(54) Title: HYBRID PLASMINOGEN ACTIVATORS

(57) Abstract

A hybrid fibrinolytic enzyme, its preparation, pharmaceutical compositions containing it and its use in the treatment of thrombotic disease.

HYBRID PLASMINOGEN ACTIVATORS

The present invention relates to a hybrid fibrinolytic
enzyme and derivatives thereof, its preparation,
pharmaceutical compositions containing it and its use in the
treatment of thrombotic disease, in particular acute
myocardial infarction.

The sequence of amino acids making up the enzyme tissue-type
plasminogen activator (t-PA) and the nucleotide sequence for
the cDNA which codes for t-PA are known (see Pennica et al.,
1983; Nature, 301, 214). t-PA is known to have fibrinolytic
activity.

As used herein, the term tissue-type plasminogen activator
(t-PA) denotes a plasminogen activator of the group having
the immunological properties defined for t-PA at the XXVIII
Meeting of the International Committee on Thrombosis and
Haemostasis, Bergamo, Italy, 27 July 1982.

The amino acid sequence of various forms of t-PA are known.
The abovementioned Nature 1983 reference discloses the
sequence for the L-chain and the mature S-chain forms of
t-PA, also discussed by Vehar et al., Biotechnology, 1984,
2, 1051-7 in which the processing of initially formed t-PA
by removal of a pro-sequence to give the S-chain form is
reported. Pohl et al., FEBS letters, 1984, Vol. 168 No.1,
29-32, refers to the N-terminal multiplicity of t-PA and
discloses the U-chain form. The numbering system for the
amino acid sequence of t-PA used herein is that described in
the Nature 1983 reference for mature (S-chain) t-PA in which
the N-terminal serine is numbered 1. By this system,
L-chain t-PA has an N-terminal glycine residue at position
-3 and U-chain t-PA has an N-terminal valine at position 4.
References to t-PA herein are understood to include all such
variant forms.

Native t-PA is composed of a B or light and an A or heavy
chain. The B-chain contains the active site of the enzyme.
The cleavage site for the conversion of t-PA from the single
to the two-chain form is located between residues arg-275
5 and ile-276. In the two-chain form the chains are held
together by a disulphide bridge formed between residues
cys-264 in the A-chain and cys-395 in the B-chain.

It has been shown (Ny, T. et al, 1984; Proc. Natl. Acad.
10 Sci. U.S.A., 81, 5355) that the A chain exhibits a number of
structural and functional domains which are homologous to
structures found in other plasma proteins: two triple
disulphide-bonded structures or kringles, a
growth-factor-like domain and a fibronectin-finger-like
15 domain.

The sequence of amino acids making up the enzyme
urokinase-type plasminogen activator (u-PA) in its single
chain and two chain forms (Verstraete, M. and Collen, D.,
20 1986; Blood, 67, 1529) and the nucleotide sequence for the
cDNA which codes for human u-PA (Holmes, W. E. et al, 1985;
Bio/technology 3, 923-929) are known. Urokinase-type
plasminogen activator is known to have fibrinolytic
activity. The two chains of u-PA are termed the A- and
25 B-chain. The B-chain contains the active site of the
enzyme. The cleavage site for the conversion of u-PA from
the single to the two chain form is located between residues
lys-158 and ile-159. In the two chain form the chains are
held together by a disulphide bridge formed between residues
30 cys-148 in the A-chain and cys-279 in the B-chain.

As used herein, the term urokinase-type plasminogen
activator (u-PA) denotes a plasminogen activator of the
group having the immunological properties defined for u-PA
35 at the XXVIII Meeting of the International Committee on
Thrombosis and Haemostasis, Bergamo, Italy, 27 July 1982.

The numbering system for the amino acid and nucleotide
sequence of u-PA used herein is that described in Holmes, W.
E. et al, 1985 (op. cit.) in which the N- terminal serine
residue is numbered 1.

5

In addition to the native forms of t-PA and u-PA described
above, various muteins and hybrids are also known, see for
example EP-A-0201153, EP-A-0233013, EP-A-0199574, WO
86/01538, EP-A-0227462, EP-A-0253582, WO 86/04351,
10 EP-A-0236040, EP-A-0200451, EP-0225286, DE 3537176, WO
87/04722, WO 90/02798, EP 0299706, WO 89/04368, WO 90/00600,
WO 90/02798 and PCT/GB91/00801 (incorporated herein by
reference).

15 References herein to t-PA and u-PA species include both
native forms and muteins.

Plasmin is a two-chain serine protease which may be obtained
by the cleavage of the single chain precursor, plasminogen,
20 at a specific internal peptide bond. The amino acid
sequence of human plasminogen is known (Wiman and Walters
(1975) Eur.J. Biochem. 50, 489-494 and 58, 539-547; Wiman
(1977) Eur. J. Biochem. 76, 129-137; Sottrup-Jensen et al.
(1978) Fibrinolysis and Thrombolysis Vol. 3, 191-209, Raven
25 Press, New York; and Sottrup-Jensen et al. (1978) Atlas of
Protein Sequence and Structure Vol. 5, Suppl. 3, p91,
National Biomedical Research Foundation, Silver Spring, MD).
A partial nucleotide sequence coding for amino acid residues
272-790 of human plasminogen has also been described
30 (Malinowski, D.P. et al., 1984, Biochemistry, 23,
4243-4250). The cleavage site of human plasminogen is
located between residues arg-560 and val-561 (according to
the sequence numbering of Sottrup-Jensen et al. (1978) Atlas
of Protein Sequence (op.cit.)). Two species of plasminogen
35 have been identified (F.J. Castellino, Chemical Reviews
Vol. 81 p431 (1981)): glu_1 which has an N-terminal glutamic
acid residue at position 1 and lys_{77} which has an N-terminal

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lysine residue at position 77. Glu-plasminogen is also
easily converted by limited plasmic digestion to other
modified forms with N-terminal valine (val_{78}) or methionine
(met_{68}) (C. Miyashita, E. Wenzel and M. Heiden, Haemostasis
5 18, supp.1 pp 7-13 (1988)). References to plasminogen
herein are understood to include all these species.

A complete nucleotide sequence has also been described
(Forsgren, M., et al., 1987, FEBS Letters 213, 254-260).
10 The nucleotide sequence predicts the existence of an extra,
previously unreported, isoleucine residue near the
N-terminus of the A-chain. This finding has been
independently confirmed (McLean, J.N., et al., 1987, Nature
330, 132-137). Accordingly all sequence numbering (amino
15 acid and nucleotide) below follows Forsgren et al. (1987).
In this numbering sequence the plasminogen cleavage site is
located between residues arg-561 and val-562 and the
N-terminal modified forms are termed met_{69}, lys_{78} and val_{79}.

20 Plasminogen has five kringle structures. The region from
the first to the last cysteine residue of each kringle
structure, residues 84 to 162, 166 to 243, 256 to 333, 358
to 435 and 462 to 541 inclusive will be referred to herein
as the K_1^P, K_2^P, K_3^P, K_4^P and K_5^P domains respectively.
25

According to the present invention there is provided a
hybrid plasminogen activator which comprises kringle 5 or
kringles 4 and 5 of plasminogen linked to the B-chain of
t-PA or u-PA via an amino acid sequence comprising,
30 respectively, the t-PA cleavage site between residues 275
and 276 and the cysteine residue 264 of t-PA or the u-PA
cleavage site between residues 158 and 159 and the cysteine
residue 148 of u-PA.

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It will be understood that by the term 'B-chain' is meant at least that portion of the B-chain containing the functional active centre of t-PA or u-PA, and preferably comprises residues 276-527 or 159-411 respectively.

5

The linking sequence of amino acids may be introduced synthetically during the preparation of the hybrid plasminogen activator (PA) and/or derived from native sequences.

10

Native plasminogen includes cysteine residues at positions 548 and 558, C-terminal to plasminogen kringle 5, which participate in the interchain disulphide bonds of the two-chain plasmin form. In the preferred embodiment these
15 residues are not present in the linking sequence.

It will be appreciated that to prevent cleavage of the plasminogen kringle(s) from the t-PA or u-PA B-chain _in vivo_, the linking sequence should be chosen so as to avoid
20 the presence of a site susceptible to trypsin-like proteolytic cleavage N-terminal to residue cys-264 of t-PA or cys-148 of u-PA, as appropriate.

Where the B-chain of t-PA is employed, the linking sequence
25 of amino acids preferably comprises t-PA residues 264 to 275 inclusive, more preferably residues 262 to 275 inclusive.

Where the B-chain of u-PA is employed, the linking sequence of amino acids preferably comprises u-PA residues 148 to 158
30 inclusive, more preferably residues 137 to 158 inclusive.

In one preferred aspect, the hybrid PA may be represented symbolically as:

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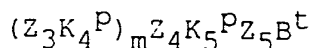
$$(Z_3K_4{}^P)_mZ_4K_5{}^PZ_5B^t$$

where B^t comprises residues 276-527 of t-PA, m is 0 or 1,
$K_4{}^P$ and $K_5{}^P$ represent kringle domains 4 and 5 derived from
5 plasminogen and each of Z_3, Z_4 and Z_5 represents, as
appropriate, an optional N-terminal amino acid sequence or a
bond or a linking sequence of amino acids which may be
introduced synthetically during the preparation of the
hybrid PA and/or derived from native plasminogen and/or t-PA
10 sequences, the sequence Z_5 comprising at least residues
cys-264 and arg-275 of t-PA.

In a second preferred aspect, the hybrid PA may be
represented symbolically as:
15

$$(Z_3K_4{}^P)_mZ_4K_5{}^PZ_5B^u$$
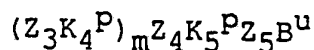
where B^u comprises residues 159-411 of u-PA and each of Z_3,
Z_4 and Z_5 represents, as appropriate, an optional N-terminal
20 amino acid sequence or a bond or a linking sequence of amino
acids which may be introduced synthetically during the
preparation of the hybrid PA and/or derived from native
plasminogen and/or u-PA sequences, the sequence Z_5
comprising at least residues cys-148 and lys-158 of u-PA and
25 m, $K_4{}^P$ and $K_5{}^P$ are as previously defined.

Where m is 1, the sequence Z_3 preferably has at its
N-terminus the sequence [GARSYQ] or [SYQ] corresponding to
the L- and S-chain forms of t-PA, and comprises some or all
30 of the native plasminogen inter-domain sequence between
plasminogen kringle domains 3 and 4, preferably plasminogen
residues 347-357.

When m is 0, the sequence Z_4 preferably has at its
35 N-terminus the sequence [GARSYQ] or [SYQ] corresponding to

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the L- and S-chain forms of t-PA, and comprises some or all
of the native plasminogen inter-domain sequence between
plasminogen kringle domains 4 and 5, preferably plasminogen
residues 443-461.

5

Where m is 1, Z_4 preferably represents the native
plasminogen inter-domain sequence between plasminogen
kringle domains 4 and 5.

10 Suitable sequences (Z_5) linking the plasminogen kringle 5
domain to the t-PA B-chain include:

        1.   [AAPSTCGLRQYSQPQFR]
        2.   [AAPSTCGLRQYSQPQFQ]
15   3.   [STCGLRQYSQPQFR]

(single letter amino acid notation) from which it can be
seen that the sequences 1 and 2 consist of residues 542-544
of plasminogen and residues 263 to 275 of t-PA linked by a
20 serine residue. The interposed serine residue can be
identified with ser-545 of plasminogen or ser-262 of t-PA.
In sequence 2, residue 275 of t-PA has been replaced by
glutamine in accordance with EP-A-0233013. The preferred
sequence 3 consists of residues 262 to 275 of t-PA.

25

The preferred sequence (Z_5) linking the plasminogen kringle
5 domain to the u-PA B-chain is:

        [AAPSFPSSPPEELKFQCGQKTLRPRFK]

30

(single letter amino acid notation) from which it can be
seen that the sequence consists of residues 542-546 of
plasminogen and residues 137 to 158 of u-PA.

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The preferred hybrid PA's of the invention have the
following structures:

    1. [GARSYQ] Plg 347-541[STCGLRQYSQPQFR]B^t
5   2. [GARSYQ] Plg 443-541[STCGLRQYSQPQFR]B^t

where Plg x-y represents residues x-y of plasminogen, B^t is
as previously defined and the symbols in brackets represent
amino acid residues according to the single letter amino
10 acid notation, including one and two chain variants, L- and
S-chain variants, and mixtures thereof.

