COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

DECLARATION IN SUPPORT OF CONVENTION OR NON-CONVENTION APPLICATION FOR A PATENT

In support of the Application made for a patent for an invention

Insert title of invention.

entitled: "POLYPEPTIDE PRODUCTION"

Insert full name(s) and address(es) of Declarant(s) being the applicant(s) or person(s) authorized to sign on behalf of an applicant company.

I / We HUGH RICHARD ILIFF PERROTT
1 YATTENDON COURT GARDENS
YATTENDON
NEWBURY
BERKSHIRE
ENGLAND

do solemnly and sincerely declare as follows :–

Cross out whichever of paragraphs 1(a) or 1(b) does not apply.

1(a) relates to application made by individual(s).

1(b) relates to application made by company; insert name of applicant company.

1. (a) ~~I am~~ / ~~We are~~ ~~the applicant~~ ..... ~~for the patent~~

or (b) I am authorized by CELLTECH LIMITED, a British Company, of 244-250 Bath Road, Slough, Berkshire, SL1 4DY, England,

the applicant..... for the patent to make this declaration on ~~its~~ / ~~their~~ behalf.

Cross out whichever of paragraphs 2(a) or 2(b) does not apply.

2(a) relates to application made by inventor(s)

2(b) relates to application made by company(s) or person(s) who are not inventor(s); insert full name(s) and address(es) of inventors.

2. (a) ~~I am~~ / ~~We are~~ ~~the actual inventor~~ ..... ~~of the invention~~

or (b) MICHAEL ANTHONY EATON, of 'Nethercote', Chinnor Road, Aston Rowant, Oxfordshire, OX9 5SH, England, RICHARD CHARLES DOMINIC TITMAS, of 26 Prince Andrew Close, Boulter's Lock, Maidenhead, Berkshire, SL6 8QR, England, and STEPHEN KEITH RHIND, of 47 Stratford Drive, Wooburn, Buckinghamshire, HP10 0QQ, England, all British Subjects

~~is~~ / are the actual inventors..... of the invention and the facts upon which the applicant.....

~~is~~ / are entitled to make the application are as follows :–

State manner in which applicant(s) derive title from inventor(s).

CELLTECH LIMITED is entitled by Contract of Employment between the Inventors as employees, and CELLTECH LIMITED as employer, as a person who would be entitled to have the patent assigned to it if a patent were granted upon an application made by the Inventors.

Cross out paragraphs 3 and 4 for non-convention applications. For convention applications insert basic country(s) followed by date(s) and basic applicant(s).

3. The basic application..... as defined by Section 141 of the Act ~~were~~ / was made

in Great Britain on the 19th September 1985

by CELLTECH LIMITED

in ..... on the .....

by .....

in ..... on the .....

by .....

4. The basic application..... referred to in paragraph 3 of this Declaration ~~were~~ / was the first application..... made in a Convention country in respect of the invention the subject of the application.

Insert place and date of signature.

Declared at SLOUGH this 30th day of April 1987

Signature of Declarant(s) (no attestation required).

HUGH RICHARD ILIFF PERROTT

Note: Initial all alterations.

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GB 30695/84 C12N 15/00
DE 31217/84 C07K 007/10

(57) Claim

1. A process for producing a product polypeptide having a C-terminal amide group, where the product polypeptide has one or more cysteine or cystine amino acid residues, comprising the step of reacting a precursor polypeptide comprising the amino acid sequence of the product polypeptide and a C-terminal glycine amino acid residue, with an amidating enzyme, characterised in that the cysteine or cystine sulphur group or groups is or are protected at least during the amidation step.

6. A polypeptide having one or more cysteine or cystine amino acid residues and a C-terminal glycine amino acid residue for use in a process according to claim 1, characterised in that the or each cysteine or cystine sulphur group or groups is or are protected.

9. A process according to claim 1 wherein the amidating enzyme preparation includes a compound capable of chelating copper.

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(57) Abstract

A process for the production of a polypeptide having a C-terminal amide group where the polypetide has one or more cysteine or cystine amino acid residues. A second polypetide comprising the amino acid sequence of the first polypeptide and a C-terminal glycine amino acid residue and where the cysteine or cystine sulphur group or groups is or are protected is reacted with an amidating enzyme capable of converting the C-terminal glycine amino acid residue to an amide.

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"Amidation of terminal carboxyl residues of cysteine or cystine-containing polypeptides".

Field of the Invention

This invention relates to a process for producing a polypeptide having a C-terminal amide group, where the polypeptide has one or more cysteine or cystine amino acid residues. comprising the step of reacting a polypeptide comprising the amino acid sequence of the polypeptide and a C-terminal glycine amino acid residue, with an amidating enzyme.

Background to the Invention :

A number of physiological, active polypeptides, especially peptide hormones, have been shown to depend upon a C-terminal amide group for their full activity. In addition, the C-terminal amide has been shown to enhance the half-life of some hormones in the circulatory system by preventing degradation from the C-terminal end by natural proteases. It is now possible, using the techniques of recombinant DNA technology, to synthesise polypeptides on an industrial scale by in vitro culture. However, polypeptides directly produced in this manner do not possess a C-terminal amide and may not therefore exhibit their full physiological activity and stability in the circulatory system. This problem has been overcome by the use of an amidating enzyme to convert an additional C-terminal amino acid residue into the necessary amide group (see for example published European Patent Application EP-A1-0134631 and EP-A1-0133282). The amidating enzyme concerned was first described and characterised by Bradbury et. al. (Bradbury, A. F. et. al. Nature (1982) 298, 686-688; Bradbury, A. F. et. al. Biochem. and Biophys. Comm. (1983) 1123(2), 372-377; Landymore-Lin, A. E. N. et. al. Biochem. And Biophys. Comm. (1983) 117(2), 289-293).

We have now discovered that such amidating enzymes do not satisfactorily amidate certain polypeptides, specifically those polypeptides which contain cysteine amino acid residues or disulphide bridges in their structure. Our research indicates that the lack of satisfactory amidation is due to precipitation of the polypeptide substrate during treatment with the amidating enzyme.

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Without prejudice it is believed that this precipitation may be caused, at least in part, by copper which in some cases has been shown to enhance the activity of the amidating enzyme. The copper present in the amidating enzyme preparation may interact with cysteine or cystine sulphur groups giving rise to polymerisation of the polypeptide, leading to dimer formation and/or aggregation, which in turn may result in precipitation of the polypeptide. In addition, ascorbic acid (Vitamin C), which is used as a further cofactor in the amidation preparation, may cause further complications. For instance, ascorbic acid may also be involved in modification of the polypeptide as a result of its interaction with cysteine or cystine sulphur groups.

It appears that the amidating activity of the enzyme is considerably reduced when the polypeptide substrate is in a precipitated form.

Summary of the Invention

According to a first aspect of the invention there is provided a process for producing a first or product polypeptide having a C-terminal amide group, where the first or product polypeptide has one or more cysteine or cystine amino acid residues, comprising the step of reacting a second or precursor polypeptide comprising the amino acid sequence of the first or product polypeptide and a C-terminal glycine amino acid residue , with an amidating enzyme, characterised in that the cysteine or cystine sulphur group or groups is or are protected at least during the amidation step.

