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Patents Act 1990

NOTICE OF ENTITLEMENT

We, CHUGAI SEIYAKU KABUSHIKI KAISHA of 5-1, Ukima 5-chome, Kita-ku, Tokyo 115, JAPAN state the following in connection with Australian Patent Application No. 75494/91:

1. We are the nominated person.

2. The nominated person is entitled to the grant of a patent as assignee of the inventors as to an undivided half share in the invention and as the assignee of Asahi Glass Co. Ltd. as to the other undivided half share in the invention, Asahi Glass Co. Ltd. being an assignee of the inventors as to said other undivided half share.

3. The nominated person is entitled to rely on the applications listed in the declaration under Article 8 of the PCT as a co-applicant of the basic convention application having consent of the other co-applicant Asahi Glass Co. Ltd..

4. The basic applications are the applications first made in a Convention country in respect of the invention.

DATED: 9 December, 1993

By PHILLIPS ORMONDE & FITZPATRICK
Patent Attorneys for the Applicant
By:

(signature) David B Fitzpatrick
....

To: The Commissioner of Patents

Our Ref: IRN 307030

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(54) Title
HYBRID CALCITONIN

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(57)

It is therefore concluded that the hybrid calcitonin of the present invention can potentially be used as an effective drug for patients who produce anti-salmon calcitonin antibodies.

CLAIM

1.   A hybrid calcitonin which is characterized in that the peptide segment on the side closer to the amino terminal end includes at least 10 amino acid residues on the corresponding side in human or human analog calcitonin and the peptide segment on the side closer to the carboxy terminal end includes at least 6 amino acid residues on the corresponding side in calcitonin derived from an animal other than humans.

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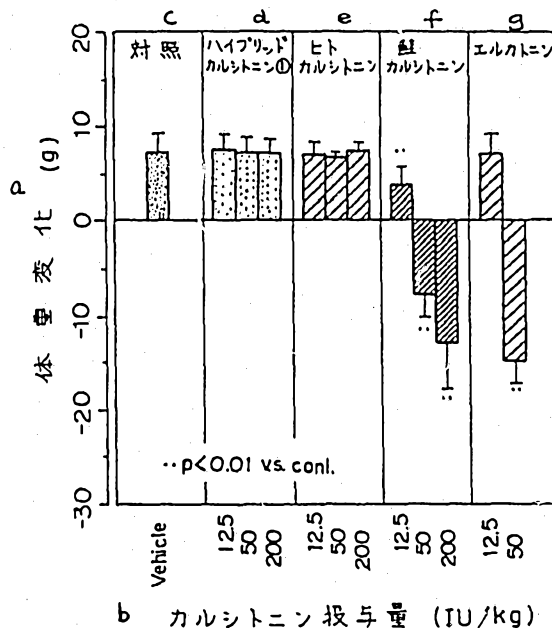
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(54) Title : HYBRID CALCITONIN

(54) 発明の名称　ハイブリッドカルシトニン

a ... change in body weight

b ... calcitonin dose

c ... control

d ... hybrid calcitonin 1

e ... human calcitonin

f ... salmon calcitonin

g ... elcatonin

(57) Abstract

A hybrid calcitonin composed of a peptide block of a human calcitonin and that of a calcitonin of an animal other than human being, such as eel, salmon or chicken. The human and non-human calcitonins may be any of the naturally occurring ones and their analogs. The hybrid calcitonin has excellent features such that not only it has a potent biological activity comparable to that of eel, salmon or chicken calcitonin but also it does not cause vomit and dysfunction of the digestive tract and is free from antigenicity.

(57) 要約

　　ヒト系カルシトニンのペプチドブロックと、ヒト以外の動物系のカルシトニン、例えば、鰻系、鮭系、あるいは鶏系のカルシトニン、のペプチドブロックとからなるハイブリッドカルシトニンが開示される。ヒト系カルシトニン及びヒト以外の動物系のカルシトニンはそれぞれ、その天然型カルシトニンあるいはそのカルシトニン類似体のいずれをも用いることができる。このようなハイブリッドカルシトニンは、鰻、鮭、鶏カルシトニンと同等の強い生物活性を有しているばかりでなく、嘔吐、消化管機能障害などを引き起こさず、しかも抗原性がないという優れた特徴を有する。
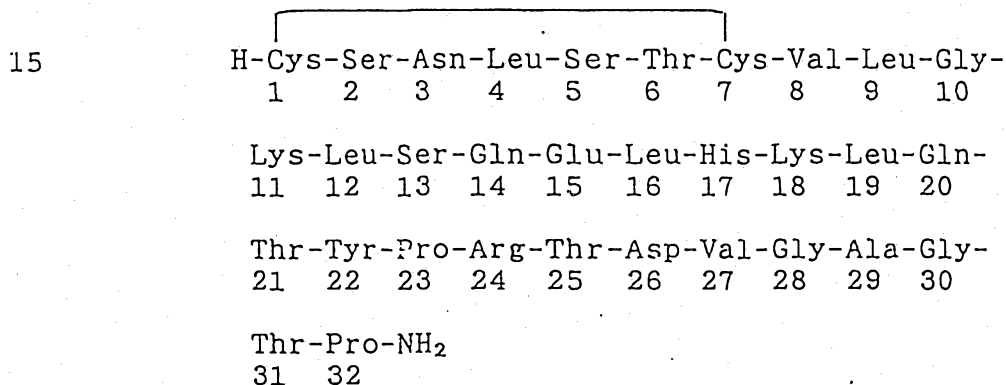
HYBRID CALCITONIN

BACKGROUND OF THE INVENTION

This invention relates to novel calcitonin analogs having biological activities.

5   Calcitonin is known to occur naturally in eel, salmon, chicken, porcine, human, bovine, sheep and rat. Whichever the origin, naturally occurring calcitonin is composed of 32 amino acids. For example, human calcitonin has the following peptide structure:

10

H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-
   1   2   3   4   5   6   7   8   9   10

Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-
11   12   13   14   15   16   17   18   19   20

Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-
21   22   23   24   25   26   27   28   29   30

Ala-Pro-NH_2
31   32

Eel calcitonin has the following peptide structure:

15

H-Cys-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-
   1   2   3   4   5   6   7   8   9   10

Lys-Leu-Ser-Gln-Glu-Leu-His-Lys-Leu-Gln-
11   12   13   14   15   16   17   18   19   20

Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-
21   22   23   24   25   26   27   28   29   30

Thr-Pro-NH_2
31   32
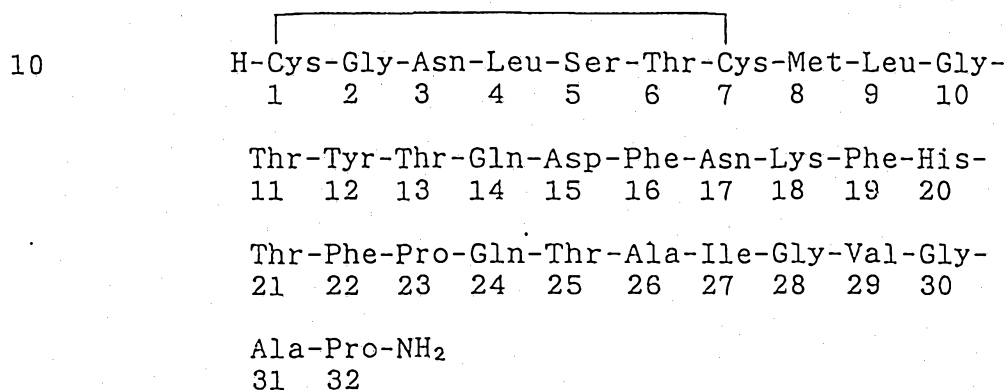
The calcitonin inherently present in humans and
20   animals is hereinafter collectively referred to as "native calcitonin".

Japanese Patent Public Disclosure Nos. 277698/1988, 284198/1988, 287800/1988, J. Biochem. Vol. 159, p. 125 (1986), Endocrinology, Vol. 117, p. 80 (1987) and J.
25   Biochem., Vol. 162, p. 399 (1987) disclose analogs to native calcitonin. Analogs to native calcitonin include: analogs having at least one of the amino acid residues in

native calcitonin replaced by another amino acid residue (substitution type); analogs having at least one of the amino acid residues in native calcitonin deleted (deletion type); analogs having at least one amino acid residue added between amino acid residues in native calcitonin or at a terminal thereof (addition type); and analogs having two or more of the substitution, deletion and addition types combined together. When two or more amino acid residues are replaced, deleted or added, they may be continuous or spaced apart in the peptide structure. These types of non-native calcitonin are hereinafter referred to as "calcitonin analogs".

Native human calcitonin is inherently present in humans but its biological activities in humans has been shown to be low. On the other hand, native calcitonins derived from animals other than humans such as salmon, eel and chicken have high biological activities for humans and hold promise for use as therapeutics for osteroporosis, neoplastic hypercalcemia and Paget's disease. Furthermore, eel calcitonin analogs have been commercially produced as therapeutics. However, native calcitonin derived from animals other than humans and their analogs cause side effects in humans such as severe nausea, disorders in the functions of the digestive tract, and antigenicity. Human calcitonin has little, if any, of these side effects.

The nausea caused by native calcitonin derived from animals other than humans and analogs thereof is described in the following references:

1) K. Takahashi, et al., "Gan to Kagaku Ryoho (Cancer and Chemotherapy)", Vol. 12, No. 10, 2004-2010 (1985);

2) J. Egawa et al., "Gan no Rinsho (Clinical Aspects of Cancer)", Vol. 30, No. 3, 251-258 (1984); and

3) G.F. Mazzuoli et al., Calcif. Tissue Int., 38, 3-8 (1986).

The disorders in the functions of the digestive tract caused by native calcitonin derived from animals other than humans and analogs thereof are described in the following references:

1)   K. Jonderko, Gut (England), <u>30</u> 430-435 (1989);

2)   K. Jonderko et al., J. Clin. Gastroenterol., <u>12</u>, 22-28 (1990); and

3)   J. Hotz et al., Digestion, <u>20</u>, 180-189 (1980).

The antigenicity of native calcitonin derived from animals other than humans and analogs thereof is described in the following references:

1)   F.R. Singer et al., J. Clinical Invest., <u>51</u>, 2331-2338 (1972);

2)   J.G. Haddad et al., J. Clinical Invest., <u>51</u> 3133-3141 (1972); and

3)   A. Grauer et al., J. Bone and Mineral Res., <u>5</u>, 387-391 (1990).

For the major biological activities of calcitonin derived from humans and from animals other than humans, the amino acid sequence at the carboxylic acid terminal is important, as described in the following references:

1)   Rene Maier et al., FEBS Letters, <u>48</u>, 68 (1974);

2)   Rene Maier et al., Clinicial Endocrinology, <u>5</u>, 3275 (1976);

3)   R.M. Epand et al., Eur. J. Brochom., <u>159</u>, 125 (1986); and

4)   D.M. Findlay et al., Endocrinology, <u>117</u>, 399 (1987).

SUMMARY OF THE INVENTION

The present invention provides a hybrid calcitonin which is characterized in that the peptide segment on the side closer to the amino terminal end includes at least 10 amino acid residues on the corresponding side in human or human analog calcitonin and the peptide segment on the side closer to the carboxy terminal end includes at least 6 amino acid residues on the corresponding side in calcitonin derived from an animal other than humans.

