+ O O +	+ O O +	+ O O +	+ O O +	+ + O +
	10^{-12}	O O O O	O O O O	O O O O	O O + +	O O + +	O O + +	+ O + +	+ + + +
		O O O O	O O O O	+ O O O	+ + O O	+ + O O	+ + O O	+ + O O	+ + + C**
	10^{-13}	O O O O	O O O O	O O O O	O O O +	O O O +	O O O +	O O O +	
		O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O +
									O O O O
	10^{-14}	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	
		O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O	O O O O
									O O O O

O = No Viral Replication; T = Toxicity (delayed toxicity with BFA and BFAP);
+ = Viral Replication; ** $TC1D_{50} = 10^{-12.6}$

Example 6: In Vivo Toxicity Study

An intraperitoneal dose response study of BFA and Compound I in mice was performed to provide and compare initial information on the toxicologic profile of BFA and the Compound I analogue as follows.

Female CD-1 mice (6/group) were employed in this investigation. BFA or Compound I was suspended in 0.5% carboxymethylcellulose (CMC). The animals were scheduled to receive BFA or Compound I, intraperitoneally, at dosage levels of 50, 100 and 400 mg/kg/day for 5 consecutive days. All doses were administered in a constant volume of 20 ml/kg. Another group of animals received 0.5% CMC (20 ml/kg) and served as a control. The animals were observed for at least an additional two weeks after which a necropsy was performed. Tissues were collected for histopathologic evaluation.

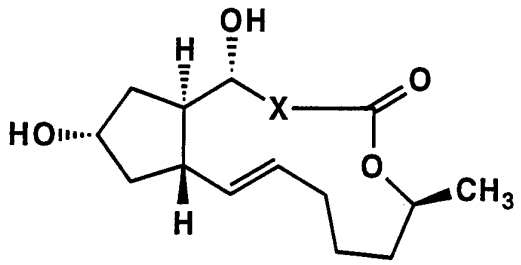
No clinical signs of toxicity were observed with either BFA or Compound I at the 50 and 100 mg/kg/day dose groups. Of the 6 animals that received 400 mg/kg of BFA, 5 died within 16 to 18 hours post dose. The one surviving animal received a second dose of BFA, 400 mg/kg and was found dead the following morning. Predeath signs included unconsciousness, decreased respiration and cyanosis. The onset of the signs was several hours post dose.

None of the 6 animals dosed with 400 mg/kg of Compound I for 5 consecutive days died during the course of the study. All animals showed transient CNS signs within 5-10 minutes post dose with recovery within 1 to 1 1/2 hours. Necropsy of animals dosed with BFA or Compound I showed no gross morphologic changes. Histopathic evaluation, performed on high dose animals only, revealed no treatment-related morphologic changes with either test compound.

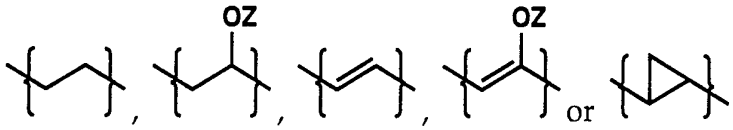
CLAIMS

We claim:

1. A compound of the formula:

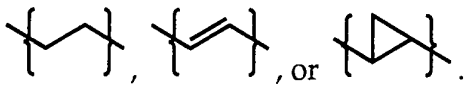


wherein X comprises

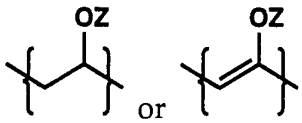


wherein Z is -H or alkyl.

2. A compound of claim 1 wherein X comprises



3. A compound of claim 1 wherein X comprises



wherein Z is -H or alkyl.

4. A method of inhibiting the transport of proteins from the endoplasmic reticulum in cells comprising administering to the individual an effective amount of a compound of claim 1.

5. A method of treating or preventing viral infection, fungal infection, tumor growth, untoward immune reactivity or pathological effects of a toxin, in an individual, which method comprises administering to the individual a compound of claim 1 in an amount effective therefor.

6. A pharmaceutical composition for treating or preventing viral infection, fungal infection, tumor growth, untoward immune reactivity or pathological effects of a toxin, which pharmaceutical composition comprises a compound of claim 1 and a pharmaceutically acceptable carrier.

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US94/01040

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07D 313/00, 493/06
US CL :514/453, 455, 468, 691. 729; 549/268, 270
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/453, 455, 468, 691. 729; 549/268, 270

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 3,896,002 (HOWARD ET AL) 22 July 1975, see entire document.	1-6
A	US, A, 4,608,078 (ACKER ET AL) 26 August 1986, see entire document.	1-6
A	US, A, 5,112,607 (HUDSON ET AL) 12 May 1992, see entire document.	1-6
A, P	US, A, 5,242,932 (GANDY ET AL) 07 September 1993, see entire document.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 APRIL 1994	Date of mailing of the international search report 16 MAY 1994
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