The hybrid PA of the invention may be derivatised to provide
pharmaceutically useful conjugates analogous to known
15 PA-containing conjugates, for example:

(a) an enzyme-protein conjugate as disclosed in EP-A-O 155
388, in which the catalytic site on the enzyme which is
responsible for fibrinolytic activity is blocked by a human
20 protein attached thereto by way of a reversible linking
group;

(b) an enzyme-protein conjugate as disclosed in EP-A-0152
736, comprising at least one optionally blocked fibrinolytic
25 enzyme linked by way of a site other than the catalytic site
responsible for fibrinolytic activity to at least one human
protein;

(c) a protein-polymer conjugate as disclosed in
30 EP-A-0183503 comprising a pharmaceutically useful protein
linked to at least one water soluble polymer by means of a
reversible linking group; or

(d) an enzyme conjugate as disclosed in EP-A-0184363
35 comprising a plurality of fibrinolytic enzymes linked

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together through the active centres thereof by means of a
removable blocking group.

The hybrid PA of the invention may take the place of a PA as
5 the enzyme or (human) protein component, as appropriate, of
any of the conjugates described above.

The above mentioned derivatives of the hybrid PA may be used
in any of the methods and compositions described hereinafter
10 for the hybrid PA itself.

In a further aspect, the invention provides a process for
preparing hybrid plasminogen activator according to the
invention which process comprises expressing DNA encoding
15 said hybrid plasminogen activator in a recombinant host cell
and recovering the hybrid plasminogen activator product.

The DNA polymer comprising a nucleotide sequence that
encodes the hybrid PA also forms part of the invention.
20

The process of the invention may be performed by
conventional recombinant techniques such as described in
Maniatis et. al., Molecular Cloning - A Laboratory Manual;
Cold Spring Harbor, 1982 and DNA Cloning vols I, II and III
25 (D.M. Glover ed., IRL Press Ltd).

In particular, the process may comprise the steps of:

i)   preparing a replicable expression vector capable, in
30   a host cell, of expressing a DNA polymer comprising a
        nucleotide sequence that encodes said hybrid
        plasminogen activator;

ii)   transforming a host cell with said vector;
35

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iii)   culturing said transformed host cell under conditions
       permitting expression of said DNA polymer to produce
       said hybrid plasminogen activator; and

5 iv)   recovering said hybrid plasminogen activator.

The invention also provides a process for preparing the DNA
polymer by the condensation of appropriate mono-, di- or
oligomeric nucleotide units.
10

The preparation may be carried out chemically,
enzymatically, or by a combination of the two methods, in
vitro or in vivo as appropriate. Thus, the DNA polymer may
be prepared by the enzymatic ligation of appropriate DNA
15 fragments, by conventional methods such as those described
by D. M. Roberts et al in Biochemistry 1985, 24, 5090-5098.

The DNA fragments may be obtained by digestion of DNA
containing the required sequences of nucleotides with
20 appropriate restriction enzymes, by chemical synthesis, by
enzymatic polymerisation, or by a combination of these
methods.

Digestion with restriction enzymes may be performed in an
25 appropriate buffer at a temperature of 20°-70°C, generally
in a volume of 50μl or less with 0.1-10μg DNA.

Enzymatic polymerisation of DNA may be carried out in vitro
using a DNA polymerase such as DNA polymerase I (Klenow
30 fragment) in an appropriate buffer containing the nucleoside
triphosphates dATP, dCTP, dGTP and dTTP as required at a
temperature of 10°-37°C, generally in a volume of 50μl or
less.

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Enzymatic ligation of DNA fragments may be carried out using
a DNA ligase such as T4 DNA ligase in an appropriate buffer
at a temperature of 4°C to ambient, generally in a volume of
50μl or less.

5

The chemical synthesis of the DNA polymer or fragments may
be carried out by conventional phosphotriester, phosphite or
phosphoramidite chemistry, using solid phase techniques such
as those described in 'Chemical and Enzymatic Synthesis of
10 Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and
A. Lang), Verlag Chemie, Weinheim (1982),or in other
scientific publications, for example M.J. Gait, H.W.D.
Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic
Acids Research, 1982, 10, 6243; B.S. Sproat and W.
15 Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D.
Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21,
719; M.D. Matteucci and M.H. Caruthers, Journal of the
American Chemical Society, 1981, 103, 3185; S.P. Adams et
al., Journal of the American Chemical Society,1983, 105,
20 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester,
Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes
et al., EMBO Journal, 1984, 3, 801. Preferably an automated
DNA synthesizer is employed.

25 The DNA polymer is preferably prepared by ligating two or
more DNA molecules which together comprise a DNA sequence
encoding the hybrid PA.

The DNA molecules may be obtained by the digestion with
30 suitable restriction enzymes of vectors carrying the
required coding sequences.

The precise structure of the DNA molecules and the way in
which they are obtained depends upon the structure of the
35 desired hybrid PA product. The design of a suitable

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strategy for the construction of the DNA molecule coding for the hybrid PA is a routine matter for the skilled worker in the art.

5 The expression of the DNA polymer encoding the hybrid PA in a recombinant host cell may be carried out by means of a replicable expression vector capable, in the host cell, of expressing the DNA polymer. The expression vector is novel and also forms part of the invention.

10

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear

15 segment with one or more DNA molecules which, together with said linear segment, encode the hybrid PA, under ligating conditions.

The ligation of the linear segment and more than one DNA

20 molecule may be carried out simultaneously or sequentially as desired.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

25

The choice of vector will be determined in part by the host, which may be a prokaryotic cell, such as E. coli or Streptomyces sp., or a eukaryotic cell, such as a mouse C127, mouse myeloma, human HeLa, Chinese hamster ovary,

30 filamentous or unicellular fungi or insect cell. The host may also be a transgenic animal. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses, derived from, for example, baculoviruses and vaccinia.

35 The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for

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restriction, polymerisation and ligation of the DNA, by
procedures described in, for example, Maniatis et al., cited
above. Polymerisation and ligation may be performed as
described above for the preparation of the DNA polymer.
5 Digestion with restriction enzymes may be performed in an
appropriate buffer at a temperature of 20°-70°C, generally
in a volume of 50μl or less with 0.1-10μg DNA.

The recombinant host cell is prepared, in accordance with
10 the invention, by transforming a host cell with a replicable
expression vector of the invention under transforming
conditions. Suitable transforming conditions are
conventional and are described in, for example, Maniatis et
al., cited above, or ''DNA Cloning'' Vol. II, D.M. Glover
15 ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the
host cell. Thus, a bacterial host such as E. coli may be
treated with a solution of $CaCl_2$ (Cohen et al, Proc. Nat.
20 Acad. Sci., 1973, 69, 2110) or with a solution comprising a
mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and
then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and
glycerol. Mammalian cells in culture may be transformed by
calcium co-precipitation of the vector DNA onto the cells.
25

The invention also extends to a host cell transformed with a
replicable expression vector of the invention.

Culturing the transformed host cell under conditions
30 permitting expression of the DNA polymer is carried out
conventionally, as described in, for example, Maniatis et al
and ''DNA Cloning'' cited above. Thus, preferably the cell
is supplied with nutrient and cultured at a temperature
below 45°C.
35

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The hybrid PA expression product is recovered by
conventional methods according to the host cell. Thus,
where the host cell is bacterial, such as E. coli it may be
lysed physically, chemically or enzymatically and the
5 protein product isolated from the resulting lysate. Where
the host cell is mammalian, the product may generally be
isolated from the nutrient medium.

The DNA polymer may be assembled into vectors designed for
10 isolation of stable transformed mammalian cell lines
expressing the hybrid PA; e.g. bovine papillomavirus vectors
or amplified vectors in chinese hamster ovary cells (DNA
cloning Vol.II D.M. Glover ed. IRL Press 1985; Kaufman, R.J.
et al., Molecular and Cellular Biology 5, 1750-1759, 1985;
15 Pavlakis G.N. and Hamer, D.H., Proceedings of the National
Academy of Sciences (USA) 80, 397-401, 1983; Goeddel, D.V.
et al., European Patent Application No. 0093619, 1983).

It will be appreciated that, depending upon the host cell,
20 the hybrid PA prepared in accordance with the invention may
be glycosylated to varying degrees. Furthermore, as
observed by Pohl et.al., Biochemistry, 1984, 23, 3701-3707,
varying degress of glycosylation may also be found in
unmodified, naturally occurring t-PA. Plasminogen also
25 exhibits varying degrees of glycosylation (Hayes M.L, J.
Biol. Chem. 254: 8768, 1979). Mutant forms of the hybrid PA
are also contemplated in which glycosylation sites are
removed by genetic engineering techniques, for example as
taught in EP-A-0225286, DE-3537176, WO 87/04722, EP-0299706
30 or WO 89/04368. The hybrid PA of the invention is
understood to include such glycosylated variations.

It will also be appreciated that, depending upon the
expression conditions, the hybrid PA prepared in accordance
35 with the invention may exist in the single or two chain
forms or mixtures thereof. The invention extends to all

such forms.

The hybrid PA of the invention comprises the B-chain of
native t-PA or u-PA linked to an A-chain comprising kringle
5 5 or kringles 4 and 5 derived from plasminogen via a linking
sequence of amino acids comprising residues 264 and 275 of
t-PA or residues 158 and 148 of u-PA.

This hybrid PA A-chain may be employed as one chain of a
10 fibrinolytically active hybrid protein such as disclosed in
EP-0 155 387. The hybrid A-chain, DNA encoding the hybrid
A-chain and a hybrid protein comprising the hybrid A-chain
linked to the B-chain of a fibrinolytically active protease,
the catalytic site of which is optionally blocked by a
15 removable blocking group, all form part of the invention.

The hybrid A-chain may be prepared by separation from the
B-chain thereof by mild reduction. Alternatively the hybrid
A-chain may be prepared by expressing DNA coding therefor in
20 a recombinant host cell and recovering the hybrid A-chain
product. The hybrid protein comprising the hybrid A-chain
linked to the B-chain of a fibrinolytically active protease
may be prepared by (a) mixing said A- and B-chains under
oxidative conditions; or (b) ligating DNA encoding said
25 A-chain to DNA encoding said B-chain and expressing the
ligated DNA in a prokaryote or eukaryote host; and
thereafter optionally blocking the catalytic site of the
hybrid protein with a removable blocking group. The
oxidation and reduction conditions are as generally
30 described in EP-A-0 155 387.

The resulting hybrid protein may be used in any of the
methods and compositions described hereinafter for the
hybrid PA itself.

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The hybrid PA of the invention or conjugate thereof can be
further derivatised such that any catalytic site essential
for fibrinolytic activity is optionally blocked by a
removable blocking group.

5

As used herein the expression 'removable blocking group'
includes groups which are removable by hydrolysis at a rate
such that the pseudo-first order rate constant for
hydrolysis is in the range of 10^{-6} sec^{-1} to 10^{-2} sec^{-1}, more
10 preferably 10^{-6} sec^{-1} to 10^{-3} sec^{-1}, in isotonic aqueous
media at pH 7.4 at 37°C.

Such blocking groups and blocking reactions are described in
European Patent No.0009879 and EP 0297882 and include acyl
15 groups such as optionally substituted benzoyl or optionally
substituted acryloyl.

Suitable optional substituents for benzoyl blocking groups
include halogen, C_{1-6} alkyl, C_{1-6} alkoxy, C_{1-6} alkanoyloxy,
20 C_{1-6} alkanoylamino, amino or p-guanidino.

Suitable optional substituents for acryloyl blocking groups
include C_{1-6} alkyl, furyl, phenyl or C_{1-6} alkylphenyl.

25 In one aspect, the removable blocking group is a
2-aminobenzoyl group substituted in the 3- or 4-position
with a halogen atom and optionally further substituted with
one or more weakly electron-withdrawing or electon-donating
groups, wherein the pseudo first order rate constant for
30 hydrolysis of the derivative is in the range 6.0 x 10^{-5} to
4.0 x 10^{-4} sec^{-1} when measured in a buffer system consisting
of 0.05M sodium phosphate, 0.1M sodium chloride, 0.01% v/v
detergent comprising polyoxyethylenesorbitan monoleate
having a molecular weight of approximately 1300, at pH 7.4
35 at 37°C.