The present invention further provides a hybrid calcitonin consisting of a peptide segment in human calcitonin and a peptide segment in calcitonin derived from an animal other than humans, the first peptide segment including the first to the n-th (n=10-28) amino

- 3 -

acid residues from the amino terminal end and the second peptide segment including the subsequent amino acid residues toward the carboxy terminal end, with the proviso that the calcitonin derived from an animal other than humans which is salmon or salmon analog calcitonin is excluded.

The present invention further provides a hybrid calcitonin consisting of a peptide segment in human calcitonin and a peptide segment in calcitonin derived from an animal other than humans, the first peptide segment comprising the first to the n-th (n=13-21) amino acid residues from the amino terminal end and the second peptide segment comprising the subsequent amino acid residues toward the carboxy terminal end, and further the first peptide segment being either a peptide segment in native human calcitonin or a peptide segment in a human calcitonin analog having no more than two amino acid residues substituted for, deleted from or added to the peptide segment in native human calcitonin.

The present invention further provides a hybrid calcitonin in which the sequence of the first to the 16th amino acid residues from the amino terminal end is the same as that of the first to the 16th amino acid residues in native human calcitonin, and the sequence of the 17th to the 32nd amino acid residues is the same as the 17th to the 32nd amino acid residues in native eel or salmon calcitonin.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing the time-dependent profile of calcium concentration in blood plasma during treatment with various types of calcitonin;

Fig. 2 is a graph showing the time-dependent profile of stimulation threshold values following administration of various types of calcitonin;

Fig. 3 is a graph showing the changes in the body weight of rats administered various types of calcitonin;

Fig. 4 is a graph showig the food uptakes of the rats administered various types of calcitonin;

- 4 -

Fig. 5 is a graph showing the relationship betweeen the concentration of salmon calcitonin and antibody binding; and

Figs. 6-9 are graphs showing the efficiency of the binding of salmon calcitonon and two hybrid calcitonins to anti-salmon calcitonin human sera.

DETAILED DESCRIPTION OF THE INVENTION

The term "human calcitonin" as used herein means both native human calcitonin and not-native human calcitonin analogs. In a similar way, the term "non-human calcitonin

or calcitonin derived from animals other than humans" means
both native calcitonin derived from animals other than
humans and non-native analogs of that non-human calcitonin.
Animals other than humans preferably include, but are

5   not limited to, salmon, eel and chicken. The number of
substituted, deleted or added amino acid residues in the
two types of calcitonin analogs is not limited to any
particular value but preferably it is no more than 5. If
a peptide segment of interest contains no more than 5 amino

10   acid residues, the number of substituted, deleted or added
amino acid residues is preferably no more than one, and if
that peptide segment contains no more than 10 amino acid
residues, the corresponding number of substituted, deleted
or added amino acid residues is preferably no more than 3.

15   In the case of the peptide segment of human cal-
citonin, the sequence of the first 10 amino acid residues
from the amino terminal end is preferably the same as that
of native human calcitonin, or it may be that the peptide
segment of a human calcitonin analog has the same amino

20   acid sequence as native human calcitonin except that the
methionine residue in position 8 is replaced by a valine
residue. As for the overall peptide segment of human cal-
citonin, a preferred analog is such that no more than two
amino acid residues are substituted for, deleted from, or

25   added to native calcitonin. The most preferred analog is
such that the peptide segment taken as a whole differs from
the native type only in the substitution at the methionine
residue in position 8 .
          In the case of the peptide segment of calcitonin

30   derived from animals other than humans, it is preferably
the same as the peptide segment of corresponding native
calcitonin, or it may be the peptide segment of a non-
human calcitonin analog that has no more than 3 amino acid
residues substituted for, deleted from or added to the

35   native type. A more preferred analog is such that the
peptide segment, taken as a whole, has a sequence in which
only one amino acid residue is substituted or deleted from
the native type.

Preferred animals other than humans are such that
the derived calcitonin has higher biological activities
for humans. Examples of such animals include fishes, such
as salmon and eel, and birds, such as chicken. Salmon and

5   eel are more preferred, with salmon being particularly
preferred.

In consideration of these facts, the hybrid
calcitonin of the present invention preferably has the
following amino acid sequence:

10   H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met(or Val)-Leu-Gly-
     1   2   3   4   5   6   7   8   9 10

Thr-Tyr-Thr-Gln-Asp(or Glu)-Phe(or Leu)-Asn(or His)-
11 12 13 14 15   16   17

Lys-Phe(or Leu or Des)-His(or Gln)-Thr-Tyr-Pro-Arg-
18 19   20   21 22 23 24

Thr-Asn(or Asp)-Thr(or Val)-Gly-Ser(or Ala)-Gly-Thr-
25 26   27   28 29   30   31 32

Pro-NH$_2$

15   In the hybrid calcitonin of the present invention,
the part of the structure of whole calcitonin derived from
animals other than humans such as salmon, eel and chicken
which causes side effects when administered to humans (e.g.
nausea, disorders in the functions of the digestive tract

20   and antigenicity) is replaced by the corresponding segment
of human calcitonin causing no such side effects, whereby
the side effects caused by the former part are reduced or
entirely eliminated. This is equivalent to saying that
the hybrid calcitonin of the present invention has the

25   pharmacologically active site of human calcitonin replaced
by the corresponding segment of non-human calcitonin in
order to enhance the desired physiological activities of
human calcitonin.

The hybrid calcitonin of the present invention can be

30   synthesized by solid-phase synthesis, using polymer resins,
or by liquid-phase synthesis, which is commonly employed
in the art of organic synthesis. The use of solid-phase

synthesis is preferred in the case of synthesizing a com-
paratively small amount of peptides. On the other hand, the
use of liquid-phase synthesis is preferred in the case of
synthesizing a large amount of peptides. In the examples

5   to be described later in this specifica-tion, peptides
were synthesized by solid-phase synthesis. However, if
the hybrid calcitonin of the present invention is to be
synthesized in a large quantity, the use of liquid-phase
synthesis, which is a well-known technique of peptide

10   sunthesis, is preferred.

       The usual procedure of liquid-phase synthesis starts
with synthesizing at least two partial peptides, each
composed of two or more amino acid residues, it then links
these partial peptides either successively and finally

15   obtains the desired peptide having the above-specified amino
acid sequence (in the case of the present invention, the
desired peptide is the hybrid calcitonin already described
above). The liquid-phase synthesis is further characterized
by performing the reaction of peptide synthesis in a liquid

20   medium, particularly in a medium such as dimethylformamide
or tetrahydrofuran. A partial peptide is typically composed
of 2 - 20, preferably 3 - 15, amino acid residues. In a
preferred case, about 2 - 10 units of these partial peptides
are synthesized and then linked successively to synthesize

25   the desired peptide.

       In the peptide synthesis described above, reactive
derivatives of amino acids are typically used as starting
materials. If the starting amino acid contains active
functional groups other than those which form peptide bonds,

30   those active functional groups are preferably protected
with protective groups, which are removed after the end
of peptide synthesis. The peptide to be produced in the
present invention has a 1-7 disulfide bond, which is formed
at any desired stage following the synthesis of a partial

35   peptide having a 1-7 sequence. However, this disulfide
bond has low stability and is preferably formed after
the end of peptide synthesis. These conditions for the
procedures of protection and deprotection and the formation

of a disulfide bond are also preferably adopted in the solid-phase synthesis to be described below.

Solid-phase synthesis is a method of synthesizing a peptide having the desired amino acid sequence by bonding amino acid residues successively onto a carrier resin. This method can be performed automatically with an automatic synthesizer.

Resins that can be used in solid-phase synthesis include chloromethyl resins, oxymethyl resins, 4-(oxymethyl)phenylacetamidomethyl resins, benzhydrylamine resins and polyacrylamide resins.

The amino acids used in these syntheses are protected amino acids, as required. Exemplary α-amino protecting groups include a carbobenzoxy group (Z), a tertiary butyl-oxycarbonyl group (Boc), a 9-fluorenylmethyloxycarbonyl group (Fmoc), a formyl group (HOC) and an acetyl group (Ac). Exemplary α-carboxy protecting groups include a benzyl group (Bzl), a tertiary butyl group (Bu^t), a methyl group (Me), an ethyl group (Et) and a phenacyl group (Pac). Groups that can be used to protect functional groups in side chains of amino acids include: a benzyl group (Bzl), a p-toluenesulfonyl group (Tos), a p-nitrophenol group (NO$_2$), a benzhydryl group (Bzh), an acetamidomethyl group (Acm), a tertiary butyl group (Bu^t), a tertiary butyloxycarbonyl group (Boc), a cyclohexyl group (CHex) and a 4-methoxy-2,3,6-trimethylbenzenesulfonyl group (Mtr). One or more of these protecting groups may be used depending on the object to be attained.

The sequential addition reaction of amino acids can be performed by dehydrative condensation using carbodiimides or with the aid of active esters. Useful carbodiimides include dicyclohexylcarbodiimide and 1-ethyl 3-(3-dimethyl-aminopropyl)carbodiimide, and useful active esters include an N-hydroxysuccinimide ester (-OSu), a pentafluorophenol ester (-OPfp) and a dihydroxobenztriazine ester (-ODhbt).

Reaction catalysts that can be used include: DMF, THF, dichloromethane, chloroform, ethyl acetate, dioxane, DMSO, N-methylpyrrolidone, pyridine and water.

Various deprotecting agents may be used depending on the type of protective groups to be eliminated and on the object to be achieved, and useful examples include: hydrogen fluoride, trifluoroacetic acid, trifluoromethanesulfonic

5 acid, ammonia/methanol, hydrogen bromide/acetic acid, hydrogen/palladium on carbon, mercury acetate, acetic acid/zinc powder, and alkali/water-methanol.
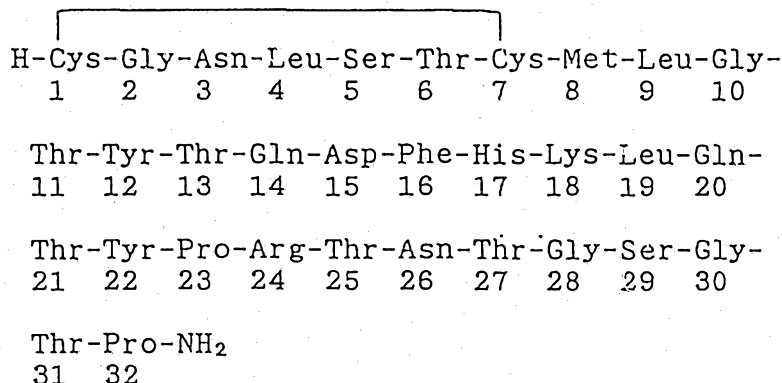
During or after synthesis, various techniques of purification can be employed, such as reverse-phase

10 chromatography, normal-phase chromatography, ion-exchange chromatography and gel filtration chromatography, and recrystallization. Disulfide bonds may be formed by oxidation with atmospheric air or potassium ferricyanide.