As used herein the term 'amidating enzyme' is used to denote an enzyme which is capable of converting the C-terminal glycine amino acid residue to an amide group.

The amidating enzyme may be derived from sources such as for example mammalian tissue or from any other suitable source.

In a particularly preferred embodiment the amidating enzyme used in the process of the invention is an α-amidating enzyme such as the α-amidating enzyme derived from porcine pituitary tissue which was described and characterised by Bradbury et al (J. loc. cit.).

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The protection of the cysteine or cystine sulphur group or groups during treatment with the amidating enzyme preparation reduces the hitherto unforeseen problem of precipitation.

The term "cysteine or cystine amino acid residues" as used herein includes derivatives thereof. Typically the cysteine or cystine group is capable of chemical modification either by derivatisation or reaction with a protecting group to yield a protected sulphur containing group.

The protection of the cysteine or cystine sulphur group employed during the amidation step may comprise irreversible protection. For instance, a cysteine or cystine sulphur group may be protected by irreversible derivatisation; for example, treatment with acrylonitrile, ethyleneimine, sulphenyl halide, or performic acid oxidation of cystine to cysteic acid. Preferably, however, the protection is reversible, and any protecting group capable of reversibly binding to the cysteine or cystine may be employed. Typically the conditions employed for protection of the cysteine or cystine sulphur group and, in the case of reversible protection, for removal of the protecting group are such that they do not have a detrimental effect on the structure of the polypeptide i.e. advantageously the final product is produced in the form of its native three-dimensional structure.

The polypeptide may be any polypeptide containing one or more cysteine or cystine amino acid residues for which a C-terminal amide group may be desirable. The process is suitable for the conversion of a C-terminal glycine amino acid residue of a polypeptide produced by recombinant DNA techniques to a C-terminal amide group. Examples of such polypeptides include calcitonins such as salmon calcitonin, human calcitonin and functionally equivalent derivatives of these calcitonins; calcitonin gene-related peptides such as human calcitonin gene-related peptide, rat calcitonin gene-related peptide and functionally equivalent derivatives of these gene-related peptides; oxytocin and functionally equivalent derivatives; and vasopressin and functionally equivalent derivatives.

The term 'polypeptide' as used herein denotes a compound comprising two or more amino acid residues and includes proteins having secondary structure.

In some cases under the conditions which are optimal for activity of the amidating enzyme (for example at a pH in the range 5 to 8, preferably about 6.8 for the preferred amidating enzyme), the polypeptide-glycine compound may be close to, or at, its isoelectric point and this may cause precipitation, reducing, in another way, the substrate available for the amidating enzyme. This problem may be overcome by using protection which introduces a net charge into the polypeptide, either by derivatisation or use of a charged protecting group. The introduction of a net charge into the protected polypeptide may modify its isoelectric point and solubility properties and thereby reduce precipitation. Suitable protecting groups which may be bound to the cysteine or cystine sulphur atoms include $-SO_3^-$ and $-SPO_3^{2-}$. Since human calcitonin-glycine and human calcitonin gene related peptide-glycine are close to their isoelectric point at the preferred amidation pH, the use of a charged protecting group, protecting the disulphide bridge, and altering the isoelectric point provides a significant improvement in the preparation of correctly C-terminal amidated polypeptides.

In a second aspect the invention provides a polypeptide having one or more cysteine or cystine amino acid residues and a C-terminal glycine amino acid residue characterised in that the or each cysteine or cystine sulphur group or groups is or are protected.

Each cysteine or cystine sulphur group may be protected by derivatisation or by binding of a protecting group. Suitable protecting groups include any protecting group capable of reversibly binding to the cysteine or cystine sulphur atom providing that it may be bound and removed under conditions which are not detrimental to the polypeptide. Preferably the protecting group is charged. Particularly preferred protecting groups are $-SO_3^-$ and $-SPO_3^{2-}$.

Examples of other suitable cysteine or cystine sulphur protecting

groups which may be used include for example benzyl groups, which may be substituted by one or more carboxyl, sulphate, phosphate, alkyl, alkoxy, nitro or halogen substituents e.g. a dinitrofluorobenzene group; a diphenylmethyl group, which may be substituted by one or more carboxyl, sulphate or phosphate substituents; a triphenylmethyl group which may be substituted by one or more carboxyl, sulphate or phosphate substituents; alkyl groups, e.g.a t-butyl group; an acyl group e.g. an acetyl group; an acyl group substituted by for example a halogen atom e.g. an iodoacetate group; a benzoyl group; a carbonate group; a carbamoyl group which may be substituted by for example an alkyl, an alkoxy or an alkoxyalkyl group; a benzhydryl group; a mono or di acetal group, e.g. a tetrahydropyranyl group or a benzylthiomethyl group; a metal e.g. mercury; a mercurial group, e.g. a mercuribenzoate group; or a mixed disulphide group e.g. an S-alkylmercapto or an S-alkylsulfenylcysteine group. For examples of other cysteine or cystine sulphur protecting groups and for methods of removing such protecting groups, if so desired, see for example "The Peptides: Analysis, Synthesis, Biology", Vol. 2 Part A (eds. Gross and Meienhofer, Academic Press (1980)) and "Chemical Modification of Proteins" (eds. Means and Feeney, Holden-Day Inc. (1971)).

It will be appreciated that a net charge may, if desired, be introduced into the above cysteine or cystine sulphur protecting groups, or any other suitable cysteine or cystine sulphur protecting group, by substitution of the protecting group with an appropriate charged substituent for example by substitution by one or more carboxyl, sulphate or phosphate substituents.

We have discovered also that if the copper which is present in the amidating enzyme preparation is present in a chelated form, improved yields of amidated product may be obtained. Chelation of copper may be used as an alternative or preferably in combination with protection of cysteine or cystine sulphur groups. It appears that chelation of the copper may diminish precipitation of the polypeptide, and may have further advantages in terms of modulating accessability of copper to the enzyme. In this latter respect, we have further discovered that whilst in some cases copper enhances the activity of the amidating enzyme,

excess copper can be detrimental to the enzyme activity. Chelation of the copper appears to overcome this difficulty.

Thus, in a third aspect the invention provides a process for producing a first polypeptide having a C-terminal amide group, where the first polypeptide has one or more cysteine or cystine amino acid residues, comprising the step of reacting a second polypeptide comprising the amino acid sequence of the first polypeptide and a C-terminal glycine amino acid residue, with an amidating enzyme, characterised in that the cysteine or cystine sulphur group or groups is or are protected at least during the amidation step, wherein the amidating enzyme preparation includes a compound capable of chelating copper.

Suitable chelating compounds include ethylenediaminetetracetic acid (EDTA) and penicillamine. A further chelating compound that may be used is bathocuproine, as a disulphonate, which especially chelates copper (I).

We have discovered that the addition of a chelating agent reduces the level of free copper, thereby reducing detrimental effects on both the polypeptide and the amidating enzyme but maintains a level of copper, in a suitable form sufficient to act as a cofactor for the enzyme.

Brief Description of the Drawings

The invention is illustrated by the following examples with reference to the accompanying drawings which are high performance liquid chromatography (HPLC) traces.

Figure 1: Trace 1 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly and porcine pituitary amidating enzyme taken at Omin incubation.

Trace 2 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly and porcine pituitary amidating enzyme taken after incubation for 420 min.