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Preferably the pseudo first order rate constant for
hydrolysis of the derivative is in the range 6.0×10^{-5} to
2.75×10^{-4} s^{-1}, preferably 6.0×10^{-5} to 2.5×10^{-4} s^{-1},
more preferably 6.0×10^{-5} to 2.0×10^{-4} s^{-1}, still more
5 preferably 6.0×10^{-5} to 1.5×10^{-4} s^{-1} and most preferably
7.0×10^{-5} to 1.5×10^{-4} s^{-1}.

Preferably, the 2-aminobenzoyl group is substituted with a
halogen atom in the 4-position.

10

Preferably, the halogen atom is fluorine, chlorine or
bromine.

When the group is further substituted, preferred groups
15 include C_{1-6} alkyl, C_{1-6} alkoxy and C_{1-6} alkenyl
substituents in the 3- or 5-positions of the ring.

Examples of the blocking group include 4-fluoro-2-
aminobenzoyl, 4-chloro-2-aminobenzoyl,
20 4-bromo-2-aminobenzoyl and p-methoxybenzoyl.

The hybrid PA and derivatives of the invention are suitably
administered in the form of a pharmaceutical composition.

25 Accordingly the present invention also provides a
pharmaceutical composition comprising a hybrid PA or
derivative of the invention in combination with a
pharmaceutically acceptable carrier.

30 The compositions according to the invention may be
formulated in accordance with routine procedures as
pharmaceutical compositions adapted for intravenous
administration to human beings.

35 Typically compositions for intravenous administration are
solutions of the sterile enzyme in sterile isotonic aqueous

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buffer. Where necessary the composition may also include a
solubilising agent to keep the hybrid PA or derivative in
solution and a local anaesthetic such as lignocaine to ease
pain at the site of injection. Generally, the hybrid PA or
5 derivative will be supplied in unit dosage form for example
as a dry powder or water free concentrate in a hermetically
sealed container such as an ampoule or sachette indicating
the quantity of protein in activity units. Where
composition comprises a derivative of the invention or where
10 the hybrid PA includes a removable blocking group, an
indication of the time within which the free protein will be
liberated may be given. Where the protein is to be
administered by infusion, it will be dispensed with an
infusion bottle containing sterile pharmaceutical grade
15 'Water for Injection' or saline. Where the protein is to be
administered by injection, it is dispensed with an ampoule
of sterile water for injection or saline. The injectable or
infusable composition will be made up by mixing the
ingredients prior to administration.
20

The quantity of material administered will depend upon the
amount of fibrinolysis required and the speed with which it
is required, the seriousness of the thromboembolic
condition and position and size of the clot. The precise
25 dose to be employed and mode of administration must per
force in view of the nature of the complaint be decided
according to the circumstances by the physician supervising
treatment. However, in general, a patient being treated for
a thrombus will generally receive a daily dose of from 0.01
30 to 10 mg/kg of body weight, such as 0.10 to 2.0mg/kg,
either by injection in for example up to five doses or by
infusion.

Within the above indicated dosage range, no adverse
35 toxicological effects are indicated with the compounds of
the invention.

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Accordingly, in a further aspect of the invention there is
provided a method of treating thrombotic diseases, which
comprises administering to the sufferer an effective
non-toxic amount of hybrid PA or derivative of the
5 invention.

In another aspect the invention provides the use of a hybrid
PA or derivative of the invention for the manufacture of a
medicament for the treatment of thrombotic diseases.
10

The invention also provides a hybrid PA or derivative of the
invention for use as an active therapeutic substance and in
particular for use in the treatment of thrombotic diseases.

15 The following Methods and Examples illustrate the invention.

-20-

I.   General Methods used in Examples

(i)   DNA cleavage

5 In general the cleavage of about 1µg of plasmid DNA or DNA
   fragments was effected using about 5 units of a restriction
   enzyme (or enzymes) in about 20µl of an appropriate buffer
   solution.

10 (ii)   Ligation of DNA fragments

   Ligation reactions were carried out as described in Maniatis
   et al, (Molecular Cloning - A Laboratory Manual, Cold Spring
   Harbor Laboratory, 1982).
15

   (iii) Transformation of plasmid DNA into E.coli HB101 cells
   used competent HB101 supplied by Gibco BRL (Paisley,
   Scotland), according to the manufacturers instructions.

20 (iv)   Plasmid preparation

   Preparation of plasmid DNA was carried out as described in
   Maniatis et al, (Molecular Cloning - A Laboratory Manual,
   Cold Spring Harbor Laboratory, (1982)).
25

   (v)   Isolation of DNA fragments from low-melting-point
   (LMP) agarose gels

   DNA fragments were isolated from LMP agarose gels as
30 described by Maniatis et al, (Molecular Cloning-A Laboratory
   Manual, Cold Spring Harbor Laboratory, 1982). Alternatively
   the excised gel band was purified using GENECLEAN^{tm},

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(Stratech Scientific, London) used according to the
manufacturers instructions.

   (vi) Oligonucleotides

5

   Oligonucleotides were made on Applied Biosystems 381A DNA
   Synthesizer according to the manufacturers instructions.
   When used in plasmid construction the oligonucleotides were
   annealed by heating together at 95°C for 5 minutes and
10 cooling slowly to room temperature. The annealed
   oligonucleotides were then ready for ligation.

   (vii) DNA sequencing by double-strand method

15 Sequencing was carried out using 'Sequenase^{TM}(United States
   Biochemical Corporation) essentially according to the
   manufacturers instructions.

   (viii) Transient expression of plasminogen activators from
20 HeLa cells

   (a)   Small-scale

   Cell preparation: cells were trypsinised and plated out at
25 approx. 2.4 x 10^5 cells per 35mm dish and incubated in 1.5ml
   growth medium (this is Hepes buffered RPM1 1640 medium
   (041-02400) containing 10% Serum (021-06010), 2% sodium
   bicarbonate solution (043-05080),; Gibco, Paisley, Scotland)
   at 37°C in a humidified incubator in an atmosphere of 5%
30 CO_2/95% air. After 72h the cells were refed, and used for
   transfection 24h later.

   Transfection procedure: Cultures were changed to Eagles MEM
   (041-01095), 10% serum (021-06010), and 1% non-essential
35 amino acids (043-01140) 3h before transfection. The
   transfections used calcium coprecipitation as described in

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'DNA Cloning' Ed. D.M. Glover (Chap. 15, C. Gorman).
Glycerol shock and 5mM butyrate treatments were used.
Plasminogen activator(s) secreted by transfected cells was
harvested in 1.0ml RPMI 1640 medium (as above, but lacking
5 serum) + 4% Soybean Peptone.

(b)   Large-scale

Cell preparation: cells were trypsinised and plated out at
10 a density of approx. 2.5 x 10^6 cells per 175cm^2 flask in
30ml growth medium (above). After 72h an extra 25ml of
growth medium was added and the cells were used for DNA
transfection 24h later (as above). 25ml of harvest medium
were used per flask.
15

Alternatively the cells were plated at a density of
approximately 2.0 x 10^6 cells per flask and 25ml of growth
medium was added after 96h incubation and the cells used as
above.
20

The two seeding rates and feed times used in the small and
large-scale protocols were designed to allow convenient
timing of experiments. Both sets of protocols allow
efficient expression of activator(s).
25

(ix) Chromogenic substrate assays

Hybrid was assayed against the chromogenic substrate S-2288
(KabiVitrum, Sweden) at a substrate concentration of 1mM in
30 0.1 M triethanolamine.HCl pH 8.0 at 25°C. An SU is defined
as the amount of activity that gives an O.D. increase at
405nm of 0.001/min in 0.5 ml substrate in a 1 cm pathlength
cell.

35 In another form of the assay, specifically designed for the
semi-quantitative assay of chromatography column fractions,
10μl of each fraction was mixed with 100μl 1mM S-2288 (as

-23-

above) in wells of a microtitre plate and the plate
incubated at 37°C until such time as yellow colour was
visible. The solutions were read at 410 nm using a Dynatech
MR700 Microplate reader.

5

(x)   Fibrinolytic activity assay

The fibrinolytic activity of hybrid plasminogen activator
solutions was measured on human plasminogen-containing
10 fibrin plates as described (Dodd, I., and Carr, K.,
Thrombosis Res. 1989 55 79-85). Dose-responses of hybrid
plasminogen activators had slightly different slopes to
those of the tissue-type plasminogen activator standard so
all activities are approximate. Activities are expressed in
15 IU with reference to the 2nd International standard for
t-PA, Lot 86/670, unless otherwise stated.

(xi)   Sodium dodecyl sulphate polyacrylamide gel
electrophoresis (SDS PAGE)

20

SDS PAGE was carried out to determine the apparent molecular
weight(s) of the hybrid plasminogen activators using
essentially the method of Laemmli (Nature 1970 227 680-685).
The activators were identified either by staining for
25 protein or by a fibrin zymography technique (Dodd, I. et al
Thromb. Haemostasis 1986, 55 94-97). Using these methods it
was generally possible to determine chain nature (sc v tc).

(xii) Rate constant determinations

30

Acyl-enzyme (ca. 20 pmol, 10µl) was added to a solution of
S-2288 (0.5ml of 1.0mM in 0.05M sodium phosphate, 0.1M NaCl,
0.01% w/v Tween 80 pH (37°C) 7.4) in a spectrophotometer
cuvette thermostatted at 37°C. Absorbance readings at

-24-

405nm were recorded at 1.0 min intervals for 30 min and
on-board software (Beckman Inc.) used to calculate the rate
of change of absorbance over each successive 1 min interval.
As deacylation proceeded in the cuvette, the rate of change
5   of A405nm increased with time.   Rate data obtained when the
absorbance exceeded a value of 0.8 were not used because of
the effect of substrate depletion. The set of rate
determinations were fitted to the following monoexponential
function:

10

$$f(t) = A_o + (A_{max} - A_o) \times (1-e^{-kt})$$

where A_o is the initial activity of the acyl-enzyme and A_{max}
is the maximum activity possible after deacylation and was
15   determined by deacylation of an aliquot of acyl-enzyme in
0.1M Tris. HCl, 20% w/v glycerol, 0.14M NaCl, 0.01% w/v
Tween 80 pH 7.4 at 37°C for 1h followed by amidolytic assay
with S-2288 under the above conditions (i.e. phosphate
buffer pH 7.4, 37°C). A and K, the first order deacylation
20   rate constant, were treated as unknowns in the fitting
process and were derived by non-linear regression analysis
on a VAX 11/750 computer.

II.   Identification of nucleotides, amino acid residues,
25   N-termini, protein domains and chain nature in the examples

(i)   Sequences

All t-PA numbering as in Pennica et al (1983) op. cit.;
30   plasminogen amino acid numbering based on Sottrup-Jensen et
al (1978) Atlas of Protein Sequence and Structure Vol. 5,
Suppl. 3, p91, National Biomedical Research Foundation,
Silver Spring, MD., but updated to include the extra amino
acid residue identified by Forsgren, M. et al (1987) FEBS

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Letters, 213, 254-260. Plasminogen nucleotide sequences as in Forsgren et al. (op.cit.).

(ii)   Protein Domains

The protein domains described in the examples have been abbreviated for convenience and are defined as follows:-

1. B^t = t-PA amino acid residues 276 to 527 inclusive.

2. K_4P = plasminogen amino acid residues 358 to 435 inclusive.

     K_5P = plasminogen amino acid residues 462 to 541 inclusive.

(iii) Chain nature

sc, indicates that the protein is in single chain form.

tc, indicates that the protein is in two chain form.

(iv)   Vectors

pTRE12 - (EP-0201153)-basic expression vector

pTRE15 - (EP-0201153)-encodes wild-type t-PA

pUC8   - a commercially available vector (Gibco-BRL) containing a multiple cloning site and a gene that confers ampicillin resistance.

Example 1

Construction of pDH55 encoding SYQ/Plasminogen 347-541/t-PA
262-527 (H55)

The construction of pDH55 was carried out as a two step
process. This plasmid comprises a cDNA encoding the t-PA
signal sequence (-35 to -1) linked to the above hybrid
plasminogen activator. The restriction sites used below
were located as follows:-

        StyI : plasminogen nucleotide 1210

        SstI : t-PA   nucleotide 1417

        BamH1 : located in SV40 polyA/t intron fragment of
                pTRE15

        BglII : t-PA   nucleotide 187

a)   Construction of the plasmid pDH55i (a holding vector
containing the sequence encoding SYQ/Plasminogen
347-541/t-PA 262-410)

Two fragments were prepared by restriction digestion and
agarose gel electrophoresis. These fragments were as
follows.