The following examples are provided for the purpose

15 of further illustrating the present invention, but are in no way to be taken as limiting.

Examples 1 - 6 illustrate the synthesis of samples of the hybrid calcitonin of the present invention, and Example 7 shows the results of analysis of amino acid composition

20 for the calcitonin samples synthesized in Examples 1. - 6. Examples 8 - 10 describe the experiments conducted to evaluate the principal activities of the hybrid calcitonin of the present invention. Examples 11 - 14 describe the experiments conducted to demonstrate elimination of the

25 side effects of calcitonin.

Example 1

Synthesis of (1-16) human/(17-32) salmon hybrid calcitonin:

```
    ┌─────────────────────────────┐
    H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-
      1   2   3   4   5   6   7   8   9   10

    Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Leu-Gln-
     11  12  13  14  15  16  17  18  19  20

    Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-
     21  22  23  24  25  26  27  28  29  30

    Thr-Pro-NH₂
     31  32
```

Synthesis was performed by a solid-phase synthesis method using an automatic synthesizer.

(1) Introducing proline onto benzhydrylamine (BHA) resin

Ten grams of BHA resin ($-NH_2$: 0.38 mmol/g) were suspended and swollen in 100 ml of DMF. After removing the supernatant, an additional 100 ml of DMF was added, followed by addition of BocProOH (15 mmol), DCC (20 mmol) and HOBT (20 mmol). The mixture was shaken overnight at room temperature. After filtration with a glass filter, the resulting resin was washed with methylene chloride.

Subsequently, 200 ml of a methylene chloride solution containing 10 v/v% acetic anhydride was added, and the mixture was shaken at room temperature for 1 h to block the residual amino groups. After completion of the reaction, the resin was washed with methylene chloride and dried under vacuum.

After drying, 50 ml of a methylene chloride solution containing 50 v/v% trifluoroacetic acid was added and the mixture was shaken at room temperature for 1 h. After filtration with a glass filter, the resulting resin was washed successively with methylene chloride, methanol, methylene chloride and triethylamine and dried under vacuum.

(2) Sequential addition reaction of amino acids

One gram of the resin obtained in (1) was packed in the column of an automatic synthesizer, and amino acids were added sequentially under the following conditions.

(i) reaction: 30 min at R.T. in solvent DMF

(ii) washing: 10 min at R.T. with DMF

(iii) Fmoc removal: 10 min at R.T. with 20 v/v% piperidine
in solvent DMF

5 (iv) washing: 10 min at R.T. with DMF

By repeating the steps (1) - (4), 31 amino acids following Pro were added. The amino acids used are listed in Table 1 below.

Table 1

Fmoc-L-Thr(But)-ODhbt	0.430 g
Fmoc-Gly-OPfp	0.367
Fmoc-L-Ser(But)-ODhbt ·	0.419
Fmoc-Gly-OPfp	0.367
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Asn-OPfp	0.412
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Arg(Mtr)-OPfp	0.641
Fmoc-L-Pro-OPfp	0.400
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Lys(Boc)-OPfp	0.503
Fmoc-L-His(Boc)-OPfp	0.510
Fmoc-L-Phe-OPfp	0.438
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-Gly-OPfp	0.367
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Met-Opfp	0.426
Fmoc-L-Cys(Trt)-OPfp	0.595
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Asn-OPfp	0.412
Fmoc-Gly-OPfp	0.367
Fmoc-L-Cys(Trt)-OPfp	0.595

After the reaction, the protective groups and the resin were removed from the peptide with hydrogen fluoride, and the peptide was washed with ether. The precipitate was dissolved in an aqueous solution of 50 v/v% acetic acid, and

5 the insoluble matter was filtered off. The filtrate was freeze-dried to obtain ca. 600 mg of crude peptide.

(3) Purification and the formation of disulfide bonds

A portion (ca. 500 mg) of the crude peptide was dissolved in water containing 0.1 v/v% TFA and the solution

10 was subjected to high-performance liquid chromatography on an ODS column.

Using solution A (acetonitrile containing 0.1 v/v% TFA) and solution B (water containing 0.1 v/v% TFA), the column was eluted with the stepwise gradient (A/B in v/v%)

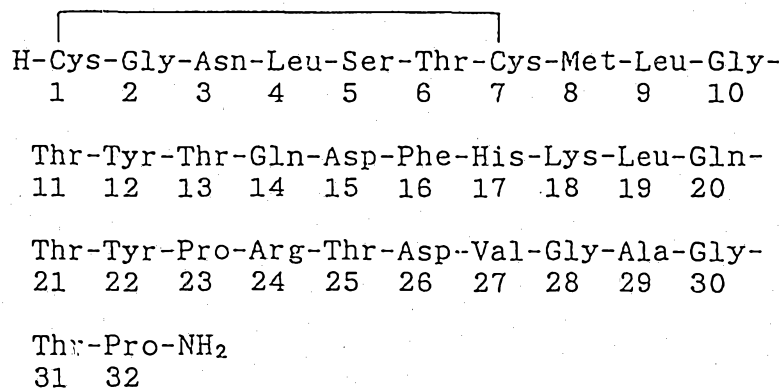15 increasing from 20 through 30 to 40%, and fractions at 30% elution were collected and freeze-dried.

The freeze-dried peptide (46 mg) was dissolved in 50 ml of 0.05 v/v% acetic acid and the pH of the solution was adjusted to 8.5 with 3 M aqueous ammonia. Subsequently, 1.5

20 ml of 0.1 M $K_3Fe(CN)_6$ was added and the mixture was stirred at room temperature for 30 min to form disulfide bonds. After adjusting the pH to 5.0 with 50% acetic acid, an anion exchange resin (Cl⁻ form) was added and the mixture was stirred for 20 min, followed by filtering off of the resin.

25 The filtrate was concentrated and subjected to high-performance liquid chromatography on an ODS column and purified again by the same procedure as described above. Fractions at 30% elution were collected and freeze-dried to obtain 28 mg of the desired peptide.

30 Example 2

Synthesis of (1-16) human/(17-32) eel hybrid calcitonin:

```
        ┌─────────────────────────────────┐
    H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-
       1    2    3    4    5    6    7    8    9   10

    Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Leu-Gln-
     11   12   13   14   15   16   17   18   19   20

    Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-
     21   22   23   24   25   26   27   28   29   30

    Thr-Pro-NH₂
     31   32
```

5   (1) Sequential addition reaction of amino acids

        One gram of the proline-incorporating BHA resin

prepared in step (1) of Example 1 was packed in the column

of an automatic synthesizer and amino acids were added

under the same reaction conditions as used in step (2) of

10   Example 1. The amino acids used are listed in Table 2

below.

Table 2

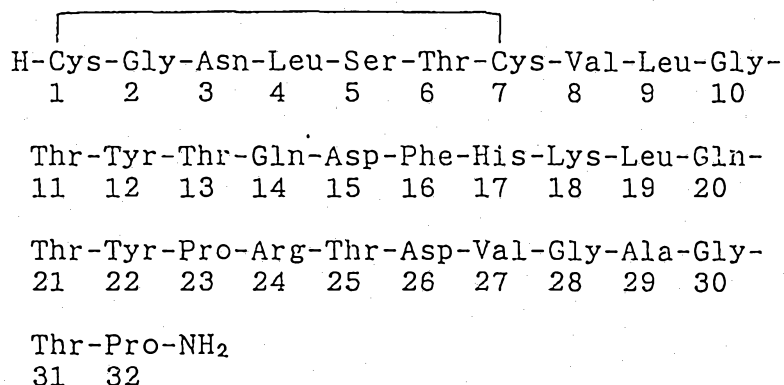|	
Fmoc-L-Thr(But)-ODhbt	0.430 g
Fmoc-Gly-OPfp	0.367
Fmoc-L-Ala-OPfp	0.378
Fmoc-Gly-OPfp	0.367
Fmoc-L-Val-OPfp	0.400
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Arg(Mtr)-OPfp	0.641
Fmoc-L-Pro-OPfp	0.400
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Lys(Boc)-OPfp	0.503
Fmoc-L-His(Boc)-OPfp	0.510
Fmoc-L-Phe-OPfp	0.438
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-Gly-OPfp	0.367
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Met-Opfp	0.426
Fmoc-L-Cys(Trt)-OPfp	0.595
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Asn-OPfp	0.412
Fmoc-Gly-OPfp	0.367
Fmoc-L-Cys(Trt)-OPfp	0.595

After the reaction, the peptide-resin was treated by the same method as described in step (2) of Example 1 to give ca. 510 mg of crude peptide.

(2) Purification and the formation of disulfide bonds

A portion (ca. 450 mg) of the crude peptide was subjected to purification and the formation of disulfide bonds by the same procedures as used in step (3) of Example 1, whereby 32 mg of purified peptide were obtained.

Example 3

Synthesis of (1-16, Met^8 → Val^8) human analog/(17-32) eel hybrid calcitonin:

```
      ┌───────────────────┐
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-
   1    2    3    4    5    6    7    8    9   10

  Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Leu-Gln-
  11   12   13   14   15   16   17   18   19   20

  Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-
  21   22   23   24   25   26   27   28   29   30

  Thr-Pro-NH₂
  31   32
```

(1) Sequential addition reaction of amino acids

One gram of the proline-incorporating BHA resin prepared in step (1) of Example 1 was packed in the column of an automatic synthesizer and amino acids were added under the same reaction conditions as used in step (2) of Example 1. The amino acids used are listed in Table 3 below.

Table 3

Fmoc-L-Thr(But)-ODhbt	0.430 g
Fmoc-Gly-OPfp	0.367
Fmoc-L-Ala-OPfp	0.378
Fmoc-Gly-OPfp	0.367
Fmoc-L-Val-OPfp	0.400
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Arg(Mtr)-OPfp	0.641
Fmoc-L-Pro-OPfp	0.400
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Lys(Boc)-OPfp	0.503
Fmoc-L-His(Boc)-OPfp	0.510
Fmoc-L-Phe-OPfp	0.438
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-Gly-OPfp	0.367
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Val-Opfp	0.400
Fmoc-L-Cys(Trt)-OPfp	0.595
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Asn-OPfp	0.412
Fmoc-Gly-OPfp	0.367
Fmoc-L-Cys(Trt)-OPfp	0.595

After the reaction, the peptide-resin was treated by the same method as described in step (2) of Example 1 to give ca. 580 mg of crude peptide.

(2) Purification and the formation of disulfide bonds

A portion (ca. 500 mg) of the crude peptide was subjected to purification and the formation of disulfide bonds by the same procedures as used in (3) of Example 1, whereby 35 mg of purified peptide were obtained.

Example 4

Synthesis of (1-16) human/(17-31, Des Leu[19]) eel analog hybrid calcitonin:

```
      ┌─────────────────────────┐
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-
   1    2    3    4    5    6    7    8    9   10

Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Gln-Thr-
 11   12   13   14   15   16   17   18   19   20

Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-Thr-
 21   22   23   24   25   26   27   28   29   30

Pro-NH₂
 31
```

(1) Sequential addition reaction of amino acids

One gram of the proline-incorporating BHA resin prepared in step (1) of Example 1 was packed in the column of an automatic synthesizer and amino acids were added under the same reaction conditions as used in step (2) of Example 1. The amino acids used are listed in Table 4 below.