Figure 2: Trace 1 shows an HPLC trace of an aliquot of a pellet obtained after centrifugation of a reaction mixture incubated for 540 min containing hCT-gly and porcine pituitary amidating enzyme.

Trace 2 shows an HPLC trace of an aliquot of a supernatant.

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obtained after centrifugation of a reaction mixture incubated for 540min containing hCT-gly and porcine pituitary amidating enzyme

Figure 3: Trace 1 shows an HPLC trace of a sample taken immediately after preparation of a reaction mixture containing hCT-gly, Cu/EDTA and ascorbic acid.

Trace 2 shows an HPLC trace of a sample taken after overnight incubation of a reaction mixture containing hCT-gly in the absence of ascorbic acid and Cu/EDTA

Trace 3 shows an HPLC trace of a sample taken after overnight incubation of a reaction mixture containing hCT-gly, Cu/EDTA and ascorbic acid

Figure 4: shows a trace run at increased sensitivity of Trace 3 shown in Figure 3.

Figure 5: Trace 1 shows an HPLC trace of an aliquot taken after incubation for 60min of a reaction mixture containing hCT and Na_2SO_3 .

Trace 2 shows an HPLC trace of an aliquot taken after incubation for 60min of a reaction mixture containing hCT-gly and Na_2SO_3 .

Figure 6: Trace 1 shows an HPLC trace of an aliquot taken immediately after preparation of a reaction mixture containing S-sulphonated hCT-gly and porcine amidating enzyme preparation .

Trace 2 shows an HPLC trace of an aliquot taken after incubation for 4hr of a reaction mixture containing S-sulphonated hCT-gly and porcine amidating enzyme preparation .

Figure 7: Trace 1 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly, Cu/EDTA and Na_3O_3PS taken after a reaction time of 30min.

Trace 2 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly, Cu/EDTA and Na_3O_3PS taken after a reaction time of 90min.

Trace 3 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly, Cu/EDTA and Na_3O_3PS taken after a reaction time of 150min.

Figure 8: Trace 1 shows an HPLC trace of an aliquot taken immediately after preparation of a reaction mixture containing hCT-gly and Na_3O_3PS.

Trace 2 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly and Na_3O_3PS taken after a reaction time of 30min.

Trace 3 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly and Na_3O_3PS taken after a reaction time of 60min.

Trace 4 shows an HPLC trace of an aliquot of a control reaction mixture

Figure 9: Trace 1 shows an HPLC trace of an aliquot taken immediately after preparation of a reaction mixture containing hCT-gly and Na_3O_3PS.

Trace 2 shows an HPLC trace of an aliquot of a reaction mixture containing hCT-gly and Na_3O_3PS taken after a reaction time of 30min.

Figure 10: shows a mass spectrum of a sample of sulphonated hCGRP.

Detailed Description of the Embodiments

Human calcitonin is a peptide hormone with possible clinical applications in the treatment of post menopausal osteoporosis and other disorders of calcium metabolism, for example Paget's disease.

A precursor to this, human calcitonyl-glycine (hCT-gly) has been made in E. coli by recombinant DNA technology (see for example published European Patent Application EP-A1-0131363). The purified precursor can be amidated under selective conditions using an amidating enzyme as described in the above introduction.

Comparative Example A

An experiment was conducted to illustrate loss of human calcitonyl-glycine substrate during an amidation reaction.

Human calcitonyl-glycine was incubated at 37°C with an amidating enzyme. The composition of the reaction mixture (210 ul) was as follows:

25mM PIPES buffer (pH 6.8)
300 mM NaCl
100 uM Cu/EDTA
1 mM ascorbic acid
2 mg/ml human calcitonyl-glycine
1000 units/ml Catalase
200 ul porcine pituitary amidating enzyme

(The porcine pituitary amidating enzyme used was partially purified according to the method of: Bradbury, A. F., and Smyth, D.G. (1985) in "Biogenetics of Neurohormonal Peptides" pp 171-186).
5 ul aliquots of the reaction mixture were taken at 0 and 420 minutes. The aliquots were analysed using HPLC. Figure 1 shows the results of these experiment and indicates clearly the loss of > 90% human calcitonyl-glycine from the solution phase of the reaction mixture over this period.

Comparative Example B

In order to show that human calcitonyl-glycine was in fact precipitated, the experiment described in Comparative Example A was repeated, and at the end of the reaction (after approximately 540 minutes) the remaining total sample (43 ul) was centrifuged (20,000 g x 5 minutes). The resulting pellet was dissolved in 50 ul of glacial acetic acid and a 10 ul aliquot was analysed by HPLC. In addition a 10 ul

aliquot of the supernatant after centrifugation was analysed by HPLC. The results of the experiment are shown in Figure 2, and indicate that the majority (71%) of human calcitonyl-glycine was present in the pellet, indicating substantial precipitation of human calcitonyl-glycine.

Comparative Example C

An experiment was conducted in order to show the precipitation of human calcitonyl-glycine in the presence and absence of amidation cofactors. Human calcitonyl-glycine was incubated at 37°C overnight in a buffer comprising 10 mM PIPES (pH 6.8) and 300 mM NaCl as a control, and in the same buffer containing additionally 0.3 mM Cu/EDTA and 3 mM ascorbic acid, each at a total volume, of 26 ul. Aliquots were centrifuged (20,000 g x 5 minutes) and analysed by HPLC. The results of the experiment are shown in Figure 3 in which Trace 1 shows 2.8 ul of the reaction mixture immediately after preparation and indicates a large peak showing the presence of human calcitonyl-glycine in the solution phase of the mixture. Trace 2 (7.5 ul) is the trace after an overnight incubation in the absence of Cu/EDTA and ascorbic acid and Trace 3 (7.5 ul) shows the trace again after an overnight incubation in the presence of Cu/EDTA and ascorbic acid.

Figure 4 shows a trace run at increased sensitivity of Trace 3 shown in Figure 3. The trace clearly shows the presence of complex multiple peaks with retention times between 2.5 and 3.0 minutes. The complex multiple peaks are taken to be polymerisation products of human calcitonyl-glycine.

Example 1

An experiment was performed to S-sulphonate human calcitonyl-glycine and human calcitonin.

50 ul of 0.1 M $NaHCO_3$ (pH 9.3) was added to 100 ul of 2.5 mg/ml human calcitonyl-glycine in water. Additions of 1M Na_2SO_3 (13 ul) and 0.01 M $CuSO_4$ (3 ul) were made and the reaction mixture was left at room temperature for 60 minutes. The reaction was judged to be completed by the disappearance of all the original human calcitonyl-glycine (retention time approximately 2.75 minutes) and the appearance of the new sulphonated derivative (retention time approximately 1.8 minutes). The relevant HPLC traces are shown in Figure 5. The upper trace shows sulphonated human calcitonin, which was treated in a similar manner.

Example 2

An experiment was conducted to show the stabilisation afforded to human calcitonyl-glycine by S-sulphonation.

S-sulphonated human calcitonyl-glycine was incubated at 37°C with a porcine amidating enzyme preparation as described in Comparative Example A. Aliquots of the reaction mixture were taken, centrifuged (20,000 g x 5 minutes) and analysed by HPLC. Figure 6 shows the resulting trace which includes a new peak (30% yield) co-migrating with S-sulphonated human calcitonin (retention time approximately 2.5 minutes) after four hours of treatment with the amidating preparation. There is no precipitation of substrate or product as judged by the initial and final peak area.