Fragment 1: was the large fragment from an SstI/BglII
digest of a pUC8 derivative containing a modified multiple
cloning site.

Structure of the modified cloning site:-

```
          HindIII                                          SstI
          ‿‿͡ ‿                                          ‿ ͡ ‿
5'   AGCTTGGGCGCCTTCATTTCCCTCCTCTCCTCCAGAAGAGCTCAAATTT
3'       ACCCGCGGAAGTAAAGGGAGGAGAGGAGGTCTTCTCGAGTTTAAA
```

```
5            BglII HindIII
             ‿‿‿͡ ‿‿͡ ‿‿
      CAGTGTGGCCAGATCTA
      GTCACACCGGTCTAGATTCGA
```

Fragment 2: was an approximately 1kb StyI/SstI fragment from
10 pTRH37 (as described in EP-A 0297 882) encoding most of K_4^P,
K_5^P and part of B^t.

These two fragments were ligated together with an
oligonucleotide linker (Linker 3) to form plasmid pDH55i.
Linker 3 (designed to encode the tripeptide SYQ and
15 amino-acid residues 347-359 of plasminogen) was formed by
annealing two oligonucleotides (A) and (B) of sequence:-

```
5'   GATCTTACCAAGCACCACCTGAGCTAACCCCTGTGGTCCAGGACTGCTAC
```
                                                          (A)
20
```
5'   CATGGTAGCAGTCCTGGACCACAGGGGTTAGCTCAGGTGGTGCTTGGTAA
```
                                                          (B)

The DNA was transformed into E.coli HB101 cells. A plasmid
25 (pDH55i) was isolated which has the structure shown in
Fig.1.

b)   Construction of the plasmid pDH55

30 Three fragments were prepared by restriction digestion and
agarose gel electrophoresis. These fragments were as
follows:-

Fragment 4: was an approximately 1kb BglII/SstI fragment
35 from pDH55i encoding the first three amino acids (SYQ) of
t-PA, residues 347-357 of plasminogen, $K_4^P K_5^P$ and part of

B^t.

Fragment 5: was an approximately 1.6kb Sst1/BamHl fragment
from pTRH37 encoding the C-terminal part of B^t and vector
5 sequences.

Fragment 6: was a BamHl/BglII fragment derived from pTRE15
encoding vector sequences and the t-PA signal sequence.
These three fragments were ligated together and transformed
10 into E.coli HB101 cells.

A plasmid was isolated which has the structure shown in
Fig.2. The plasmid, when introduced into HeLa cells,
directed the expression of a novel plasminogen activator.
15

Example 2

Purification and Characterisation of H55 Protein

20 Conditioned medium from twenty 175cm^2 HeLa cultures
transfected with the plasmid pDH55 was centrifuged at
approximately 9000g for 30 min. The supernatant (480ml) was
buffer-exchanged into PBS'A' (Dulbecco)/0.01% Tween 80 pH
7.4 using a column (i.d., 90mm;h,226 mm) of Sephadex G25 and
25 a 710ml fraction eluting immediately after the void volume
of the column was obtained. The 710 ml fraction was then
purified in a similar way to that described for t-PA (Dodd,
I. et al FEBS Lett., (1986) 209 13-17). The zinc chelate
and lysine Sepharose Fast Flow columns had volumes of 90ml
30 and 10ml respectively. Protein H55 was dissociated from the
lysine Sepharose column using a 0.5M arginine-containing
buffer; peak H55 - containing fractions were identified by a
microtitre plate S2288 assay and were pooled and were
ultrafiltered using a membrane with a nominal molecular
35 weight cut off of 10,000 (YM10, Amicon) to a final volume of

2.2ml. This retentate was regarded as the H55 product.

Fibrin plate assay showed that the original, conditioned
harvest medium contained approximately 17000 IU and that the
5 product contained approximately 22000 IU. This difference
is believed to be within the natural error in the assay.

Analysis of the product by SDS PAGE followed by protein
staining showed a major band at approx apparent M_r 60,000
10 (non-reduced) or two major bands at M_r 36,000 and 31,000
(reduced). The M_r 36,000 band is known to be the t-PA
B-chain moiety; the 31,000 band is presumed to be the
$K_4^P+K_5^P$ moiety. These results suggest the majority of the
material is in the two-chain form.
15
Analysis of the retentate by SDS PAGE followed by fibrin
zymography indicated a major (approximately 95%)
fibrinolytic species at apparent M_r 60,000; so the
fibrinolytic activity and the major stainable band
20 co-migrate on SDS PAGE.

Example 3

Synthesis of N,N-dimethyl-4-aminobenzoyl two chain H55
25 (DAB-H55)

1.0ml of the ultrafiltered retentate described in Example 2
(containing 9900 IU;10,000 SU; nominal 0.8nmoles); was mixed
with 0.48ml 0.02M Tris/0.2M NaCl/0.2M arginine/0.01% Tween
30 80 pH 7.0 (Buffer A) and 7.5µl 20mM 4' amidinophenyl-N,N-
dimethyl-4- aminobenzoate. HCl (dissolved in DMSO). The
mixture was incubated for 1h at 25°C and the S2288 activity
of the solution measured; only 2% of the input activity
remained. The mixture was buffer-exchanged into Buffer A
35 using a prepacked column (PD10) of Sephadex G25. The final
product had a volume of 2.5ml. Fibrin plate assay showed

-30-

the solution contained 8000 IU; the S2288 activity was 450
SU. These figures indicate that the activity is being
regenerated during the longer incubation on the fibrin
plates i.e., that deacylation is occurring.

5

Example 4

Construction of pDH56 encoding SYQ/Plasminogen 443-541/t-PA
262-527 (H56)

10

The construction of pDH56 was carried out as a two step
process. This plasmid comprises a cDNA encoding the t-PA
signal sequence (-35 to -1) linked to the above hybrid
plasminogen activator. The restriction sites used during
15 construction were as follows:-

        HinFl: plasminogen nucleotide 1498
        AlwNl: t-PA   nucleotide 1130
        Sstl : t-PA   nucleotide 1417
20   BamHl: located in SV40 polyA/t intron
                 fragment of pTRE15
        BglII: t-PA   nucleotide 187

    a)   Construction of the plasmid pDH56i (a holding vector
25 containing the sequence encoding SYQ/Plasminogen
    443-541/t-PA 262-410)

    Three fragments were prepared by restriction digestion and
    agarose gel electrophoresis. These fragments were as
30 follows:-

    Fragment 1: was from an Sstl/BglII digest of a pUC8
    derivative containing a modified linker region. (as
    described in Example 1).

35

Fragment 7: was an approximately 411bp HinF1/AlwN1 fragment
from pTRH37 (as described in EP-A-0297 882) encoding part of
the $K_4^P-K_5^P$ bridge, the whole of K_5^P and part of B^t.

5 Fragment 8: was an approximately 292bp AlwN1/Sst1 fragment
from pTRH37 (as described in EP-A-0297 882) encoding part of
B^t.

These three fragments were ligated together with an
10 oligonucleotide linker (Linker 9) to form plasmid pDH56i.
Linker 9 (designed to encode the tripeptide SYQ and
amino-acid residues 443-455 of plasminogen) was formed by
annealing two oligonucleotides C and D of sequence:-

15 5'   (C)
GATCTTACCAAGTAGCACCTCCGCCTGTTGTCCTGCTTCCAGATGTAGAG 3'

5'   (D)
AGTCTCTACATCTGGAAGCAGGACAACAGGCGGAGGTGCTACTTGGTAA 3'
20

The DNA was transformed into E.coli HB101 cells. A plasmid
(pDH56i) was isolated which has the structure shown in
Fig.3.

25 b)   Construction of the plasmid pDH56

Three fragments were prepared by restriction digestion and
agarose gel electrophoresis. These fragments were as
follows:-
30

Fragment 10: was an approximately 750bp BglII/Sst1 fragment
from pDH56i encoding the first three amino acids of t-PA
(SYQ), residues 443 to 461 of plasminogen, K_5^P and part of
B^t.

35

Fragment 5: was an approximately 1.6kb Sst1/BamH1 fragment
from pTRH37 encoding the C-terminal part of B^t and vector

-32-

sequences.   (As described in Example 1)

Fragment 6: was a BamH1/BglII fragment derived from pTRE15
encoding vector sequences and the t-PA signal sequence. (As
5 described in Example 1)

These three fragments were ligated together and transformed
into E.coli HB101 cells.

10 A plasmid was isolated (pDH56) which has the structure shown
in Fig.4. The plasmid, when introduced into HeLa cells,
directed the expression of a novel plasminogen activator.

Example 5

15

Purification and characterisation of H56 protein

Two purifications were carried out, each resulting in a
batch of H56 that was characterised extensively. Both
20 purifications were experimental in that it was not clear
which affinity columns were best employed; this led to H56
protein not adsorbing to columns and thus rather complicated
purification schemes (not helped by affinity matrices
performing poorly). For these reasons, the following
25 descriptions indicate the generality of the purifications -
factors considered unimportant for the actual purification
of H56 are not detailed (e.g. a batch of lysine Sepharose
used on one occasion did not adsorb the H56 protein; the
reason is not known).

30

(a)   Approximately 500ml conditioned medium from HeLa
cultures transfected with the plasmid pDH56 was centrifuged
at approximately 9000g for 30 min. The supernatant was
buffer-exchanged into PBS 'A' (Dulbecco)/0.01% Tween 80 pH

-33-

7.4 (PBS/TW) using a column of Sephadex G25 (Vt 1500ml) and the 780 ml sample eluting immediately after the void volume of the column was retained.

5 The 780ml sample was chromatographed on zinc chelate Sepharose (see Example 2) and aminohexyl Sepharose 4B (AH Sepharose; Sigma chem.Co.). The latter chromatography was carried out as follows.

10 The column (i.d., 15 mm; h, 45 mm; Vt, 8.0 ml) was equilibrated with PBS/TW. The imidazole-eluted fraction from the zinc chelate column was applied and was washed through with PBS/TW. H56 protein was desorbed using 0.02M Tris/0.5M NaCl/0.5M L-arginine/0.01% Tween 80 pH 7.0. All 15 parts of the chromatography were at 4°C at approximately 100 cm h^{-1}. Active fractions (containing H56) were identified using S2288 and then concentrated by stirred-cell ultrafiltration (YM10, Amicon Ltd). The ultrafiltered retentate was regarded as the product.
20

The product showed a dose-response relationship on human fibrin plates slightly different to that of t-PA and exhibited a single major band of fibrinolytic activity at apparent M$_r$ 40,000 on SDS PAGE followed by fibrin 25 zymography. This band had the same M$_r$ as a doublet, possibly triplet, of polypeptides detected after probing Western blots of H56 with a monoclonal directed at the B-chain of t-PA (ESP2, BioScot, U.K.) or an anti-t-PA B chain Ig G preparation (Dodd, I. et al, Thrombos. 30 Haemostos., 1986 55 94).

(b)   Approximately 500ml conditioned media was prepared and chromatographed on Sephadex G25 and zinc chelate Sepharose essentially as described in (a). Some of the H56 was 35 partially purified on AH-Sepharose and p-aminobenzamidine Sepharose CL4B (pABA Sepharose; Pierce Chem. Co.) resulting in three active fractions. These were pooled, ultra

filtered (YM10) and the retentate clarified by
centrifugation (10,000g/10min). The supernatant was
buffer-exchanged into PBS/TW using Sephadex G25 (PD10) and
then purified on freshly autoclaved pABA Sepharose (Vt 11ml)
5 using the same protocol as for AH Sepharose described in
(a). Material that was eluted from the pABA Sepharose
column by the 0.5M arginine buffer was concentrated
(stirred-cell ultrafiltration; YM10) and buffer-exchanged
into 0.05M sodium phosphate/0.1M sodium chloride/10mg ml^{-1}
10 mannitol/50μM E-amino caproic acid/0.01% Tween 80 pH7.4
(Sephadex G25, PD10). The buffer-exchanged material was
regarded as the product.

The dose-response of the product on fibrin plates was
15 approximately parallel to that of t-PA; the product
contained approximately 3000 IU/ml. SDS PAGE/fibrin
zymography and Western blotting studies revealed similar
pictures to those obtained for product (a). SDS PAGE
(non-reduced) followed by silver staining also showed a
20 major band in the approximate M_r 40,000 region.