Table 4

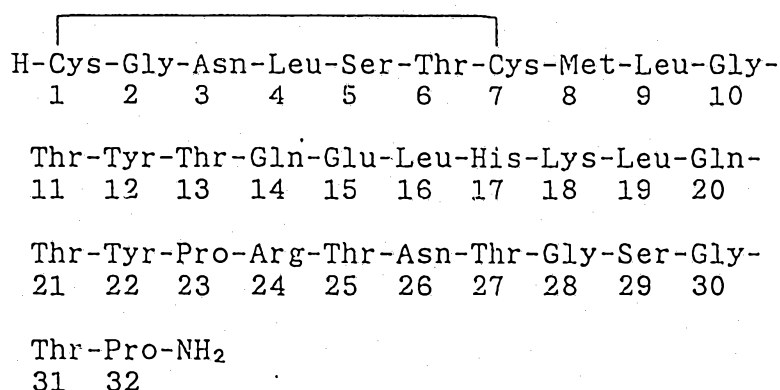|	
Fmoc-L-Thr(But)-ODhbt	0.430 g
Fmoc-Gly-OPfp	0.367
Fmoc-L-Ala-OPfp	0.378
Fmoc-Gly-OPfp	0.367
Fmoc-L-Val-OPfp	0.400
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Arg(Mtr)-OPfp	0.641
Fmoc-L-Pro-OPfp	0.400
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Lys(Boc)-OPfp	0.503
Fmoc-L-His(Boc)-OPfp	0.510
Fmoc-L-Phe-OPfp	0.438
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-Gly-OPfp	0.367
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Met-Opfp	0.426
Fmoc-L-Cys(Trt)-OPfp	0.595
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Asn-OPfp	0.412
Fmoc-Gly-OPfp	0.367
Fmoc-L-Cys(Trt)-OPfp	0.595

After the reaction, the peptide-resin was treated by the same method as described in step (2) of Example 1 to obtain ca. 520 mg of crude peptide.

(2) Purification and the formation of disulfide bonds

5   A portion (ca. 450 mg) of the crude peptide was subjected to purification and the formation of disulfide bonds by the same procedures as used in step (3) of Example 1, whereby 23 mg of purified peptide were obtained.

Example 5

10   Synthesis of (1-13) human/(14-32) salmon hybrid calcitonin:

```
       ┌─────────────────────┐
   H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-
      1   2   3   4   5   6   7   8   9   10

   Thr-Tyr-Thr-Gln-Glu-Leu-His-Lys-Leu-Gln-
    11  12  13  14  15  16  17  18  19  20

   Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-
    21  22  23  24  25  26  27  28  29  30
```

15
```
   Thr-Pro-NH₂
    31  32
```

Synthesis was performed by a solid-phase synthesis method using an automatic synthesizer.

(1) Introducing proline into benzhydrylamine (BHA) resin

Ten grams of BHA resin (-NH$_2$: 0.38 mmol/g) was 20 suspended and swollen in 100 ml of DMF. After removing the supernatant, an additional 100 ml of DMF were added, followed by addition of BocProOH (19 mmol), DCC (19 mmol) and HOBT (19 mmol). The mixture was shaken overnight at room temperature. After filtration with a glass filter, 25 the resulting resin was washed with methylene chloride. Subsequently, 200 ml of a methylene chloride solution containing 10 v/v% acetic anhydride was added and the mixture was stirred at room temperature for 1 h to block the residual amino groups. After completion of the 30 reaction, the mixture was washed with methylene chloride and dried under vacuum.

After drying, 50 ml of a methylene chloride solution containing 50 v/v% trifluoroacetic acid was added and the

mixture was stirred at room temperature for 1 h. After
filtration with a glass filter, the resulting resin was
washed successively with methylene chloride, methanol,
methylene chloride and triethylamine, and dried under
vacuum.

A portion of the resulting resin was analyzed with
an amino acid analyzer and it was found that 0.31 mmol of
proline had been incorporated per gram of the resin.

(2) Sequential addition reaction of amino acids

One gram of the resin obtained in step (1) was packed
in the column of an automatic synthesizer and amino acids
were added sequentially under the following conditions.

(i)   addition reaction: 30 min at R.T. in solvent DMF

(ii) washing: 10 min at R.T. with DMF

(iii) Fmoc removal: 10 min at R.T. with 20 v/v% piperidine
                     in solvent DMF

(iv) washing: 10 min at R.T. with DMF

By repeating the steps (1) - (4), 31 amino acids
following Pro were added. The amino acids used are listed
in Table 5 below.

Table 5

Fmoc-L-Thr(But)-ODhbt	0.430 g
Fmoc-Gly-OPfp	0.367
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-Gly-OPfp	0.367
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Asn-OPfp	0.412
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Arg(Mtr)-OPfp	0.641
Fmoc-L-Pro-OPfp	0.400
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Lys(Boc)-OPfp	0.503
Fmoc-L-His(Boc)-OPfp	0.510
Fmoc-L-Leu-Opfp	0.411
Fmoc-L-Glu(OBut)-OPfp	0.468
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-Gly-OPfp	0.367
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Met-Opfp	0.426
Fmoc-L-Cys(Trt)-OH	0.464
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Asn-OPfp	0.412
Fmoc-Gly-OPfp	0.367
Fmoc-L-Cys(Trt)-OH	0.464

-    After the reaction, the resin and the protective
groups were removed from the peptide with hydrogen fluoride
and the peptide was washed with ether. The precipitate was
dissolved in an aqueous solution of 50 v/v% acetic acid and
5   the insoluble matter was filtered off. The filtrate was
freeze-dried to obtain ca. 720 mg of crude peptide.
(3) Purification and the formation of disulfide bonds.

     A portion (ca. 700 mg) of the crude peptide was
dissolved in water containing 0.1 v/v% TFA and the solution
10   was subjected to high-performance liquid chromatography on
an ODS column.

     Using a solvent system consisting of water and
acetonitrile containing 0.1 % TFA, the column was eluted
with a stepwise gradient of acetonitrile increasing from
15   20 v/v% through 30 v/v% to 40 v/v%, and fractions at
30 v/v% elution were collected and freeze-dried (primary
purification).

     The freeze-dried peptide (ca. 80 mg) was dissolved
in 80 ml of 0.05 v/v% acetic acid and the pH of the solution
20   was adjusted to 8.5 with 3 M aqueous ammonia. Subsequently,
1.5 ml of 0.1 M $K_3Fe(CN)_6$ was added and the mixture was
stirred at room temperature for 30 min to form disulfide
bonds. After adjusting the pH to 5.0 with 50 v/v% acetic
acid, an anion exchange resin (Cl^- form) was added and the
25   mixture was stirred for 20 min, followed by filtering off
the resin.

     The filtrate was concentrated and subjected again
to high-performance liquid chromatography on an ODS column,
which was eluted with the same solvent system as used in
30   the primary purification at stepwise density gradients of
acetonitrile from 25, 27 through 30 to 35 v/v% (secondary
purification). Fractions at 30 v/v% elution were collected
and freeze-dried to obtain 53 mg of the titled peptide.
Example 6

35   Synthesis of (1-21) human/(22-32) salmon hybrid
calcitonin:

```
       ┌──────────────────────────┐
   H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-Gly-
      1    2    3    4    5    6    7    8    9   10
```

Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-
11 12 13 14 15 16 17 18 19 20

Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-Gly-
21 22 23 24 25 26 27 28 29 30

Thr-Pro-NH$_2$
31 32

One gram of the proline-incorporating BHA resin prepared in step (1) of Example 5 was packed in the column of an automatic synthesizer and amino acids were added under the same reaction conditions as used in step (2) of Example 5. The amino acids used are listed in Table 6 below.

Table 6

Fmoc-L-Thr(But)-ODhbt	0.430 g
Fmoc-Gly-OPfp	0.367
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-Gly-OPfp	0.367
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Asn-OPfp	0.412
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Arg(Mtr)-OPfp	0.641
Fmoc-L-Pro-OPfp	0.400
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-His(Boc)-OPfp	0.510
Fmoc-L-Phe-OPfp	0.438
Fmoc-L-Lys(Boc)-OPfp	0.503
Fmoc-L-Asn-OPfp	0.412
Fmoc-L-Phe-OPfp	0.438
Fmoc-L-Asp(OBut)-OPfp	0.457
Fmoc-L-Gln-OPfp	0.423
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Tyr(But)-OPfp	0.495
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-Gly-OPfp	0.367
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Met-Opfp	0.426
Fmoc-L-Cys(Trt)-OPfp	0.595
Fmoc-L-Thr(But)-ODhbt	0.430
Fmoc-L-Ser(But)-ODhbt	0.419
Fmoc-L-Leu-OPfp	0.411
Fmoc-L-Asn-OPfp	0.412
Fmoc-Gly-OPfp	0.367
Fmoc-L-Cys(Trt)-OPfp	0.595

- After the reaction, the peptide-resin was treated by the same method as used in step (2) of Example 5 to obtain ca. 860 mg of crude peptide.

(2) Purification and the formation of disulfide bonds

5   A portion (ca. 850 mg) of the crude peptide was subjected to purification and the formation of disulfide bonds by the same procedures as used in step (3) of Example 5.

The resulting filtrate was concentrated and subjected

10   again to high-performance liquid chromatography on an ODS column, which was eluted with the same solvent system as used in the primary purification at stepwise density gradients of acetonitrile from 24 to 35 v/v% through 26, 28 and 30 v/v% (secondary purification). Fractions at 28 v/v%

15   elution were collected and freeze-dried to obtain 66 mg of the desired peptide.

Example 7 Analysis of amino acid composition

Each of purified hybrid calcitonin prepared in Examples 1 - 6 was hydrolyzed at 150 °C for 1 h in the

20   presence of 6 N HCl, and the amino acid composition was analyzed with an amino acid analyzer. The results are shown in Table 7.

Table 7

Amino acid	Example 1	Example 2	Example 3	Example 4	Example 5	Example 6
Asx	3.14(3)	3.12(3)	3.12(3)	3.17(3)	2.11(2)	4.50(4)
Thr	6.43(7)	5.32(6)	5.44(6)	5.65(6)	6.37(7)	6.86(7)
Ser	1.79(2)	0.85(1)	0.82(1)	0.84(1)	1.71(2)	1.64(2)
Glx	2.34(2)	2.26(2)	2.35(2)	2.28(2)	3.49(3)	1.00(1)
Gly	4.14(4)	4.20(4)	4.14(4)	4.08(4)	4.01(4)	4.57(4)
Ala	0 (0)	1.01(1)	1.02(1)	0.96(1)	0 (0)	0 (0)
Val	0 (0)	1.07(1)	2.01(2)	1.12(1)	0 (0)	0 (0)
Met	0.97(1)	0.98(1)	0 (0)	0.90(1)	1.01(1)	0.93(1)
Leu	3.15(3)	3.11(3)	3.10(3)	2.03(2)	4.28(4)	2.00(2)
Tyr	2.02(2)	2.06(2)	2.02(2)	2.06(2)	2.12(2)	2.01(2)
Phe	1.04(1)	1.06(1)	1.04(1)	1.07(1)	0 (0)	2.07(2)
His	1.02(1)	1.03(1)	1.12(1)	1.08(1)	1.05(1)	0.80(1)
Lys	1.03(1)	1.04(1)	1.02(1)	1.06(1)	1.05(1)	0.92(1)
Arg	1.01(1)	1.00(1)	0.98(1)	0.99(1)	0.99(1)	0.85(1)
Pro	1.97(2)	1.92(2)	1.95(2)	1.69(2)	1.84(2)	2.06(2)

Note: The theoretical values are parenthesized.