The specific details of the HPLC determinations carried out in the above experiments were as follows:

Column:	Hypersil ODS 10 x 0.2 cm			
% A :	10 mM NH_4HCO_3			
% B :	CH_3CN			
Gradient:	15-50% B over 5 Min.			
Flow Rate:	1 ml/Min.			
Detection:	Fig. 1	1.1	AUFS,	A_{225}
	Fig. 2	1.5	AUFS,	A_{225}
	Fig. 3	0.2	AUFS,	A_{225}
	Fig. 4	0.025	AUFS,	A_{225}
	Fig. 5 & 6	0.05	AUFS,	A_{225}

Example 3

An experiment was carried out to thiophosphorylate hCT-gly and to determine the effect of varying experimental conditions.

40 ul of 0.5M Na_3O_3 PS and 10 ul of 100 mM Cu/EDTA solution were added to 100 ul of a 0.6 mg/ml solution of hCT-gly which had been dried down. A final volume of 200 ul was obtained by adding 150 ul of 100 mM Tris buffer. Samples of 20 ul were analysed after reaction times of 30 minutes, 90 minutes and 150 minutes. As can be seen from the trace [Fig. 7] the product did not revert back to the starting material with time, but remained steady.

The reaction was repeated, using 10 ul of 10 mM Cu/EDTA solution instead of 10 ul of 100 mM Cu/EDTA solution. This decrease in Cu/EDTA appeared to have little effect on the reaction. A control was also carried out i.e.; reaction mixture minus Na_3O_3 PS and as can be seen from the trace [Fig 8] the reaction has gone to completion after 100 minutes.

In the next experiment, the amount of Na_3O_3PS added was decreased i.e 10 mM final Na_3O_3 PS instead of 100mM final Na_3O_3 PS. Like the decrease in Cu/EDTA, this appears to have had no great effect on the reaction [Fig. 9]. From these last two experiments it can be said that cupric ions are definitely required in order for the reaction to take place and that varying the amounts of Cu/EDTA and Na_3O_3PS has had very little effect on the reaction.

The thiophosphate protected hCT-gly is then amidated substantially as described in Example 2.

The specific details of the HPLC determinations are as described above in Example 2.

Example 4

SULPHONATION of hCGRP

A reaction mixture consisting of 14.4 mg of PENINSULAR, CRUDE CGRP; Tris (200 mM, 10 ml); and Na_2SO_3 (1M; 500ul); Cu/EDTA (10 mM; 100ul) was left to stand at room temperature and aliquots removed for injection onto Synchropak CM 300

A = HPLC water

B = 2M NaCl/20mM (P) pH 6.00

(i.e. 116.88g of NaCl in 1 litre HPLC H_2O + 2ml H_3PO_3, pH - 6.00 with dilute NaOH)

C = MeCN

T = 0 - 30%; %B = 1 - 20%; %C = 10 - 10

T = 30 - 50%; %B = 20 - 90%; %C = 10 - 10

Flow = 3.0 ml min^{-1}; wavelength = 230 nm

The reaction was complete after 330 minutes. The peak at retention time 20.3 minutes was collected and freeze dried. The residue was dissolved in ca 3 ml 0.1m HOAC and loaded onto Sephadex G-10 for desalting. The column was eluted. The first peak was collected and freeze-dried. A sample of the resulting residue was sent for mass spectrum analysis.

The mass spectrum (shown in Figure 10) was run at acidic pH and gave a very weak protonated molecular ion in positive ion mode with a small fragment ion showing loss of one sulphate group 80 amu lower.

It will be understood that the present invention is described by way of example only and modification of details may be made within the scope of the invention.

CLAIMS:

1. A process for producing a product polypeptide having a C-terminal amide group, where the product polypeptide has one or more cysteine or cystine amino acid residues, comprising the step of reacting a precursor polypeptide comprising the amino acid sequence of the product polypeptide and a C-terminal glycine amino acid residue, with an amidating enzyme, characterised in that the cysteine or cystine sulphur group or groups is or are protected at least during the amidation step.

2. A process according to Claim 1 wherein the cysteine or cystine sulphur group or groups of the precursor polypeptide is or are reversibly protected.

3. A process according to Claim 1 wherein the cysteine or cystine sulphur group or groups of the precursor polypeptide is or are protected by a sulphite or a thiosulphate group.

4. A process according to Claim 1 wherein the cysteine or cystine sulphur group or groups of the precursor polypeptide is or are protected by a charged protecting group.

5. A process according to any of the preceding claims wherein the product polypeptide is human calcitonin of human calcitonin gene-related peptide.

6. A polypeptide having one or more cysteine or cystine amino acid residues and a C-terminal glycine amino acid residue ~~for use~~ when used in a process according to claim 1, characterised in that the or each cysteine or cystine sulphur group or groups is or are protected.

7. A precursor polypeptide according to Claim 6 where the or each cysteine or cystine sulphur group is or are protected by a sulphite or thiosulphite group.

8. A precursor polypeptide according to Claim 6 or Claim 7 wherein precursor polypeptide is human calcitonin-gly or human calcitonin gene related peptide-gly.

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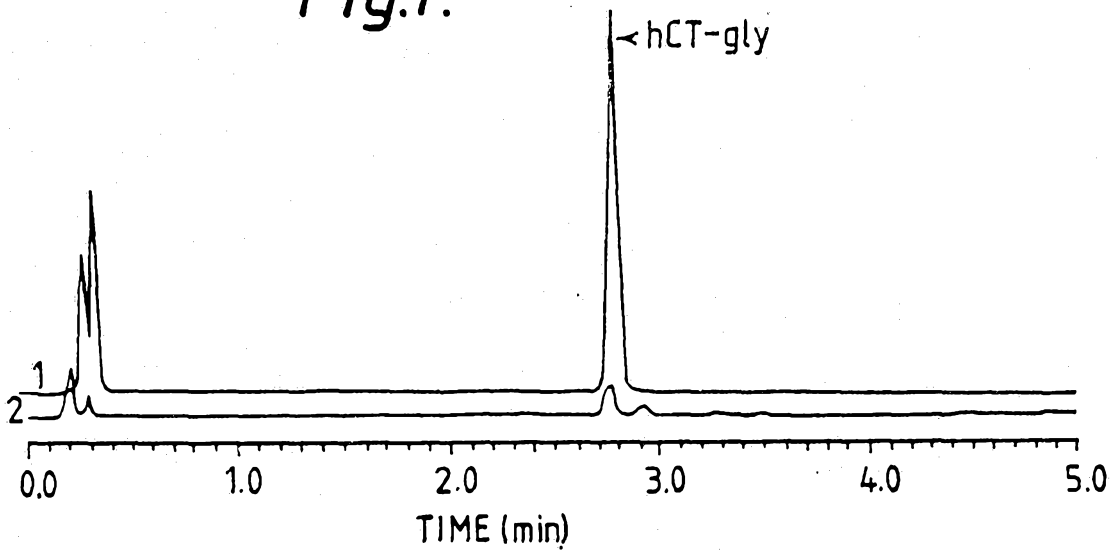
9. A process according to claim 1 wherein the amidating enzyme preparation includes a compound capable of chelating copper.