Example 6

Synthesis of N,N-dimethyl-4-aminobenzoyl two-chain H56 (DAB
25 H56)

2.1ml H56 (1200 IU/ml) from Example 5 (a) was mixed at 25°C
with 6μl 4'-amidinophenyl-N,N-dimethyl-4-aminobenzoate.HCl
(AP-DAB; 2mM in DMSO) and incubated at 25°C. Additional
30 aliquots of 20mM AP-DAB were added at 30 min (2μl) and 60
min (3μl). At 90 min 6 per cent of the original amidolytic
activity remained. The mixture was buffer-exchanged into
0.02M Tris/0.2M NaCl/0.2M L-arginine/0.01% Tween 80 pH 7.0
(2.5ml) and aliquoted and stored at -40°C.
35

'In-cuvette' deacylation (see General Methods,
(xii))revealed that 90 per cent of the product was in the

-35-

acylated (DAB) form. Under the conditions of the
deacylation the material had a deacylation half-life of 105
min.

5 <u>Example 7</u>

<u>Expression of H55 protein in dhfr⁻Chinese hamster ovary
(CHO) cells</u>

10 The strategy used for expression of H55 in CHO DXB11 cells,
using an amplifiable dhfr vector, has been described
previously in EPA 0297 882.

The cDNA encoding H55 was recovered from pDH55 as a 3.2kb
15 BamHI/MluI fragment. This fragment was subcloned into
pTRH11 (EPA 0297 882) replacing the original 4.1kb
MluI/BamHI fragment (which encoded protein H204). The new
plasmid was called pDH17. In pDH17, the hybrid and dhfr
transcription cassettes are opposed i.e. converge at their
20 3' ends.

A second plasmid, pDH16, was also prepared. In this plasmid
the whole XhoI fragment carrying the H55 transcription
cassette (including RSVLTR and SV40 elements : depicted in
25 EPA 0297 882, pTRH71) is reversed with respect to that in
pDH17. The transcription cassettes for dhfr and H55 are
therefore transcribed in tandem.

<u>Cell preparation</u>
30
CHO cells were trypsinised and plated out at 6 x 10^5 per 90
mm dish and left in growth medium [Hams F12 nutrient media
(041-1765) with 1% stock penicillin/streptomycin (043-5070)
and 10% foetal calf serum (013-6290); Gibco, Paisley,
35 Scotland] at 37°C in a humidified incubator, in an
atmosphere of 5% CO_2/95% air. After 18 hrs the growth
medium was replaced with transfection medium [Eagles MEM

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(041-1095) with 1% non-essential amino acids (043-1140), 1%
stock penicillin/streptomycin (043-5070), and 10% newborn
calf serum (021-6010); Gibco, Paisley, Scotland]. After a
further 2 hrs the cells were used for DNA transfection.

5

Transfection Procedure

The transfection procedure, carried out in transfection
medium, used calcium coprecipitation and glycerol shock as
10 described in DNA Cloning Volume II (Ed. D.M. Glover; chapter
6, C. Gorman). Following transfection the cells were
maintained in growth medium for 48 hrs under growth
conditions (as above), prior to the selection procedure.

15 Selection

Forty eight hours post-transfection the cells were medium
changed into selective medium [αMEM (041-2561) with 2%
stock glutamine (043-5030), 1% stock penicillin/streptomycin
20 (043-5070) and 10% dialysed foetal calf serum (063-6300);
Gibco, Paisley, Scotland]. The cells were maintained in
selective medium for 8-10 days until colonies of dhfr+ cells
appeared.

25 Isolated colonies were grown to confluency in 25 cm^2 flasks
and harvested in serum-free medium for 24 hours.
Fibrinolytically active protein was detected by fibrin plate
assay.

30 Example 8

Synthesis of 4-anisoyl-t-PA 1-3/plasminogen 443-541/t-PA
262-527

35 Purified H56 protein from Example 5 (nominal 0.34 nmoles) in
0.05M NaH$_2$PO$_4$.2H$_2$0/0.1M NaCl/0.01% Tween 80/10 mgml^{-1}

-37-

mannitol/50μM E-ACA pH 7.4 (0.84ml) was treated with
4-amidinophenyl-4-anisate. HCl (6.8 nmoles; 6.8μl) at 25°C.
After 90 min the amidolytic activity of the preparation had
decreased to 5% of the original activity.

5

The material was buffer-exchanged using Sephadex G25 (PD10)
into 0.02M Tris/0.2M NaCl/0.2M L-Arginine/0.01% Tween 80 pH
7.0 and stored at -40°C.

10 Example 9

Synthesis of 4-anisoyl-t-PA 1-3/plasminogen 347-541/t-PA
262-527

15 Purified H55 protein from Example 2 (nominal 0.16 nmoles) in
0.02M Tris/0.5M NaCl/0.5M L-Arginine/0.01% Tween 80 pH 7.4
(0.3ml) was treated with 4-amidinophenyl-4-anisate. HCl (3.2
nmoles; 3.2μl) at 25°C. After 90 min the amidolytic
activity of the preparation had decreased to <2% of the
20 original activity.

The material was buffer-exchanged into 0.02M Tris/0.2M
NaCl/0.2M L-Arginine/0.01% Tween 80 pH 7.0 using Sephadex
G25 (PD10) and stored at -40°C.

25

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Example 10

The elements used in expressing H56 are:

5   pAcCL29 Vector: this is based on pAcYM1 (an expression
vector in which a unique Bam H1 cloning site has been
positioned so as to maximise expression using the
polyhedrin promoter: Matsura, Y, Possee R.D. Overton, H.A.
and Bishop D.H.L [1987] J.Gen.Virol 68. 1233-1250).

10

pAcCL29 (Livingstone, C. and Jones I (1989) NAR 17. 2366)
was derived from pAcYM1 as follows; an approximately 5Kb
EcoRI-XhoI fragment coding for all the signals necessary
for efficient expression and recombination were removed

15   from pAcYM1, blunt ended and ligated into in-filled EcoRI
Hind III sites in pUC118 (Vieira. J. and Messing J. [1987]
Meth. Enzy. 153 3-11).

Wild type virus is: Autographa californica nuclear

20   polyhedrosis virus (AcNPV).

Cell line : IPLB Sf21 derived from Spodoptera frugiperda.
(Vaughan, J.L., Goodwin, R.H., Thompkins, G.J. and
McCawley, P. 1977: In Vitro 13, 213-217)

25

Method

An approx. 2.5 kb Hind III - Bam HI (Hind III located at
5' end of untranslated region, Bam HI located 3' to coding

30   region) fragment from pDH56 (Example 4) was cloned into
the Bam HI site of the baculovirus transfer vector pAcCL29
using the following linker:

5'   GATCCGATATCA   3'
35   3'   GCTATAGTTCGA 5'

to give pDB769.

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This new recombinant baculovirus was used to infect Sf 21
cells and express the H56 gene as a fibrinolytically
active product; standard methods were used (Summers, M.D.
and Smith, G.E., 1987: "A Manual of Methods for
5 Baculovirus Vectors and Insect Cell Culture Procedures",
Texas Agricultural Experiment Station Bulletin No. 1555,
and modifications described in Page, M.J. and Murphy,
V.F., 1988: in J.M. Walker (ed) Methods in Molecular
Biology, Vol. 5).
10

Example 11

Expression of H55 in E.coli

15 (a) : Construction of the E.coli Expression Vector pDB525

The tac expression vector pDB525 was derived from pKK223-3
(Pharmacia).
The 3.28 kb SphI-Sca I fragment of pKK223-3 was replaced
20 with the equivalent fragment from pAT153 (Twigg, A.J. and
Sherratt, D.J. (1980) Nature, 283, 216-218) to render the
plasmid non-mobilisable; this new vector was called
PTR550. PTR550 was restricted with EagI and a 1.7kb
blunt-ended EcoRI fragment from ptac-1-Iq, encoding the
25 lacIq gene, was ligated in to give pDB525 (figure 5A).
The presence of the lacIq gene (Calos, M.P. (1978) Nature,
274, 762-765) ensures tight repression of the tac promoter
under non-induced conditions.

30 (b) : Construction of the H55 Protein E.coli expression
vector pDB549

(i) Construction of pTR545
The approximately 7.8kb fragment of BstEII-BglII-cut
35   pTRH37 (European Patent Application 0297882) was
ligated with oligonucleotides 1 and 2
1) 5' GTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGAGGCCT
ACTAGGCCAAGCTTA 3'

2)   5' GATCTAAGCTTGGCCTAGTAGGCCTCACGGTCGCATGTTGTCACGAATC
      CAGTCTAGGTAGTTG 3'

to give pTR545.

This places a convenient HindIII site 3' to the H37
sequence in this plasmid (figure 5A).

(ii) Construction of pDB546

The 0.89 kb EcoRI-HindIII fragment of pTR545,
encoding most of the H37 B chain was inserted into
the expression vector pDB525 between the EcoRI and
HindIII sites to make pDB546. (Figure 5A)

(iii) Construction of pDB545

The approximately 1kb NcoI-Sst I fragment from pTRH37
encoding K_4PK_5P and part of the B chain of t-PA was
ligated into EcoR1-Sst1-cut pUC19 together with
oligonucleotides 3 and 4

3)   5' ATTTCATGTCTTACCAAGCACCACCTGAGCTAACCCCTGTGGTCCAGG
      ACTGCTAC 3'

4)   5' CATGGTAGCAGTCCTGGACCACAGGGGTTAGCTCAGGTGGTGCTTGGT
      AAGACATG 3'

to give pDB545 (Figure 5B).

(iv) Construction of pDB549

The approximately 1kb EcoRI fragment of pDB545 was
ligated into EcoRI-cut and phosphatased pDB546 to
give pDB549. (Figure 5C).

(c) : Expression of H55 protein in E.coli

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The H55 expression plasmid pDB549 was transformed into
E.coli HB101. The transformed host was grown in L-Broth
at 37°C to an OD$_{550}$ of 0.8-1.0 and expression was induced
5   with 1mM IPTG (isopropyl-β-D-thiogalactopyranoside).
Incubation was continued for a further 3 hours. The cells
were harvested and disrupted by sonication (Heat Systems-
Ultrasonics; 50 x 50%. 5 second pulses at 70W). The
insoluble fraction containing H55 protein was separated by
10   centrifugation at 11,000g for 15 minutes at 4°C. It was
subsequently renatured following the protocol described by
van Kimmenade, A et al; (1988), Eur. J. Biochem.; 173,
105-114. Analysis of this refolded material using the
fibrin zymography technique (Dodd et al; (1986) Thromb.
15   Heam; 55 (1) 94-97) showed it to be fibrinolytically
active. The apparent molecular weight observed was
consisent with the predicted structure.

Example 12
20

Construction of pDH55U encoding SYO/Plasminogen 347-546/u-
PA 137-411 (H55U)

Construction of the plasmid pDH55U was accomplished by
25   substituting a 620bp MluI-BstXI fragment from pDH55
(Example 1) (MluI site located in RSVLTR promoter, BstXI
site at nucleotide 1209 in plasminogen cDNA sequence) for
the analogous MluI-BstXI fragment in pTRH25 (EP A 370
711).
30

The novel hybrid was expressed using HeLa cell system
(Methods). Fibrinolytically active protein, as determined
by fibrin plate assay, was recovered from the HeLa cell
harvest medium.
35

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Example 13

Construction of pDH56U encoding SYO/plasminogen 443-546/u-
PA 137-411 (H56U)

An approximately 5.9 kb MluI - BspMII (MluI in RSVLTR,
BspMII at nucleotide 1060 in u-PA cDNA) fragment and a 593
bp AvaII - BspMII (AvaII at 1693 in plasminogen, BspMII at
1060 in u-PA) fragment were isolated from pDH55U (Example
12). These were ligated with a 816 bp MluI - AvaII (MluI
in RSVLTR, AvaII at 1693 in plasminogen) fragment from
pDH56 (Example 4) to give pDH56U. Fibrinolytically active
protein was expressed as for H55U.

Example 14

Amplificiation of expression of H55 in CHO cells

The transfections described in Example 7 were carried out
with either 10 or 20μg of pDH16 or pDH17. All were then
selected as described in Example 7 and were amplified as
follows.

Those dishes transfected with 20μg plasmid DNA were grown
to confluency and these cells were amplified in
methotrexate as mass cultures. Twenty colonies were
isolated from dishes transfected with 10μg plasmid DNA (10
for each plasmid) and these were grown to confluency in 25
cm^2 flasks and harvested in serum-free medium for 24hrs.
Fibrinolytically active protein was detected by fibrin
plate assay and the four clones with the highest activity
were chosen for amplification in methotrexate.