Example 8   Assaying biological activities

The six novel calcitonins prepared in Examples 1 - 6
were dissolved in a 0.1 M sodium acetate buffer solution
(pH 4.2) containing 0.1% BSA (bovine serum albumin) and :

5   injected into pre-fasted (24 h) SD male rats (4-wk old)
through the tail vein. One hour later, the concentration
of serum calcium was measured by the OCPC method (Calcium C
- Test Wako of Wako Pure Chemical Industries, Ltd.)
The calcitonin activity that caused a 10% drop in the

10   concentration of serum calcium was determined to be 10 mU,
and the number of units per milligram of calcitonin was
designated "specific activity". The results are shown in
Table 8.

Table 8

Novel calcitonin	Specific activity (U/mg)
Example 1	2106*
2	2639
3	2262
4	2611
5	2575
6	1792

*   Average for two measurements

Example 9   Efficacy of calcitonins against hypercalcemia
Method:

25   Vitamin D_3 (5 mg/kg) was administered orally to
5-wk old SD male rats for 4 consecutive days to construct
experimental models of hypercalcemic rats. On day 5, (1-16)
human/(17-32) salmon hybrid calcitonin (hybrid calcitonin
1), salmon calcitonin and human calcitonin were injected

30   subcutaneously, each in an amount of 4 IU/kg. Blood was
sampled at given time intervals and the concentration of
calcium in plasma was measured.
Results and Discussion:

The time-dependent profiles of plasma calcium

35   concentrations for the test calcitonins are shown in Fig. 1.
As one can see from Fig. 1, all of the hybrid calcitonin 1,
salmon calcitonin and human calcitonin tested caused rapid

reduction in the elevated blood calcium concentration. It
is therefore clear that the hybrid calcitonin of the present
invention is as effective as salmon calcitonin and human
calcitonin against hypercalcemia.

Example 10   Analgesic effect of calcitonins
Method:
i)   Animal

Ten male rabbits (Kbl:JW) weighing 2.7 - 3.2 kg were
used.

ii)   Implantation of a guide cannula for intraventricular
administration

The rabbits were secured in a stereotaxic apparatus
under anesthesia with pentobarbital (30 mg/kg, i.v.). After
the scalp was incised, a guide cannula for intraventricular
administration (Plastic Products) was inserted into the
left lateral ventricle of each animal and fixed with dental
cement (stereotaxic coordinates from bregma: posterior,
4.0 mm; lateral, 5.5 mm; ventral, 5.5 mm). After 1-wk
convalescence, the rabbits were subjected to the experiment
described below. After the experiment, methylene blue was
injected to verify the cannula placement.
iii) Antinociception test

The rabbits were retained with a rabbit retainer and
a hole having a diameter of 1 mm and a depth 1 mm was bored
into both lateral sides of an upper major incisor with a
dentist's drill. A stimulating electrode was fitted into
each hole and electric stimulation (5 msec, 5 Hz, 3 sec
duration) was applied at various voltages from an electric
stimulator (Nihon Kohden Corp.) to measure the stimulation
threshold values (V) that induced licking responses (licking
and the movement of lips and lower jaw) in the animals.
Measurements were conducted one hour before drug administra-
tion and, immediately after the administration, as well as
0.5 h, 1.0 h, 1.5 h, 2.0 h, 3.0 h and 4.0 h after the
administration.

The drugs tested were (1-16) human/(17-32) salmon
hybrid calcitonin (hybrid calcitonin 1), salmon calcitonin
and human calcitonin, each of which was administered

intraventricularly in an amount equivalent to 8 IU/kg.
Physiological saline was used as a control.
Results and Discussion:

The time-dependent profiles of stimulation threshold
5   for the test calcitonins are shown in Fig. 2. As one can
see from Fig. 2, the three calcitonins caused comparable
increases in threshold value that peaked 0.5 - 1.5 h after
drug administration. It is therefore calculated that the
hybrid calcitonin of the present invention is as effective
10   as salmon calcitonin and human calcitonin in analgesic
action.

Example 11   Effects of calcitonins on the inhibition of body
              weight gain and appetite
Method:
15   Rats were injected intramuscularly with (1-16)
human/(17-32) salmon hybrid calcitonin (hybrid calcitonin
1), human calcitonin and elcatonin, and their body weight
and food consumptions were measured 24 h later.
Results:
20   The changes in the body weight of rats administered
various calcitonins are shown in Fig. 3, and their food
consumptions in Fig. 4. Each of the calcitonins was
administered in three different doses: 12.5 IU/kg, 50 IU/kg
and 200 IU/kg. For the body weight changes, the measured
25   values were averaged for 5 rats, and for the food consump-
tion, the measured values for 5 rats were added together.
Discussion:

As is clear from Figs. 3 and 4, salmon calcitonin and
elcatonin exhibited dose-dependent effects on the inhibition
30   of body weight gain and appetite, whereas hybrid calcitonin
1 and human calcitonin caused no differences in body weight
and food consumption. It can therefore be concluded that
the hybrid calcitonin of the present invention causes as
little effects on body weight gain and appetite as human
35   calcitonin.

Example 12   Effects of calcitonins on gastric emptying time
Method:

Female beagles fasted from the previous day were
injected with (1-16) human/(17-32) salmon hybrid calcitonin
(hybrid calcitonin 1), salmon calcitonin, human calcitonin
and physiological saline through one cephalic vein. One
hour later, enteric aspirin tablets (containing 200 mg of
the active ingredient) were administered orally together
with 25 ml of water. Thereafter, blood samples were taken
from the other cephalic vein at given time intervals, and
the plasma obtained in the usual manner was stored at -20 °C
until measurement.

As it passes through the intestinal wall, most of the
ingested aspirin is metabolized and enters the blood stream
as salicylic acid. Hence, salicylic acid in plasma was
determined as a marker for aspirin. Ethanol (200 µl) was
added to 50 µl of plasma and the mixture was centrifuged at
10,000 rpm x 1 min, with the resulting supernatant being
subjected to HPLC.

Results:

The delaying action of each calcitonin on the gastric
emptying time is summarized in Table 9. The difference
between the time from calcitonin administration to the
absorption of aspirin and the time from physiological saline
administration to the absorption of aspirin was calculated
for each animal to determine the delay in gastric emptying
time.

Table 9

Calcitonin	Dose, μg (IU)/kg		Lag time, h			Delay time, h		
Hybrid calcitonin 1	0.6	(1.2)	1.9	4.8	7.7	0.0	3.2	6.6
	10	(21.0)	5.1	18.0	21.4	3.8	16.3	20.3
. . . .
Salmon calcitonin	0.1	(0.4)	2.0	2.0		0.3	0.7	
	0.3	(1.2)	2.7	4.5	11.1	0.0	3.9	10.0
	0.6	(2.4)	22.0	30.0	45.0	19.4	29.4	43.1
. . . .
Human calcitonin	6	(1.2)	3.0	3.9	4.0	1.3	2.6	2.9
	100	(20.0)	5.0	6.9		4.4	5.0	

Notes Lag time: time from aspirin administration to its absorption

Delay time: difference in time to aspirin absorption between calcitonin and physiological saline

Discussion:

As Table 9 shows, salmon calcitonin had a very strong action in delaying the gastric emptying time, whereas human calcitonin was weak in such action. Hybrid calcitonin 1 was more similar to human calcitonin than salmon calcitonin in delaying to gastric emptying time. It can therefore be concluded that the hybrid calcitonin 1 of the present invention can be administered in a higher dose of activity units than salmon calcitonin.

Example 13 Effects of calcitonins on gastrointestinal motility in conscious dog.

Method:

A healthy beagle dog weighing about 10 kg was anesthetized with an i.v. injection of pentobarbital sodium and the abdominal cavity was opened under aseptic conditions.

Extraluminal force transducers were sutured onto the serosa of the gastric antrum stomach, duodenum and jejunum to measure circular muscle contraction, as reported previously (Itoh et al. Gastroenterol Jpn. 12, 275 1977).

- The lead wires of these transducers were out of the abdominal cavity and then brought out through a skin incision made between the scapulae.

5 After the abdominal surgery, a 5-cm <u>longitudinal</u> skin incision was made in the right Frontal neck to expose the external jugular vein.

A Silastic tube (French size 8.5, Dow corning, Midland, MI) was placed into the superior vena cava through the vein and sutured onto the adjacent skin as a route for 10 the i.v. injection of test drugs.

After the operation, a jacket protector was placed on the dog to protect the lead wires and the Silastic tube.

The dog was housed in individual experimental cage, and given commercial dog food at 5:00 P.M. and water was 15 given freely.

The gastrointestinal motor activety was recorded on a thermal pen writing recorder (WR-3101, Graphtic, Tokyo, Japan) by connecting the lead wires of the transducers to the connecting cables from the amplifiers (UG-5, Nihon 20 Kohden, Tokyo, Japan).

About two weeks postoperatively, gastrointestinal contractile activity could be divided into two main patterns of activity, interdigestive and digestive states.

In the interdigestive state, IMC(interdigestive 25 migrating motor complex) were seen to occur at regular intervals of 100 to 120 min. in the gastric antrum, and migrated through the duodenum and jejunum at a constant velocity.

In all animals, feeding disrupted the regular IMC 30 pattern. Experiments were carried out during interdigestive state. The drug was dissolved into 0.9 % saline and administered through the indwelling Silastic tube for about 10 seconds in a volume of 0.3 ml/kg.

35 15 Min after the end of the IMC in the gastric antrum, which were subsequently flushed in with 0/9 % saline.

The time until the next IMC is occurred from administration of the drug was measured, and used as a index for inhibitory activity of the drug on gastrointestinal motility.

The results are shown in Table 10.

Results:

Table 10

Salmon calcitonin	Dose, µg/kg (U/kg)	0.1 (0.4)	0.3 (1.2)	1.0 (4.0)	3 (12)	
	IMC delay	0	1	2	3	

Human calcitonin	Dose, µg/kg (U/kg)	100 (20)	300 (60)	1000 (200)		
	IMC delay	0	0.5	0.5		

Hybrid calcitonin 1	Dose, µg/kg (U/kg)	0.03 (0.06)	0.1 (0.21)	1.0 (2.1)	3.0 (6.3)	100 (210)
	IMC delay	0	0.5	1	1	1

Hybrid calcitonin 2	Dose, µg/kg (U/kg)	0.3 (0.5)	1.0 (1.8)	3.0 (5.4)	10 (18)	30 (54)	100 (180)
	IMC delay	0	0.5	1	1	1	1

Notes: 1. The indexes of "IMC delay" mean as follows.

    0 ... Next IMC occurred within 180 min after administration.

    0.5 ... Next IMC occurred more than 180 min but within 300 min after administration.