10. A process according to Claim 9 wherein the compound capable of chelating copper is ethylenediaminetetracetic acid.

DATED THIS &day&
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Patent Attorneys for the Applicant
CELLTECH LIMITED

AUSTRALIAN PATENT OFFICE

Fig.1.


hCT-gly
1
2
0.0
1.0
2.0
3.0
4.0
5.0
TIME (min)

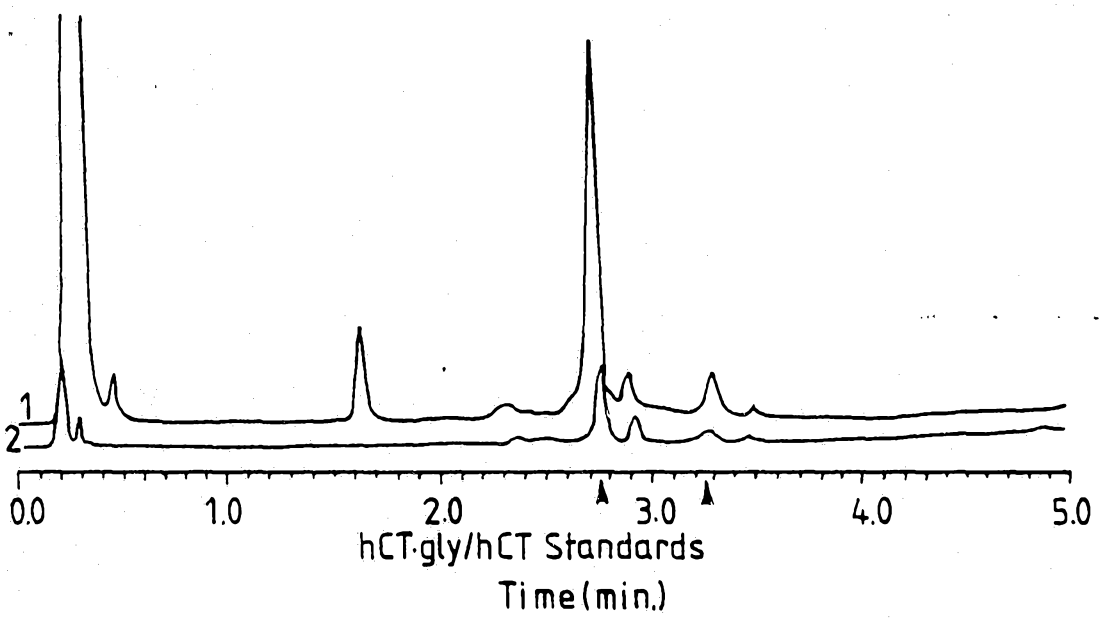

1
2
0.0
1.0
2.0
3.0
4.0
5.0
hCT-gly/hCT Standards
Time (min.)

Fig.2.

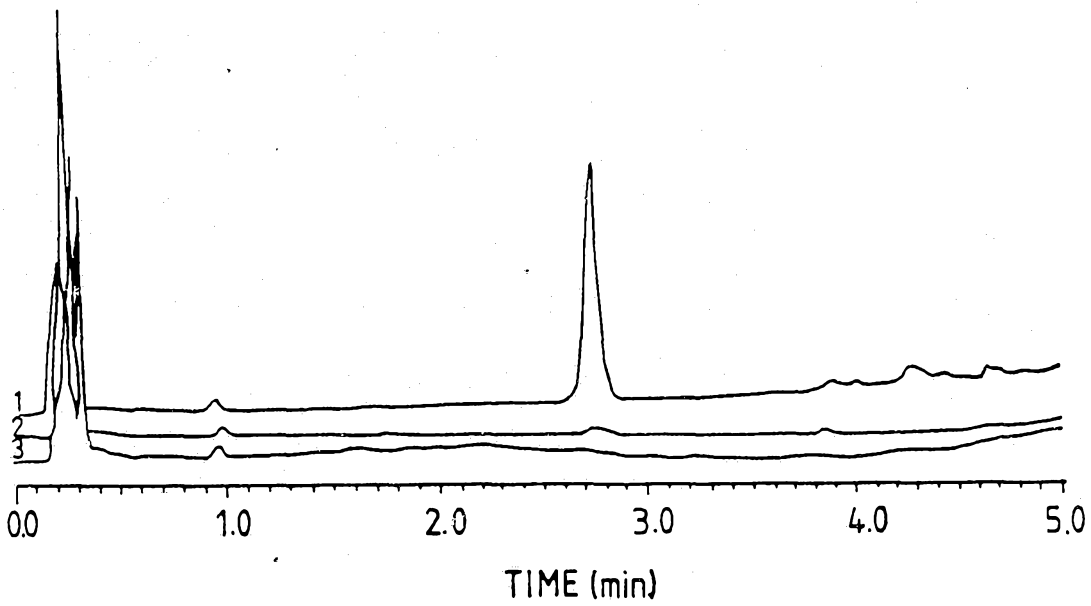


Fig. 3.

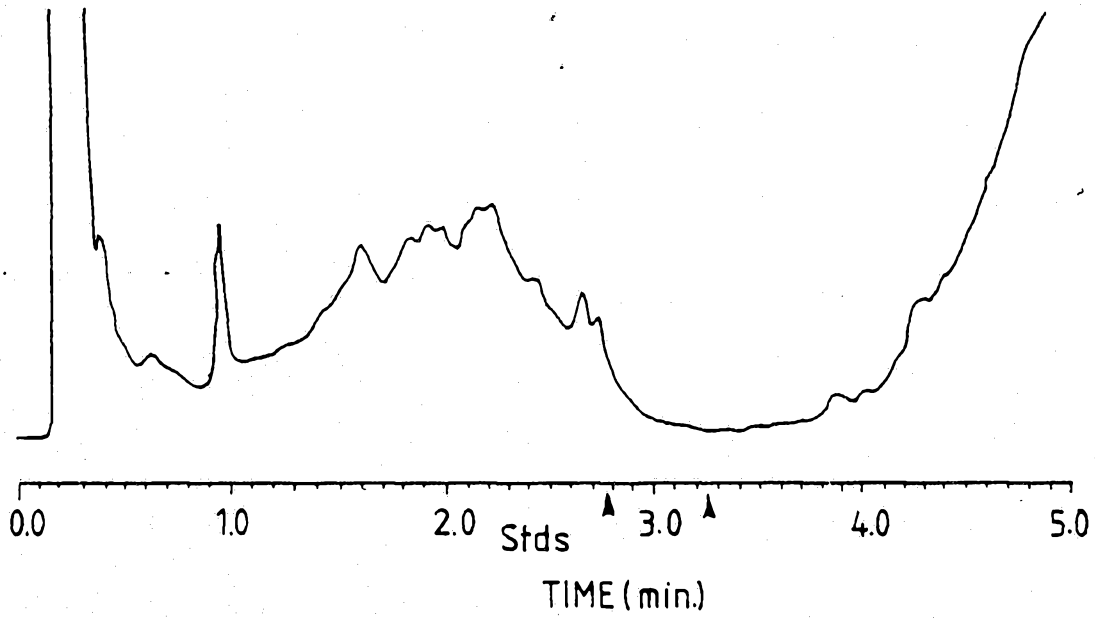


Fig. 4.