The methotrexate concentration was initially 0.05 μM and
was increased stepwise to 5 or 10 μM. At 0.1 μM
methotrexate the best cell line, as judged by activity on
a fibrin plate, was the pDH17-transfected mass culture
(17MC). At 1μM methotrexate the 3 best cell lines (the

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pDH16-and pDH17-transfected mass cultures [16MC and 17MC]
and the pDH17-transfected clone [17.1]) were sub-cloned,
giving 12 sub-clones per cell line. The best cell line
from these 36 sub-clones plus the 3 parental lines was
sub-clone #1 isolated from 16MC i.e., 16MC.1. This sub-
clone along with the 6 next best sub-clones, plus the 3
parental lines, were amplified to 5 μM and, for 16MC, to
10 μM methotrexate. At these methotrexate concentrations
the 2 best cell lines were the sub-clone 16MC.1 at 5μM and
the parental mass culture 16MC at 10μM methotrexate.

Example 15

Purification of protein H55U

Approx 45ml conditioned media from HeLa cells transfected
with the plasmid pDH55U (see Example 12) and harvested as
described in `General Methods' was centrifuged at 9000g
for 30 min and stored at -40°C for 2 months. It was then
thawed and the H55U protein isolated by chromagraphy on
Benzamidine Sepharose 6B (Pharmacia). The details are as
follows.

A column (i.d., 16 mm; h, 12mm) of Benzamidine Sepharose
was equilibrated with PBS `A' (Dulbecco)/0.01% Tween 80.
The conditioned media was applied to the column and was
washed through with equilibration buffer followed by 0.02M
Tris/0.5M NaCl/0.01% Tween 80 pH 7.4. H55U was then
dissociated from the matrix by washing with 0.02M
Tris/0.5M NaCl/0.5M L-arginine/0.01% Tween 80 pH7.4. The
chromatography was at 4°C at a flow rate of 100 cmh^{-1}.
The eluant from the column was collected as discrete
fractions. Fractions containing the protein H55U were
identified using the microtitre-plate based chromogenic
substrate assay (General example (ix)) except that S2444
was used instead of S2288. The most active fractions were
pooled and were ultrafiltered (YM10, Amicon Ltd) to 2.0 ml
(the `product').

Assay of the product by fibrin plate assay with reference
to a u-PA standard showed it contained 100 IU/ml.
Analysis by SDS PAGE followed by fibrin zymography showed
5   a single major species at apparent M_r approx. 60,000.

Example 16

Purification of protein H56U
10

Approx 45ml conditioned media from HeLa cells transfected
with the plasmid pDH56U (see Example 13) and harvested as
described in 'General Methods' was centrifuged at 9000g
for 30 min and stored at -40°C for 2 months. It was then
15   thawed and the H56U protein isolated by chromatography on
Benzamidine Sepharose 6B (Pharmacia). The details are as
follows.

A column (i.d., 16mm; h, 15mm) of Benzamidine Sepharose
20   was equialibrated with PBS 'A' (Dulbecco)/0.01% Tween 80.
The conditional media was applied to the column and was
washed through with equilibration buffer followed by 0.02M
Tris/0.5M NaCl/0.01% Tween 80 pH 7.4. H56U was then
dissociated from the matrix by washing with 0.02M
25   Tris/0.5M NaCl/0.5M L-arginine/0.01% Tween 80 pH 7.4. The
chromatography was at 4°C at a flow rate of 100 cm h^{-1}.
The eluant from the column was collected as discrete
fractions. Fractions containing the protein H56U were
identified using th microtitre-plate based chromogenic
30   substrate assay (General example (ix)) except that S2444
was used instead of S2288. The most active fractions were
pooled and were ultrafiltered (YM10, Amicon Ltd) to 2.9 ml
(the 'product').

35   Assay of the product by fibrin plate assay with reference
to a u-PA standard showed it contained 47 IU/ml. Analysis
by SDS PAGE followed by fibrin zymography showed a single
major species at apparent M_r approx. 45,000.

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Example 17

Purification of protein H56 from Spodoptera frugiperda 21
cells

2.0l serum-free conditioned media from a culture of Sf21
cells that had been infected with recombinant Baculovirus
encoding H56 (see Example 10) was clarified by filtration
(Whatman No 1) and then adjusted to pH 5.0 using HCl.

H56 was purifed from the media using two chromatography
columns in series.

First, the media was passed down a column (i.d., 41mm; h,
38mm) of S-Sepharose Fast Flow that had been equilibrated
in 20mM succinate, 10mM EACA pH 5.0. the media was washed
through with equilibration buffer followed by a gradient
(in equilibration buffer) of 0 to 1M NaCl. A final rinse
with the 1M NaCl-containing buffer was then carried out.

The eluant was fractionated and assayed using the S2288
chromogenic substrate assay. The peak H56-containing
fractions were those collected during the development of
the NaCl gradient and were pooled. The pH of this pool
was adjusted to 7.0 using NaOH.

The pH 7.0 - adjusted pool was then chromatographed on a
column (i.d., 16mm; h, 55mm) of zinc chelate Sepharose
that had been equilibrated in PBS `A' (Dulbecco)/0.01%
Tween 80. After application of the pool from the S-
Sepharose the zinc chelate was washed with equilibration
buffer followed by 0.02M sodium phosphate/0.3M NaCl/0.01%
Tween 80 pH 7.4 and then 0.02M sodium phosphate/0.3M
NaCl/0.05M imidazole/0.01% Tween 80 pH 7.4.

H56-containing fractions were identified by S2288
substrate assay and were pooled. The pool had a volume of

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44 ml and contained 100,000 IU by fibrin plate assay with
reference to a u-PA standard curve. This particular pool
was not analysed by SDS PAGE. However, other purified H56
batches, prepared under almost identical conditions, were
5   analysed under non-reducing conditions. On silver-
staining, a single major band at apparent M_r approximately
40,000 was evident.

Example 18
10

Purification of protein H55 from CHO cells

Two methods were used to purify H55 from CHO cells (see
Example 14).
15

(1)   500ml serum-free conditioned media from the CHO mass
culture 17MC (amplified to 100nM methotrexate) was
applied to zinc chelate Sepharose (Vt = 9ml) and
lysine Sepharose (Vt = 2ml) essentially as described
20   (Dodd, I. et al (1986) FEBS Lett., 209 13-17). The
H55 protein was desorbed from the lysine - Sepharose
column using a 0.5M L-arginine - containing buffer
and was then concentrated by ultrafiltration using a
10,000 molecular weight cut-off membrane (Centriprep-
25   10, Amicon Ltd). The ultrafiltered retentate had a
volume of 0.95 ml and contained 4,800 IU by fibrin
plate assay.

(2)   The second purification example relates to 12 litres
30   conditioned media from the CHO cell line 16MC.1
(Example 14).

All glassware and columns were autoclaved prior to
use, all buffers were 0.2μ sterile filtered. The
35   chromatography system used (A Waters 650 advanced
protein purification system) was sanitised with 2M
NaOH.500ml of lysine Sepharose Fast Flow was packed
into an Amicon industrial glass 70mm column and

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packed at linear flow rate of 300 cm.h^{-1} with
phosphate-buffered saline (Dulbecco A). Upon
completion of packing (determined when the bed height
remains constant) the column was equilibrated with a
5   further 4 bed volumes of phosphate-buffered saline
(PBS).Conditioned media (12L) that had been filtered
through an 8μ polypropylene filter was then applied
at a linear velocity of 100cmh^{-1} .

10 The column was then washed with PBS containing 1M
NaCl at a linear velocity of 100cmh^{-1} for 5 bed
volumes. The column was then eluted using a linear
gradient of 0-10mM EACA in PBS/1M NaCl (over 6 bed
volumes). Elution was monitored by following 280nm
15 adsorption of the protein eluted by the EACA. 73mg,
as determined by fibrin plate analysis, of H55 was
eluted in 1L of solution.

The eluted pool from above was combined with a
20 similar run to give a solution containing 132mg of
H55 in 2.5L. This material was concentrated using an
Amicon 8400 stirred ultrafiltration cell containing
an Amicon YM-30 ultrafiltration membrane. The
ultrafiltration was performed at 50 psi using
25 nitrogen to pressurise the system. The
ultrafiltration was continued until the solution was
reduced in volume to 50ml when 300ml of 0.5M
arginine, 0.2M NaCl, 20mM Tris/HCl, 0.01% Tween 80pH
7.4 buffer was added. Ultrafiltration was continued
30 until the volume was again reduced to 50ml when a
further 300ml of the above buffer was added. The
volume was reduced to 25ml containing 120mg of H55.
Ultrafiltration/diafiltration gave a yield of
91%.Upon completion of diafiltration the material was
35 stored frozen at -40°C.

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In the figures:

Fig. 1   Plasmid pDH55i

5   Fig. 2   Plasmid pDH55
           signal = t-PA signal sequence

Fig. 3   Plasmid pDH56i

10   Fig. 4   Plasmid pDH56
           signal = t-PA signal sequence.

Fig. 5A Construction of plasmid pDB546

15   Fig. 5B Construction of plasmid pDB545.

Fig. 5C Construction of plasmid pDB549.

Claims-A

1.   A hybrid plasminogen activator (PA) which comprises
kringle 5 or kringles 4 and 5 of plasminogen linked to the
5 B-chain of t-PA or u-PA via an amino acid sequence
comprising, respectively, the t-PA cleavage site between
residues 275 and 276 and the cysteine residue 264 of t-PA or
the u-PA cleavage site between residues 158 and 159 and the
cysteine residue 148 of u-PA.
10

2.   A hybrid plasminogen activator according to claim 1 of
the formula: -

$$(Z_3 K_4^P)_m Z_4 K_5^P Z_5 B^t$$
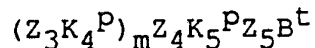
15 where B^t comprises residues 276-527 of t-PA, m is 0 or 1,
K_4^P and K_5^P represent kringle domains 4 and 5 derived from
plasminogen and each of Z_3, Z_4 and Z_5 represents, as
appropriate, an optional N-terminal amino acid sequence or a
bond or a linking sequence of amino acids which may be
20 introduced synthetically during the preparation of the
hybrid PA and/or derived from native plasminogen and/or t-PA
sequences, the sequence Z_5 comprising at least residues
cys-264 and arg-275 of t-PA.

25 3.   A hybrid plasminogen activator according to claim 2
wherein m is 1, Z_4 represents the native plasminogen inter-
domain sequence between plasminogen kringle domains 4 and 5
and Z_3 has at its N-terminus the sequence [GARSYQ] or [SYQ]
corresponding to the L- and S-chain forms of t-PA, and
30 comprises some or all of the native plasminogen inter-domain
sequence between plasminogen kringle domains 3 and 4.

4.   A hybrid plasminogen activator according to claim 3
wherein Z_3 comprises plasminogen residues 347-357.
35

5.   A hybrid plasminogen activator according to claim 2
wherein m is 0 and Z_4 has at its N-terminus the sequence
[GARSYQ] or [SYQ] corresponding to the L- and S-chain forms
of t-PA, and comprises some or all of the native plasminogen
inter-domain sequence between plasminogen kringle domains 4
and 5.

6.   A hybrid plasminogen activator according to claim 5
wherein Z_4 comprises plasminogen residues 443-461.

7.   A hybrid plasminogen activator according to any one of
claims 2 to 6 wherein Z_5 is selected from:

    1.   [AAPSTCGLRQYSQPQFR]
    2.   [AAPSTCGLRQYSQPQFQ]
    3.   [STCGLRQYSQPQFR]

(single letter amino acid notation)

8.   GARSYQ/plasminogen 347-541/t-PA 262-527 including one
and two chain variants, gly_{-3} and ser_1 variants, and
mixtures thereof.

9.   GARSYQ/plasminogen 443-541/t-PA 262-527 including one
and two chain variants, gly_{-3} and ser_1 variants, and
mixtures thereof.

10.   GARSYQ/plasminogen 347-546/u-PA 137-411 including one
and two chain variants, gly_{-3} and ser_1 variants, and
mixtures thereof.

11.   GARSYQ/plasminogen 443-546/u-PA 137-411 including one
and two chain variants, gly_{-3} and ser_1 variants, and
mixtures thereof.

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12.   A hybrid plasminogen activator according to any one of claims 1 to 11 expressed using chinese hamster ovary, HeLa, E. coli or Spodoptera frugiperda cells.