    1 ... Next IMC occurred one day after administration.

    2 ... Next IMC occurred two days after administration.

    3 ... Next IMC occurred three days after administration.

  2. Hybrid calcitonin 1 was (1-16) human/(17-32) salmon calcitonin, and hybrid calcitonin 2 was (1-21) human/(22-32) salmon calcitonin.

Discussion:

As reported in Table 10, salmon calcitonin

administered i.v. does-dependently inhibited the occurrence
of the next IMC in conscious dog.

At a dose of 3 μg/kg, it delayed the occurrence of
the next IMC 3 days. On the other hand, human calitonin had
5   only a slight effect on the IMC.

Although hybrid calcitonins also inhibited the IMC,
these compounds delayed the occurrence of the next IMC only
one day even at the highest dosage examined (100 μg/kg).

Our results definitely demonstrate that hybrid
10   calcitonins had a weaker effect on gastrointestinal motility
in conscious dogs than salmon calcitonin.

These results also indicated that hybrid calcitonins
will probably cause less adverse effects on gastrointestinal
tract than salmon calcitonin.

15   Example 14   Cross reaction between anti-salmon calcitonin
                  human antibody and hybrid calcitonin.
Method:

Salmon calcitonin was synthesized by an ordinary
procedure of solid-phase synthesis. Salmon calcitonin
20   labelled with ^{125}I was prepared by the Tejedor method
(Tejedor, F. and Ballesta, J.P.G.; Analytical Biochemistry,
127, 143-149 (1982)).

Four types of human sera that had been verified to
contain antibody after administration of salmon calcitonin
25   were furnished from Dr. Frederick R. Singer (CEDERS-SINAI
MEDICAL CENTER). The antibody titres of those sera against
salmon calcitonin were as follows:

No. 1	1 : 4000	No. 2	1 : 8000
No. 3	1 : 8000	No. 4	1 : 2000

30   1)   Determining the ratio of serum dilution

A 0.1 M borate buffer solution (pH 8.0) containing
0.5% BSA, 0.9% NaCl, 0.1% NaN₃ and 0.05% Tween 20 was used
as a reaction buffer. This reaction buffer, anti-salmon
calcitonin human serum (diluted with the reaction buffer to
35   a final dilution of 100 - 6,400) and a solution of labelled
calcitonin were charged into an assist tube (Spitz type of
Salschted Inc. measuring 12 x 75 mm) in the respective
volums shown below, stirred thoroughly, and left to stand

overnight at room temperature.

		*1	*2
i)	Reaction buffer	300 µℓ	400 µℓ
ii)	Anti-salmon calcitonin human serum	100 µℓ	0 µℓ
iii)	Solution of labelled calcitonin (≈ 20,000 cpm)	100 µℓ	100 µℓ

*1 Total binding (B) *2 Non-specific binding (NSB)

The reaction solution was mixed well with 500 µl of 0.2% BGG (bovine γ-globulin, Sigma), further mixed with 1 ml of 25% PEG #6000, stirred thoroughly and thereafter left to stand at room temperature for 15 min. The reaction solution was then centrifuged with a bucket type centrifuge (05RP-22 of Hitachi) at 3,000 rpm for 10 min at 4°C. After removing the supernatant by aspiration, the radioactivity level of the residue was measured with a gamma-counter. The results are shown in Fig. 5.

2) Reaction of competition between ^{125}I labelled salmon calcitonin and salmon calcitonin or hybrid calcitonin.

The reaction buffer, various types of calcitonin, anti-salmon calcitonin human serum and the solution of labelled calcitonin were charged into an assist tube in the respective volumes indicated below and subsequently treated in the same way as in the determination of serum dilution.

		*1	*2	*3
i)	Reaction buffer	200 µℓ	400 µℓ	300 µℓ
ii)	calcitonin	100 µℓ	0 µℓ	0 µℓ
iii)	Anti-salmon calcitonin human serum	100 µℓ	0 µℓ	100 µℓ
iv)	Solution of labelled calcitonin (≈ 20,000 cpm)	100 µℓ	100 µℓ	100 µℓ
		(B')	(NSB)	(Bo')

The two hybrid calcitonins used were: 1. (1-16) human/(17-32) salmon calcitonin; 2. (1-21) human/(22-32) salmon calcitonin.

The results are shown in Figs. 6 - 9.

Results and Discussion:

Investigation was made as to whether ^{125}I labelled salmon calcitonin would compete with salmon calcitonin for

anti-salmon calcitonin human serum No. 1 (Fig. 5). Similar
curves were obtained when anti-salmon calcitonin human sera
Nos. 2 - 4 were used. These results indicate that salmon
calcitonin started to compete with the labelled salmon
calcitonin in a dose of at least 1 ng/ml.

Experiments with the four types of sera demonstrated
that neither hybrid calcitonin 1 nor 2 was recognized by the
anti-salmon calcitonin human sera (Figs. 6 - 9).

It is therefore concluded that the hybrid calcitonin
of the present invention can potentially be used as an
effective drug for patients who produce anti-salmon
calcitonin antibodies.

The hybrid calcitonin of the present invention
has the outstanding advantage that it exhibits as strong
biological activities as salmon, eel and chicken cal-
citonins while causing no side effects including nauseation,
disorders in the functions of the digestive tract and
antigenicity. It also has a process advantage in that
side reactions that would otherwise be encountered during
synthesis can be prevented by changing Met^8 in human
calcitonin to Val^8.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1.   A hybrid calcitonin which is characterized in that the peptide segment on the side closer to the amino terminal end includes at least 10 amino acid residues on the corresponding side in human or human analog calcitonin and the peptide segment on the side closer to the carboxy terminal end includes at least 6 amino acid residues on the corresponding side in calcitonin derived from an animal other than humans.

2.   A hybrid calcitonin according to claim 1 wherein the human calcitonin is native human calcitonin or an analog thereof and the calcitonin derived from an animal other than humans is native calcitonin or an analog thereof.

3.   A hybrid calcitonin according to claim 1 wherein the non-human calcitonin is derived from eel, salmon or chicken.

4.   A hybrid calcitonin consisting of a peptide segment in human calcitonin and a peptide segment in calcitonin derived from an animal other than humans, the first peptide segment including the first to the n-th (n=10-28) amino acid residues from the amino terminal end and the second peptide segment including the subsequent amino acid residues toward the carboxy terminal end, with the proviso that the calcitonin derived from an animal other than humans which is salmon or salmon analog calcitonin is excluded.

5.   A hybrid calcitonin consisting of a peptide segment in human calcitonin and a peptide segment in calcitonin derived from an animal other than humans, the first peptide segment including the first to the n-th (n=13-21) amino acid residues from the amino terminal end and the second peptide segment including the subsequent amino acid residues toward the carboxy terminal end, and further the first peptide segment being either a peptide segment in native human calcitonin or a peptide segment in a human calcitonin analog having no more than

- 39 -

two amino acid residues substituted for, deleted from or added to the peptide segment in native human calcitonin.

6.   A hybrid calcitonin according to claim 5 wherein the peptide segment in a human calcitonin analog has a valine residue substituted for the 8th methionine residue in native human calcitonin.

7.   A hybrid calcitonin according to claim 5 wherein the second peptide segment includes the n-th (n=14-22) and the subsequent amino acid residues from the amino terminal end of eel, salmon or chicken calcitonin and is either a peptide segment in native eel, salmon or chicken calcitonin or a peptide segment in a calcitonin analog having no more than three amino acid residues substituted for, deleted from or added to the peptide segment in native eel, salmon or chicken calcitonin.

8.   A hybrid calcitonin according to claim 7 wherein the second peptide segment is a peptide segment in native eel or salmon calcitonin or a peptide segment in a calcitonin analog having one amino acid residue substituted for or deleted from the peptide segment in native eel or salmon calcitonin.

9.   A hybrid calcitonin in which the sequence of the first to the 16th amino acid residues from the amino terminal end is the same as that of the first to the 16th amino acid residues in native human calcitonin, and the sequence of the 17th to the 32nd amino acid residues is the same as the 17th to the 32nd amino acid residues in native eel or salmon calcitonin.

10.   A hybrid calcitonin having the following structural formula:

```
        ┌──────────────────────────────┐
        │                              │
    H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met(or Val)-Leu-Gly-
        1   2   3   4   5   6   7   8            9   10

    Thr-Tyr-Thr-Gln-Asp(or Glu)-Phe(or Leu)-Asn(or His)-
    11  12  13  14  15           16          17

    Lys-Phe(or Leu or Des)-His(or Gln)-Thr-Tyr-Pro-Arg-
    18  19                 20          21  22  23  24

    Thr-Asn(or Asp)-Thr(or Val)-Gly-Ser(or Ala)-Gly-Thr-
    25  26          27          28  29           30  31

    Pro-NH₂ .
    32
```

11.   A hybrid calcitonin according to claim 10 which
has the following structural formula:

```
        ┌──────────────────────────────┐
        │                              │
    H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-

    Gly-Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Leu-

    Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-

    Gly-Thr-Pro-NH₂ .
```

12.   A hybrid calcitonin according to claim 10 which
has one of the following structural formulas:

```
        ┌──────────────────────────────┐
        │                              │
    H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-

    Gly-Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Leu-

    Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-

    Gly-Thr-Pro-NH₂ ;
```

```
 ┌─────────────────────────┐
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Val-Leu-

Gly-Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Leu-

Gln-Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-

Gly-Thr-Pro-NH₂ ;
```

```
 ┌─────────────────────────┐
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-

Gly-Thr-Tyr-Thr-Gln-Asp-Phe-His-Lys-Gln-

Thr-Tyr-Pro-Arg-Thr-Asp-Val-Gly-Ala-Gly-

Thr-Pro-NH₂ ;
```

```
 ┌─────────────────────────┐
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-

Gly-Thr-Tyr-Thr-Gln-Glu-Leu-His-Lys-Leu-

Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-

Gly-Thr-Pro-NH₂ ; and
```

```
 ┌─────────────────────────┐
H-Cys-Gly-Asn-Leu-Ser-Thr-Cys-Met-Leu-

Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-

His-Thr-Tyr-Pro-Arg-Thr-Asn-Thr-Gly-Ser-

Gly-Thr-Pro-NH₂ .
```

13.   A process for the preparation of the hybrid
calcitonin as claimed in any one of claims 1 to 12, which
process is characterized in that the peptide having a
sequence of said hybrid calcitonin is synthesized by
solid-phase synthesis or liquid-phase synthesis and the
disulfide bond of said peptide is formed at any desired
stage of the synthesis of said hybrid calcitonin.

14. A process according to claim 13 wherein the peptide having a sequence of said hybrid calcitonin is completely synthesized except that the disulfide bond is not formed and then the disulfide bond is formed.

15. A hybrid calcitonin substantially as hereinbefore described with reference to any one of the examples.

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DATED: 9 December, 1993

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PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
CHUGAI SEIYAKU KABUSHIKI KAISHA

David B Fitzpatrick

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HYBRID CALCITONIN

ABSTRACT OF THE DISCLOSURE

The improved hybrid calcitonin consists of a peptide segment in human calcitonin and a peptide segment in

5   calcitonin derived from animals other than humans, such as eel, salmon and chicken. Each of the human or human analog calcitonins and the calcitonins derived from animals other than humans may be either native calcitonin or an analog thereof. The hybrid calcitonin has the outstanding

10   advantage that it exhibits biological activities as strong as eel, salmon and chicken calcitonin while causing no side effects including nausea, disorders in the functions of the digestive tract or antigenicity.