SUBSTITUTE SHEET

Fig.5.

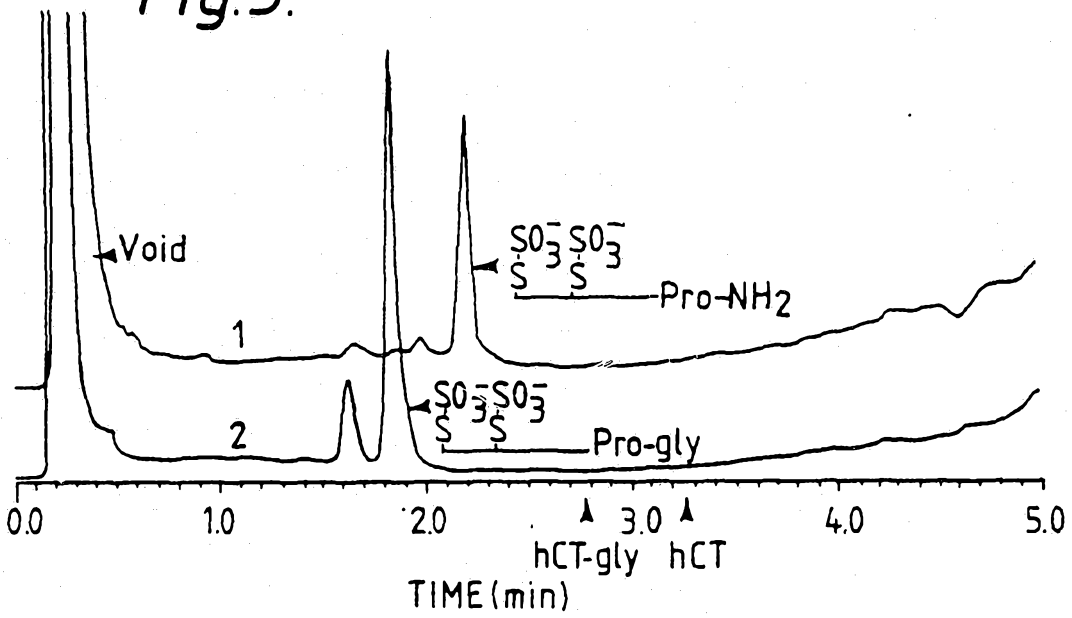

Void
1
2
Pro-NH2
Pro-gly
0.0
1.0
2.0
3.0
4.0
5.0
hCT-gly
hCT
TIME(min)

Fig.6.

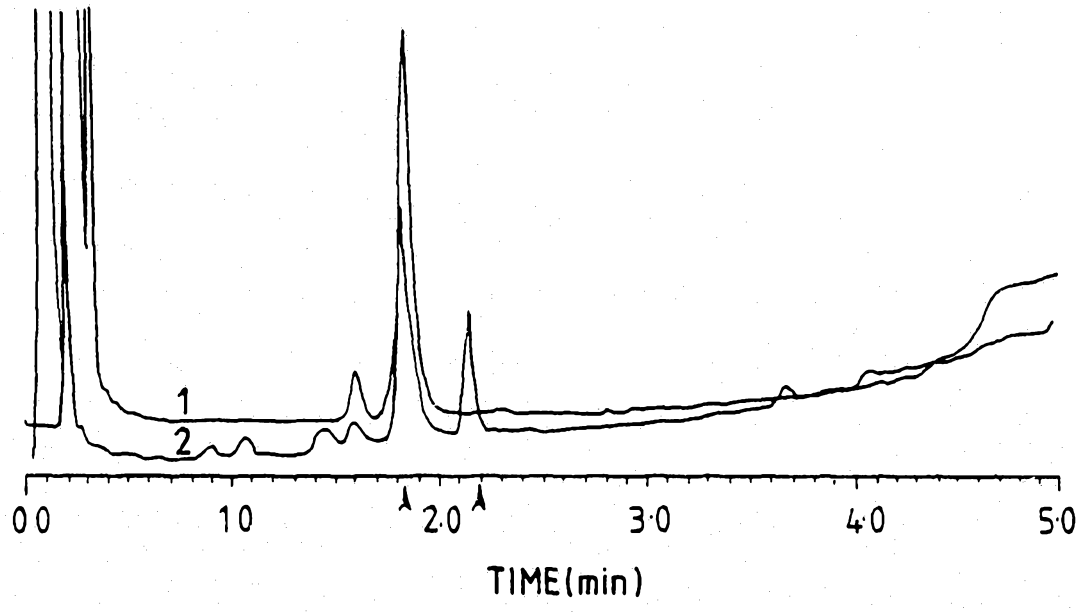
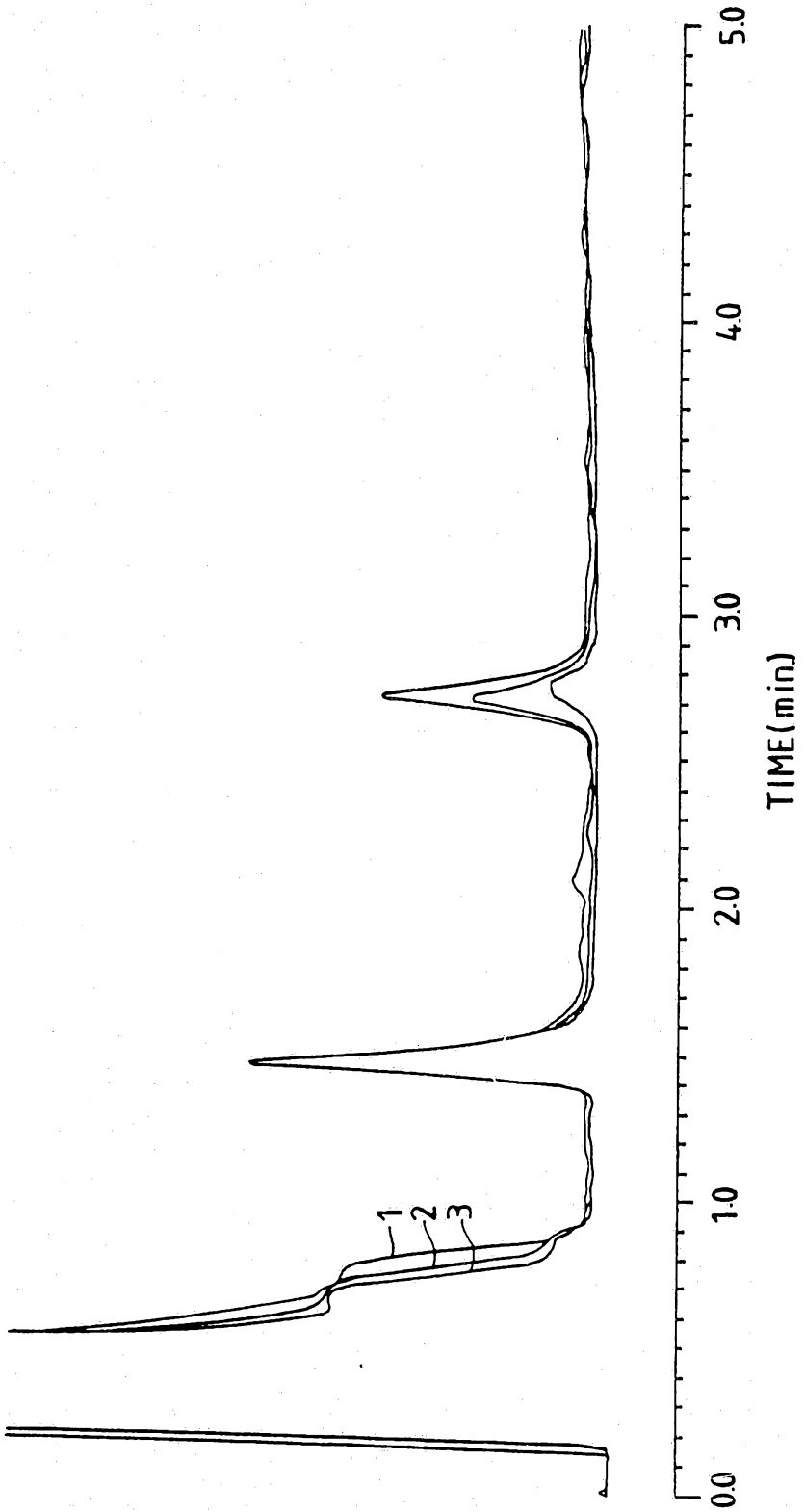