5 13.   A hybrid plasminogen activator according to any preceding claim where the catalytic site essential for fibrinolytic activity is blocked by a removable blocking group.

10 14.   N,N-Dimethyl-4-aminobenzoyl two chain SYQ/plasminogen 347-541/t-PA 262-527.

15.   4-Anisoyl SYQ/plasminogen 347-541/t-PA 262-527.

15 16.   4-Anisoyl SYQ/plasminogen 443-541/t-PA 262-527.

17.   A pharmaceutical composition comprising a hybrid plasminogen activator according to any preceding claim in combination with a pharmaceutically acceptable carrier.
20
18.   A hybrid plasminogen activator according to any of claims 1 to 16 for use as an active therapeutic substance.

19.   A hybrid plasminogen activator according to any of 25 claims 1 to 16 for use in the treatment of thrombotic diseases.

20.   Use of a hybrid plasminogen activator according to any of claims 1 to 16 for the manufacture of a medicament for 30 the treatment of thrombotic diseases.

21.   A method of treating thrombotic diseases which comprises administering to the sufferer an effective non-toxic amount of a hybrid plasminogen activator according to 35 any of claims 1 to 16.

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22.   A process for preparing a hybrid plasminogen activator
according to claim 1 or 13 which process comprises
expressing DNA encoding said hybrid plasminogen activator in
a recombinant host cell and recovering the hybrid
5 plasminogen activator product, and thereafter optionally
blocking the catalytic site essential for fibrinolytic
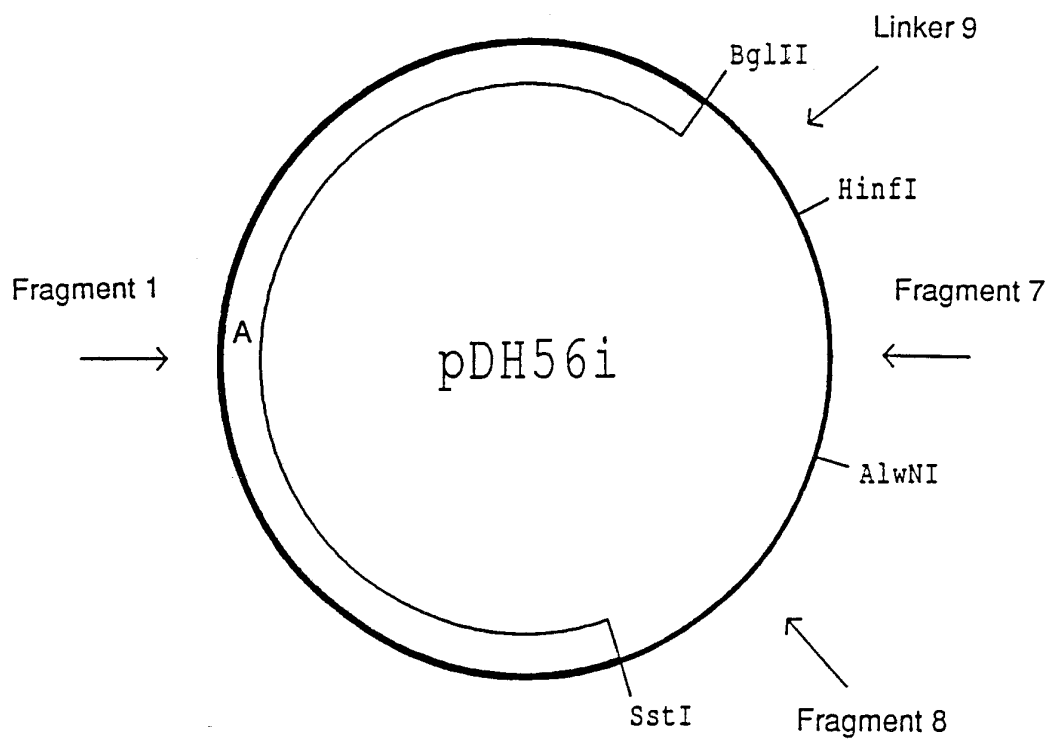activity with a removable blocking group.

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Fig. 1

A - Vector

Fig. 2

Fragment 6

pDH55

BamHI — A

BglII

C   Fragment 4

SstI

B

D

Fragment 5
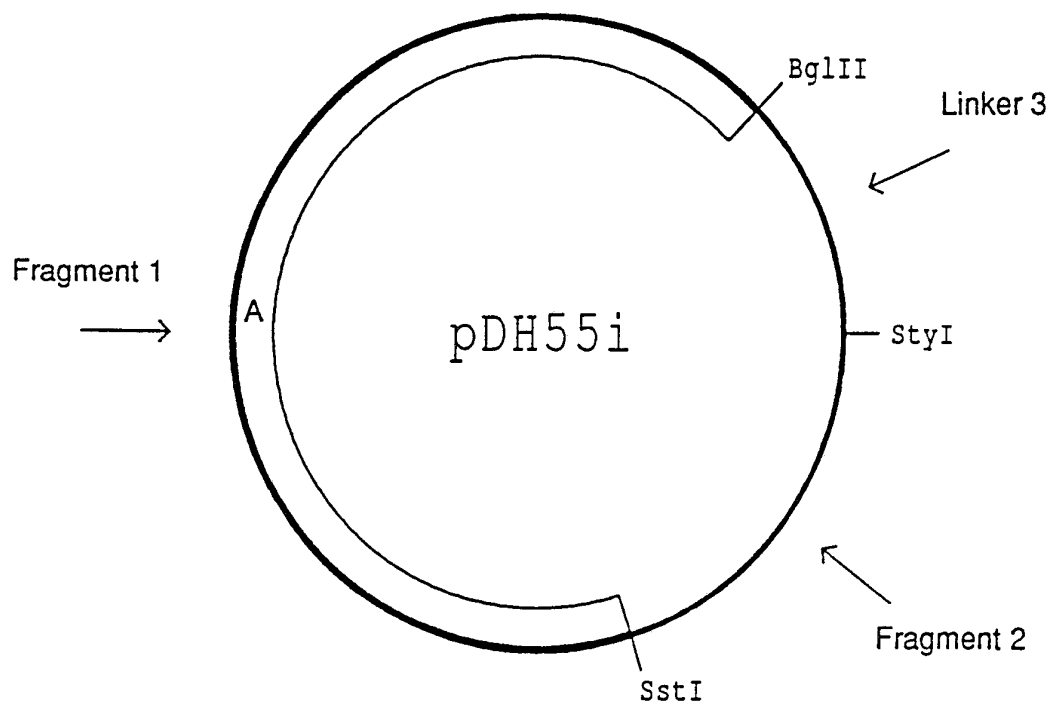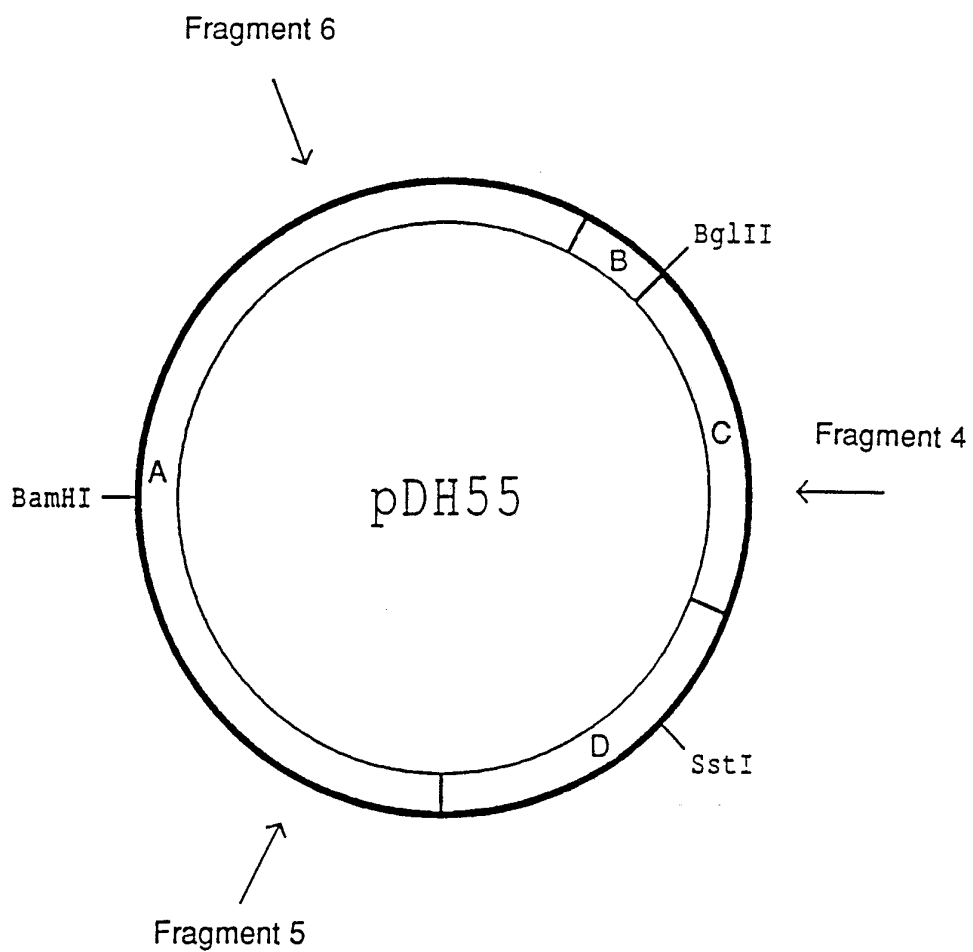
A - Vector
B - Signal
C - (K4K5)p
D - Bt