Fig. 1
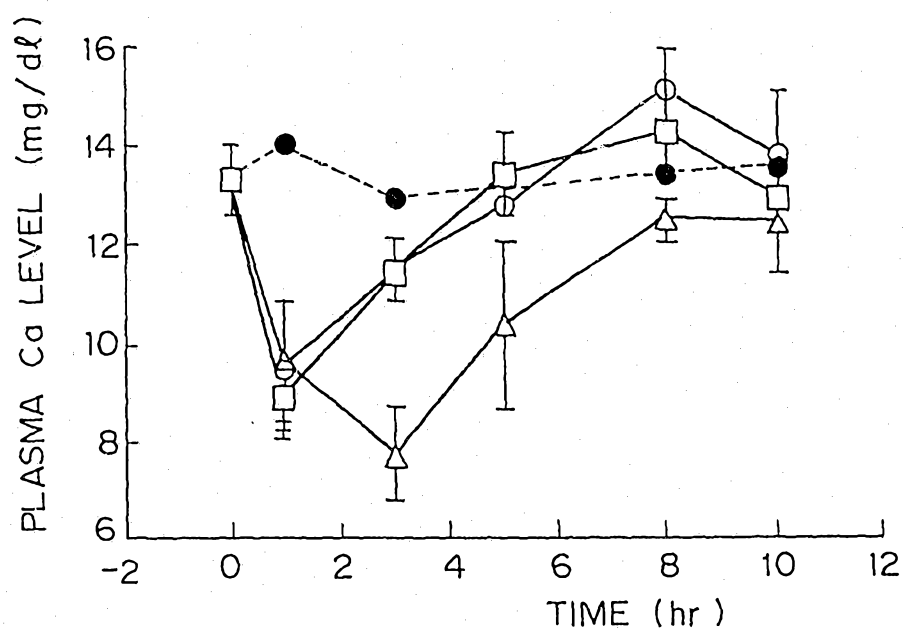
CONTROL

HYBRID CALCITONIN 1

SALMON CALCITONIN

HUMAN CALCITONIN

Fig. 2
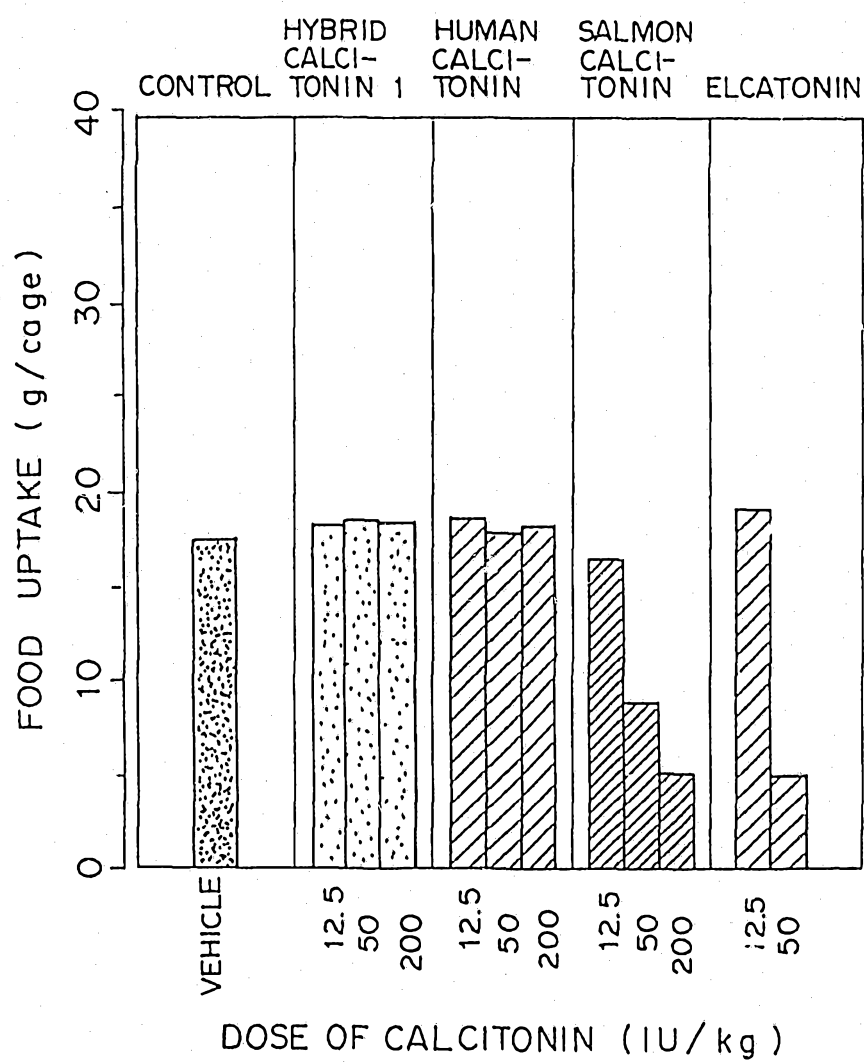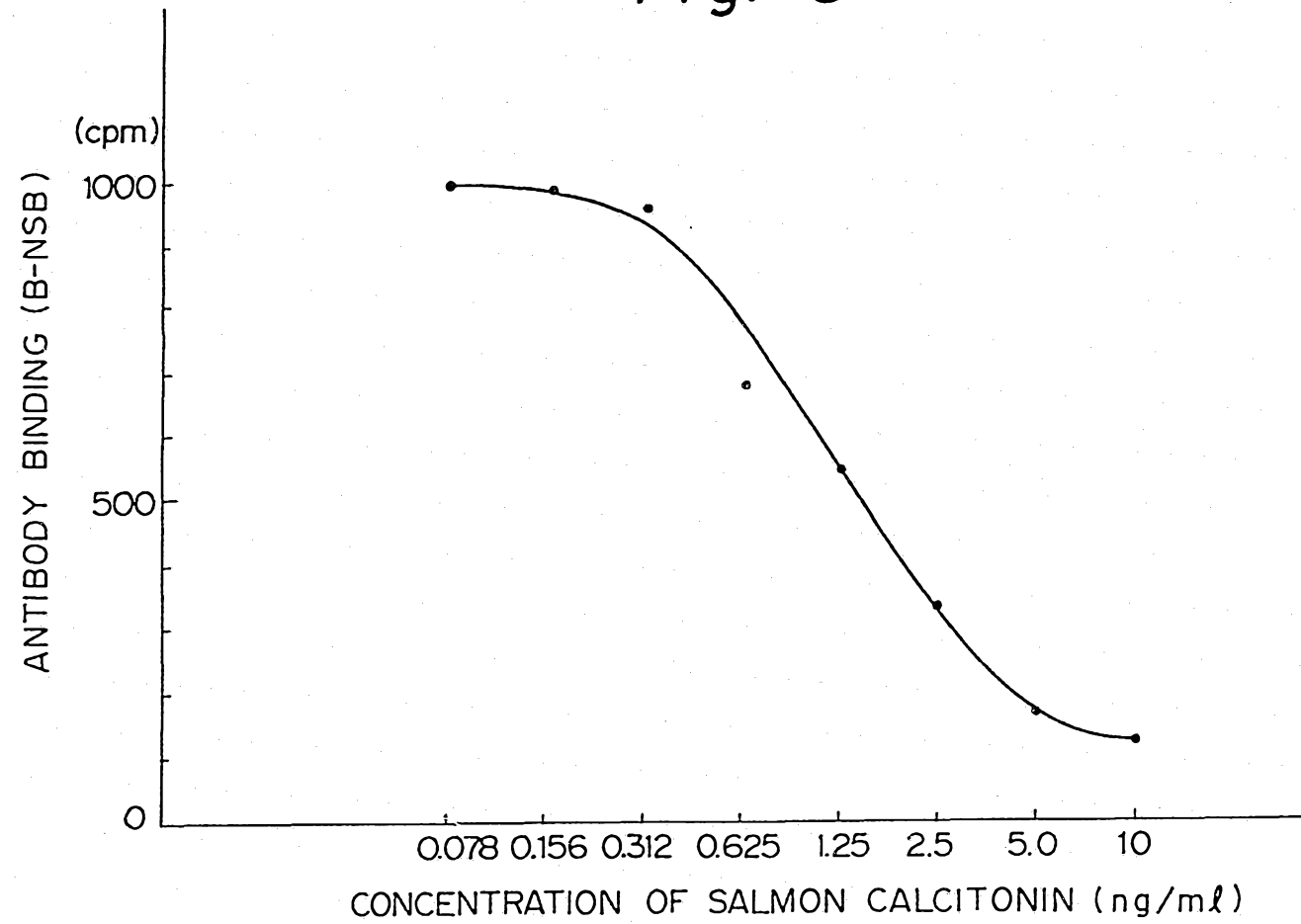
TIME AFTER INTRAVENTRICULAR
ADMINISTRATION (h r)

--□-- PHYSIOLOGICAL SALINE
--■-- SALMON CALCITONIN
—○— HUMAN CALCITONIN
—●—HYBRID CALCITONIN 1

Fig. 3

HYBRID   HUMAN   SALMON
CALCI-   CALCI- CALCI-
CONTROL TONIN 1   TONIN   TONIN   ELCATONIN

CHANGE IN BODY WEIGHT (g)

p<0.01 V.S. CONL

VEHICLE

12.5   50   200   12.5   50   200   12.5   50   200   12.5   50

DOSE OF CALCITONIN (IU/kg)