1
2
0 0
1 0
2·0
3·0
4·0
5·0
TIME(min)

Fig.7.



SUBSTITUTE

Fig.8.

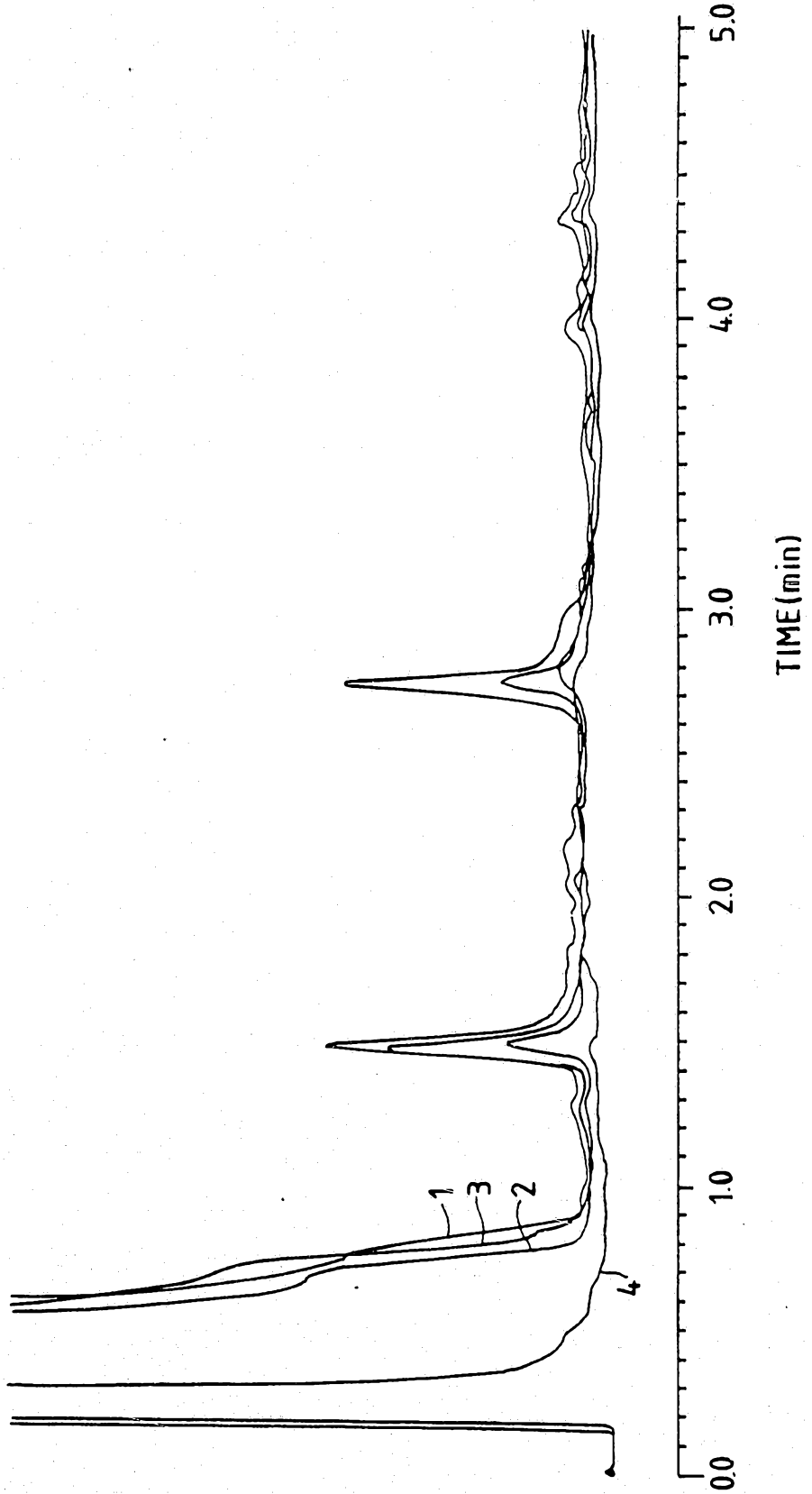


Fig.9.