Fig. 3

A - Vector

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Fig. 4

Fragment 6

Fragment 5
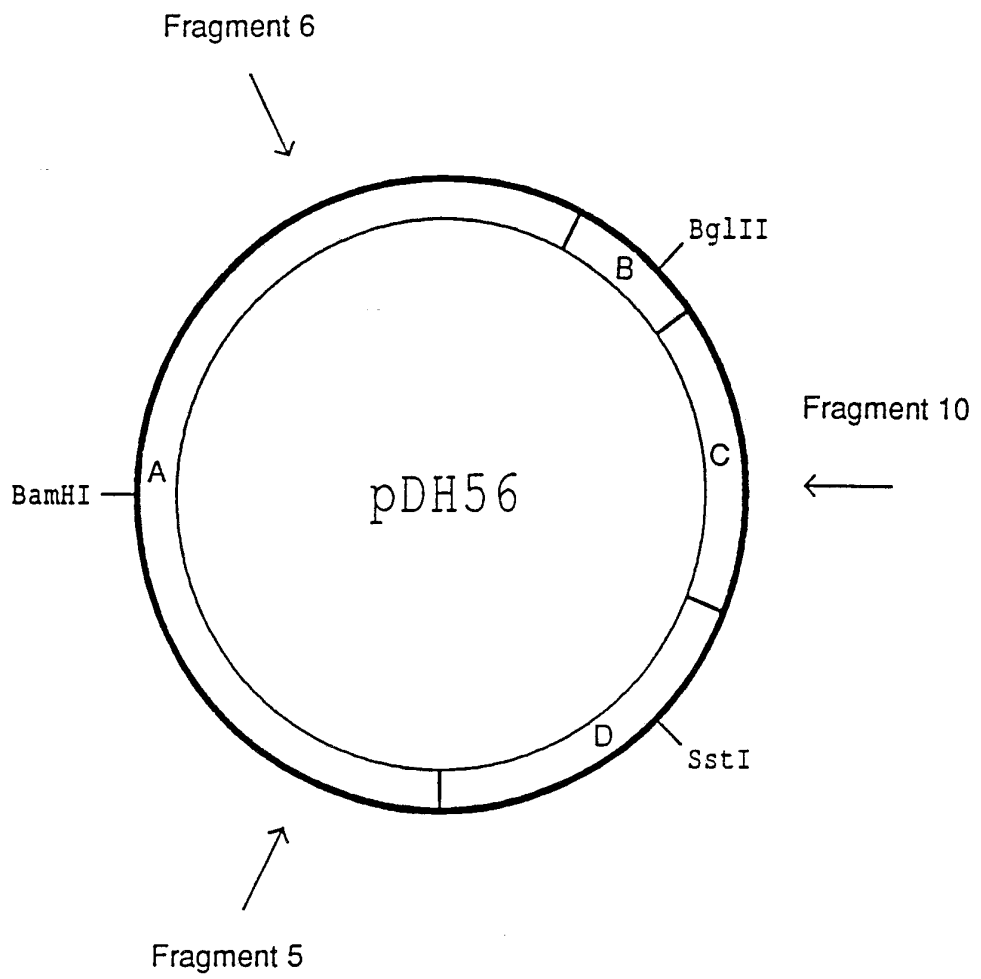
A - Vector
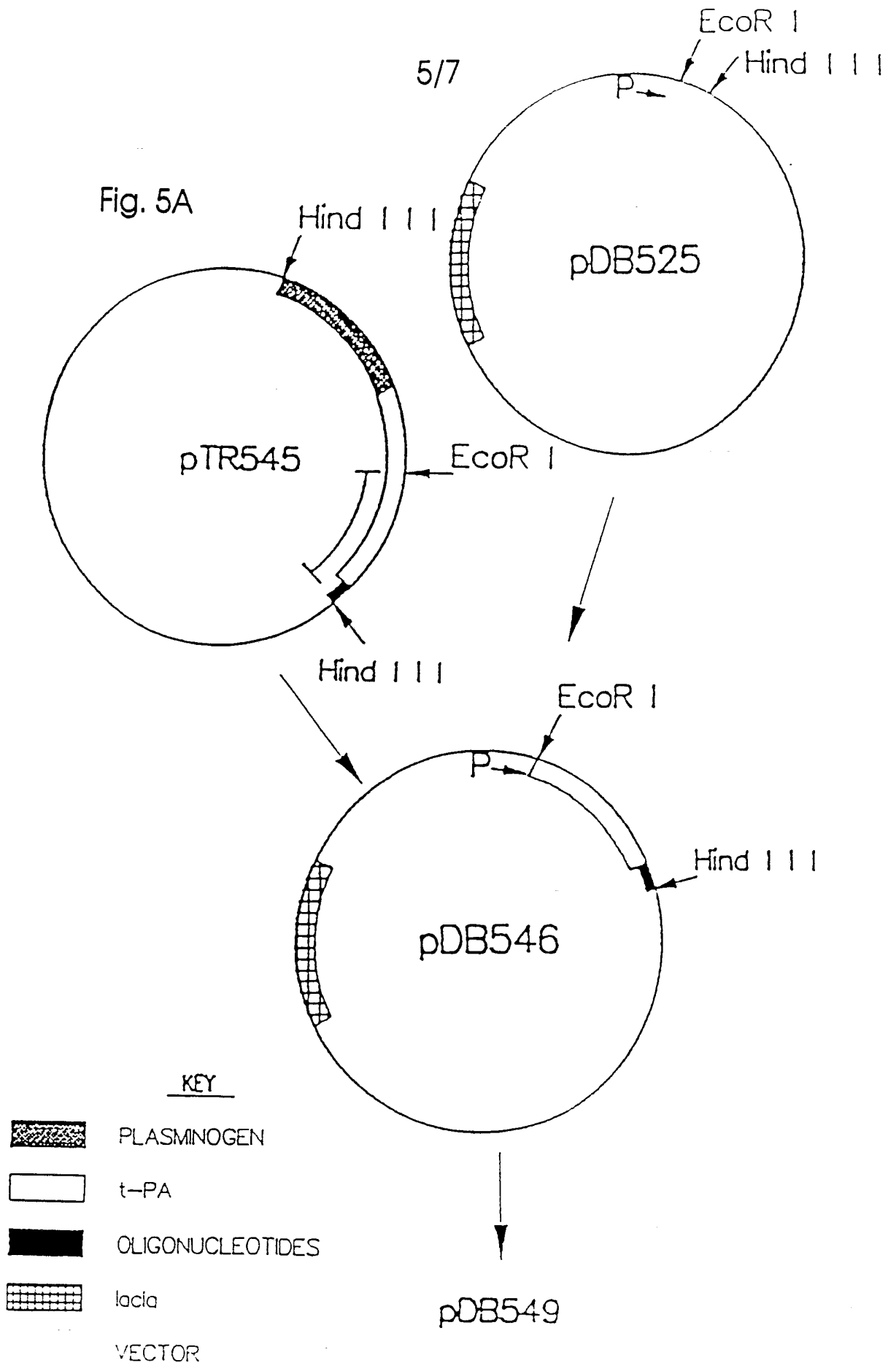B - Signal
C - (K5)p
D - Bt

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Fig. 5A

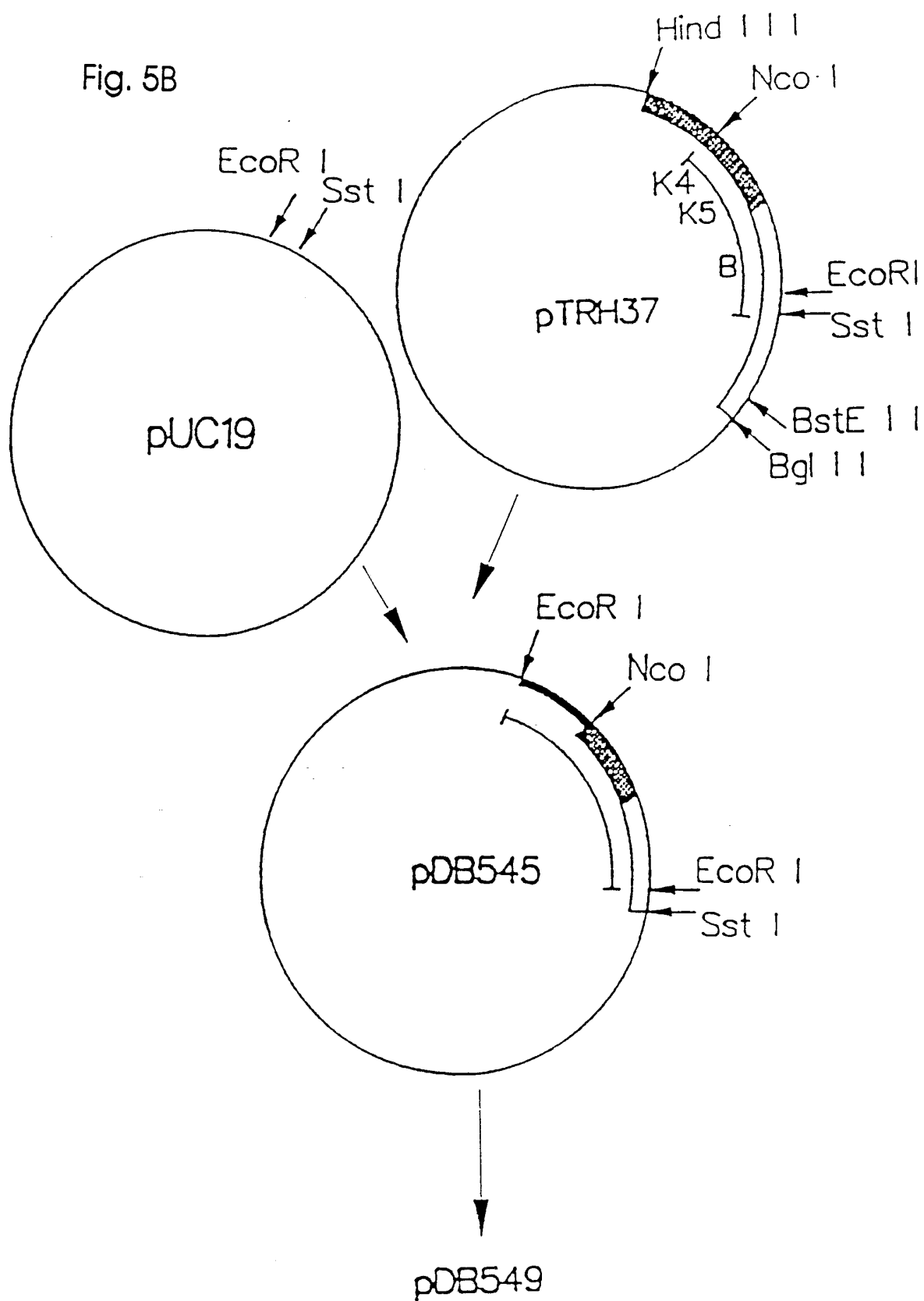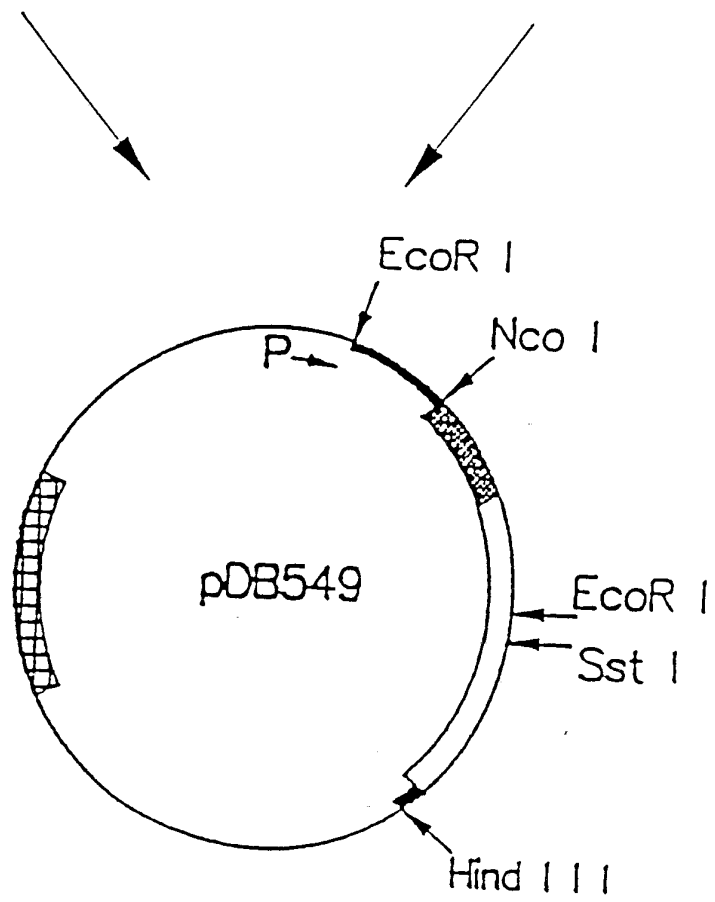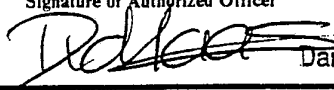Fig. 5B

Fig. 5C

pDB545   pDB546

INTERNATIONAL SEARCH REPORT

International Application No **PCT/GB 91/01455**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

```
Int.Cl.5      C 12 N  15/62     C 12 N  9/72     A 61 K  37/547
  C 12 N  15/58
```

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 N A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP,A,0297882 (BEECHAM GROUP LTD) 4 January 1989, see abstract; claims	1-22
Y	EP,A,0293934 (ZYMOGENETICS INC.) 7 December 1988, see abstract; claims	1-12,17 -22
Y	EP,A,0292326 (BEECHAM GROUP PLC) 23 November 1988, see abstract; claims	1-22
Y	Protein Engineering, volume 1, no. 6, December 1987, IRL Press LTD, (Oxford, GB) T.J.R. Harris: "Second-generation plasminogen activators", pages 449-458, see page 455, right-hand column, 4th paragraph - page 456, right-hand column, 1st paragraph	1-12,17 -22
Y	WO,A,8805081 (CETUS CORPORATION) 14 July 1988, see abstract; claims -/-	1-12,17 -22

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"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)

"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

"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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"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.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26-11-1991	17. 12. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Danielle van der Haas

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8910401 (COLLABORATIVE RESEARCH INC.) 2 November 1989, see abstract; claims ---	1-12,17 -22
Y	EP,A,0379890 (FUJISAWA PHARMACEUTICAL CO.) 1 August 1990, see abstract; claims ---	1-12,17 -22
P,Y	EP,A,0397366 (BOARD OF REGENTS OF THE UNIVERSITY OF OKLAHOMA) 14 November 1990, see abstract; claims ---	1-12,17 -22
A	WO,A,9009437 (BOEHRINGER MANNHEIM GmbH) 23 August 1990, see claims ---	3
A	EP,A,0290118 (BEECHAM GROUP PLC) 09 November 1988, see abstract; claims -----	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers   because they relate to subject matter not required to be searched by this Authority, namely:

    Although claim 21 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claim numbers   because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers   because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple Inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 10/12/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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