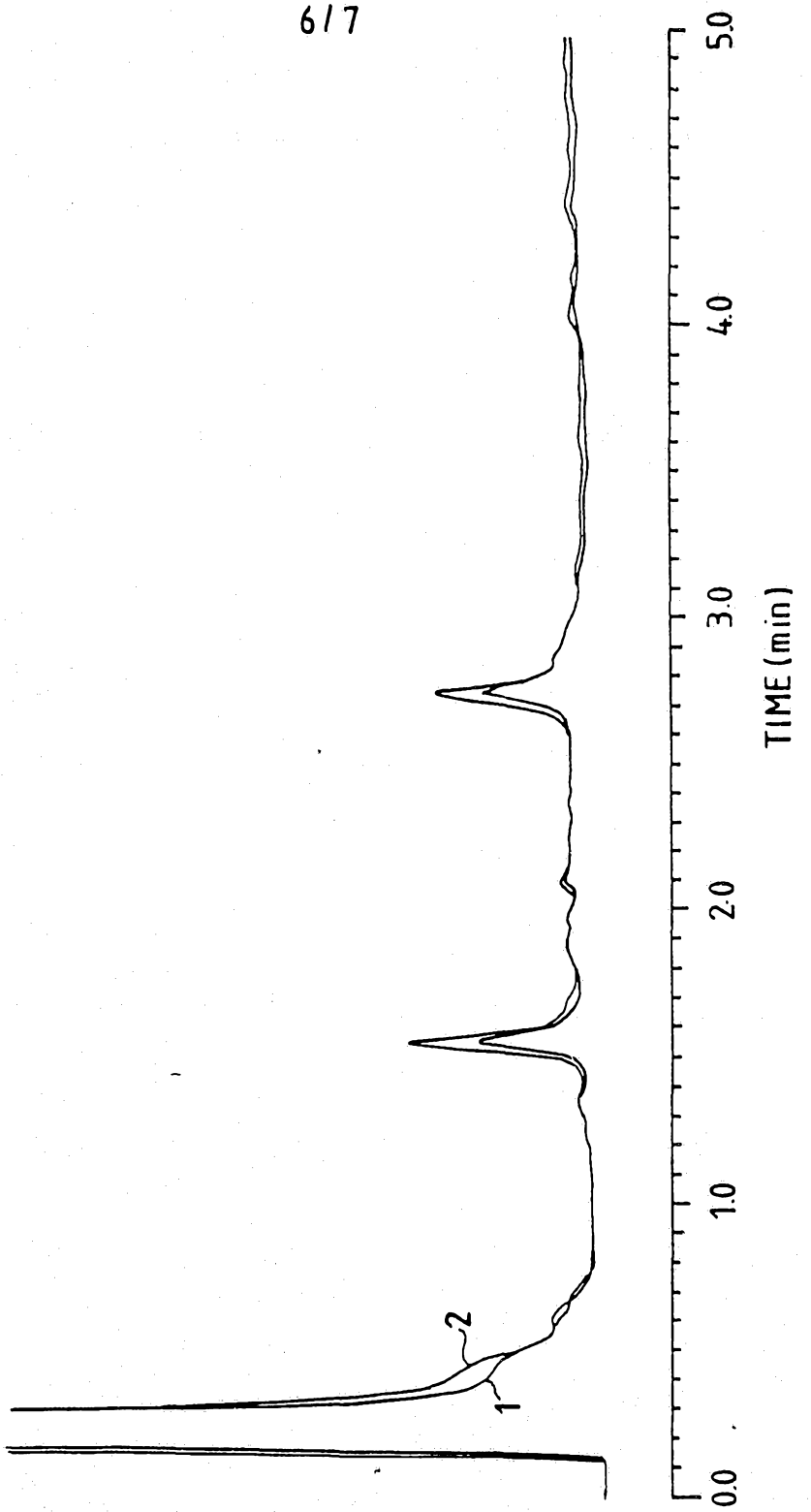
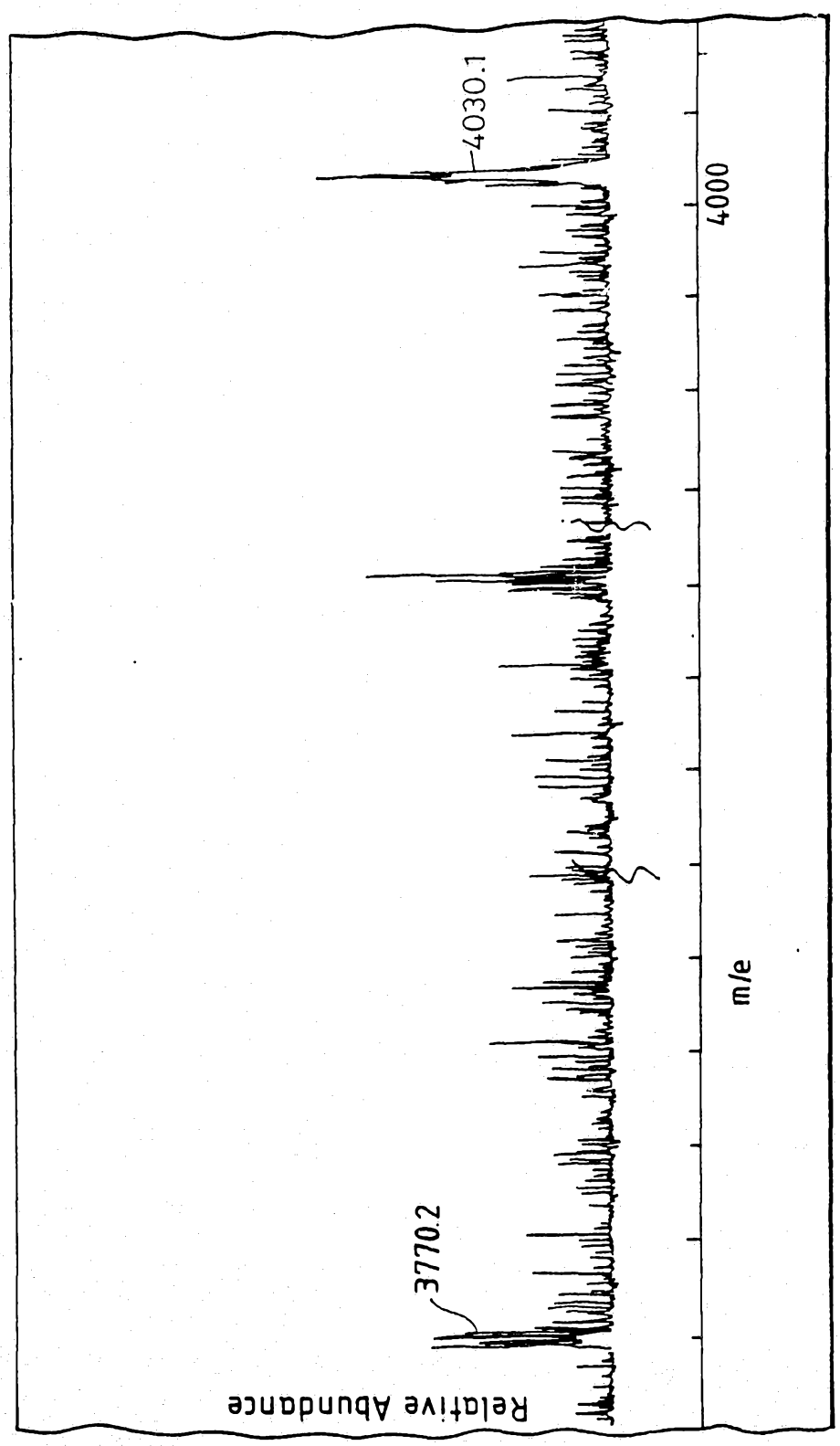


Fig.10.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 86/00559

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

IPC[4]: C 12 P 21/02; C 07 K 1/06; C 07 K 7/36

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 P C 07 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 [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	EP, A, 0134631 (CRAIG AND EDBROOKE) 20 March 1985 see page 4, lines 12-19 (cited in the application)	1-5
A	EP, A, 0133282 (HOECHST AG) 20 February 1985 see page 5, lines 10-15 (cited in the application)	1-4
A	Nature, volume 298, 12 August 1982, A.F. Bradbury et al.: "Mechanism of C-terminal amide formation by pituitary enzymes", pages 686-688 see the whole article (cited in the application)	1-4
A	FEBS Letters, volume 152, no. 2, February 1983, I. Husain et al.: "Formation of the COOH-terminal amide group of thyrotropin-releasing factor" pages 277-281 see the whole article	1-4

* Special categories of cited documents: [14]

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

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"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
2 January 1987	29 JAN 1987
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 86/00559 (SA 14582)

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 12/01/87

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0134631	20/03/85	GB-A-	2141430	19/12/84
		WO-A-	8500043	03/01/85
		AU-A-	3069584	11/01/85
		JP-T-	60501562	19/09/85
EP-A- 0133282	20/02/85	DE-A-	3327007	07/02/85
		AU-A-	3121784	31/01/85
		JP-A-	60049798	19/03/85