Fig. 4

Fig. 5

Fig. 6

① SERUM NO.1

CONCENTRATION OF CALCITONIN (ng/ml)

$$B/Bo = \frac{B' - NSB}{Bo' - NSB} \times 100 \,(\%)$$
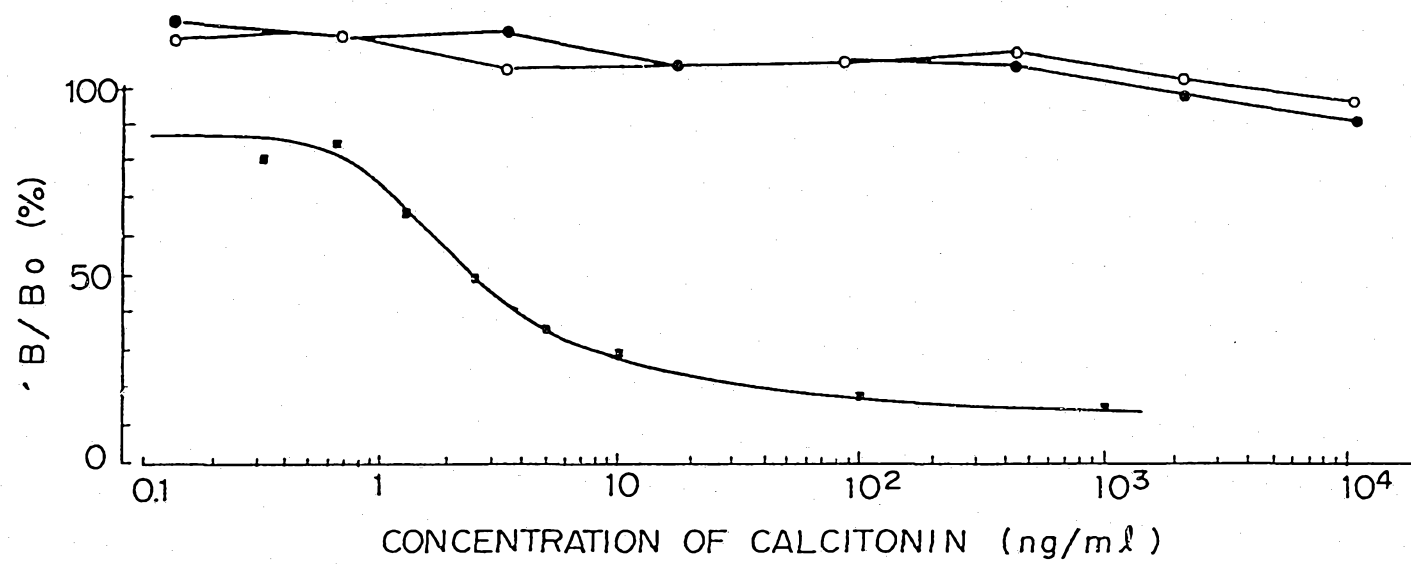
HYBRID CALCITONIN 1
──○──

HYBRID CALCITONIN 2
──●──

SALMON CALCITONIN
──■──

Fig. 7

② SERUM NO.2
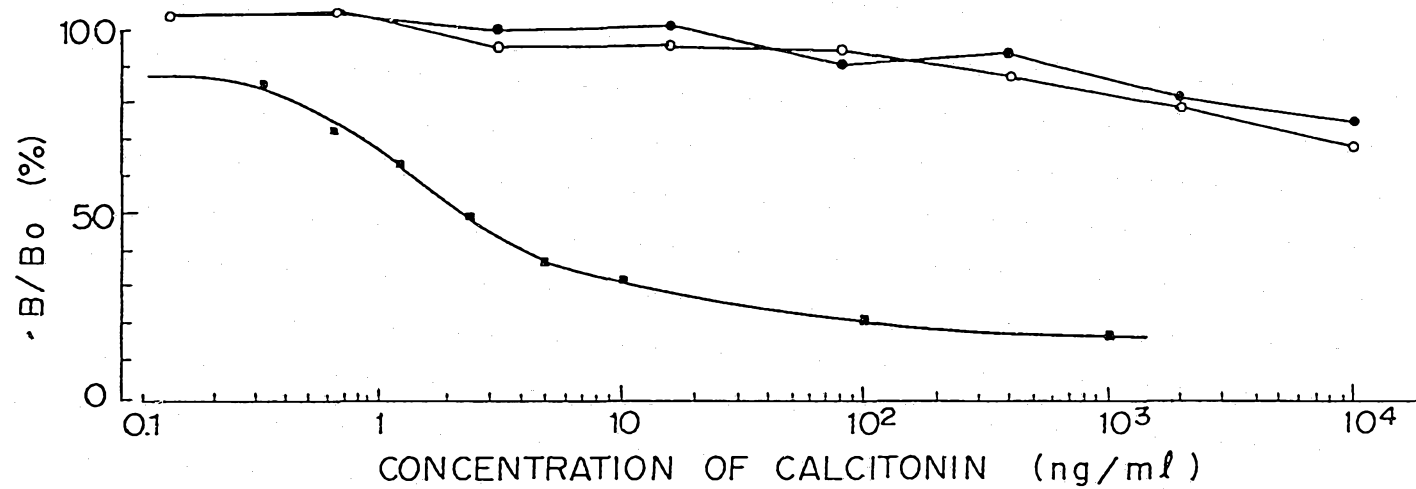
HYBRID CALCITONIN 1
—o—

$B/Bo = \dfrac{B'-NSB}{Bo'-NSB} \times 100(\%)$

HYBRID CALCITONIN 2
—●—

SALMON CALCITONIN
—■—

Fig. 8

③ SERUM NO.3

CONCENTRATION OF CALCITONIN

$B/Bo = \dfrac{B' - NSB}{Bo' - NSB} \times 100 \, (\%)$
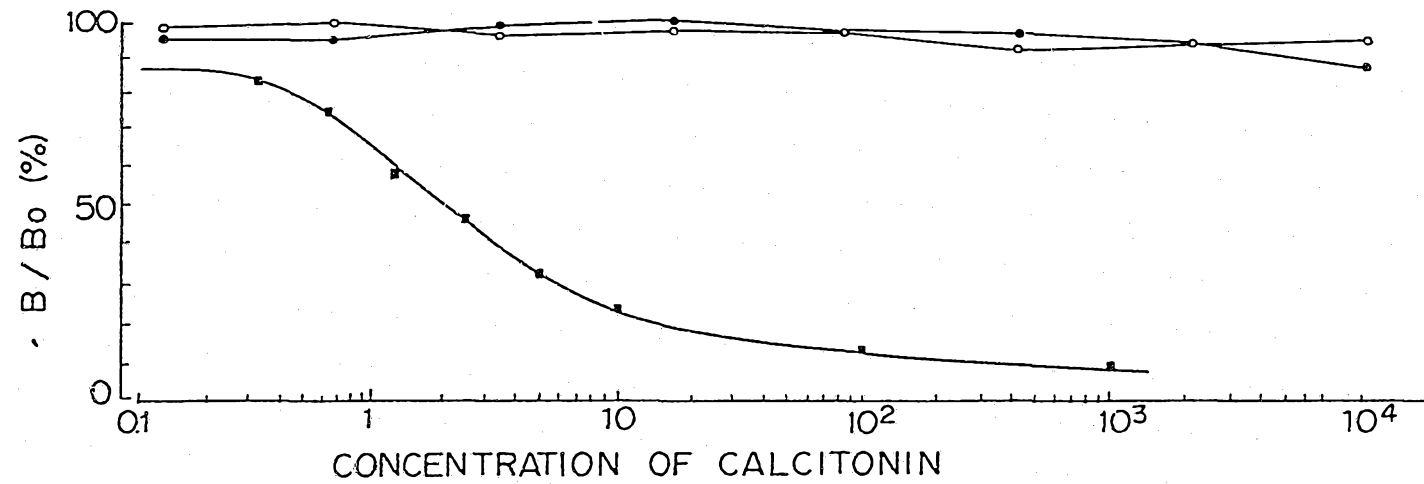
HYBRID CALCITONIN 1
——o——

HYBRID CALCITONIN 2
——●——

SALMON CALCITONIN
——■——

Fig. 9

④ SERUM NO. 4

$$B/Bo = \frac{B'-NSB}{Bo'-NSB} \times 100 \ (\%)$$
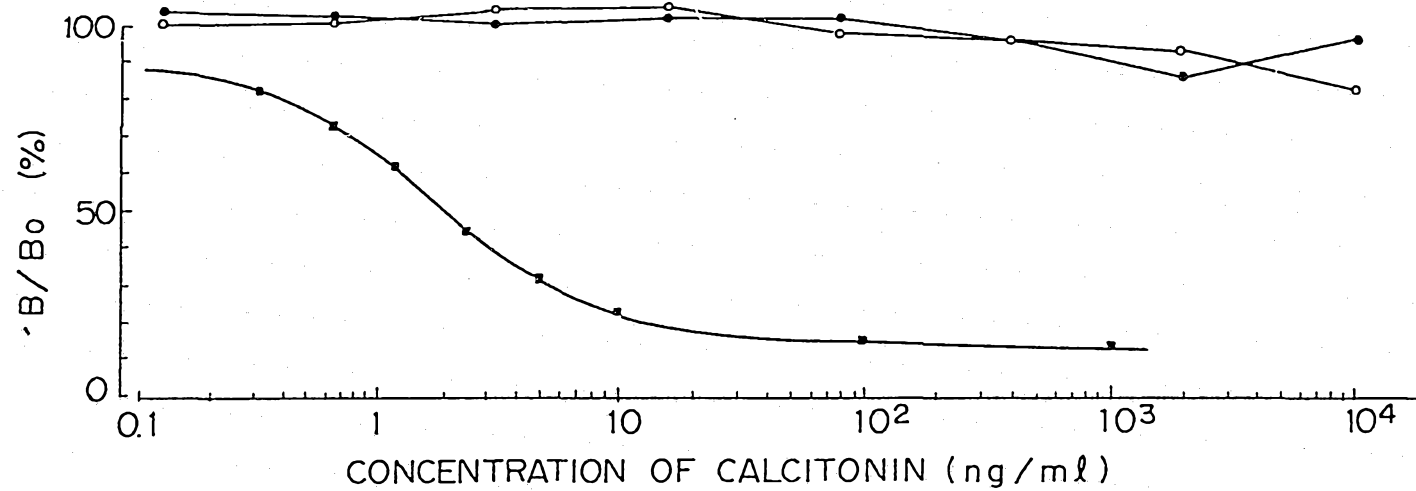
HYBRID CALCITONIN 1
——○——

HYBRID CALCITONIN 2
——●——

SALMON CALCITONIN
——■——

INTERNATIONAL SEARCH REPORT

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 C07K7/36, 1/00, A61K37/30

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	C07K7/36, 1/00, A61K37/30

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 *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	FEBS Lett., Vol.48, No.1 (1974), Rene Maier et al. "Analogs of human calcitonin, I. Influence of modifications in amino acid positions 29 and 31 on hypocalcemic activity in the rat" pp.68-71	1-15
X	Calcif. Tissue Res., Vol.21, Suppl. (1976), R. Maier "Analogs of human calcitonin" pp.317-320	1-15

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* 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
June 24, 1991 (24. 06. 91)	July 8, 1991 (08. 07. 91)

International Searching Authority	Signature of Authorized Officer
Japanese Patent Office	

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

国 際 調 査 報 告

I. 発明の属する分野の分類

国際特許分類 (IPC)

Int. Cℓ'
C07K7/36, 1/00, A61K37/30

II. 国際調査を行った分野

調 査 を 行 っ た 最 小 限 資 料

分 類 体 系	分 類 記 号
IPC	C07K7/36, 1/00, A61K37/30

最小限資料以外の資料で調査を行ったもの

III. 関連する技術に関する文献

引用文献の カテゴリー※	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	請求の範囲の番号
X	FEBS Lett.,第48巻, 第1号 (1974) Rene Maier, et. al「Analogs of human calcitonin I. Influence of moditications in amino acid positions 29 and 31 on hypocalcemic activity in the rat」 pp. 68-71	1-15
X	Calcif. Tissue Res.,第21巻, Suppl. (1976) R. Maier「Analogs of human calcitonin」 pp. 317-320	1-15

÷引用文献のカテゴリー

「A」特に関連のある文献ではなく、一般的技術水準を示すもの

「E」先行文献ではあるが、国際出願日以後に公表されたもの

「L」優先権主張に疑義を提起する文献又は他の文献の発行日 若しくは他の特別な理由を確立するために引用する文献 (理由を付す)

「O」口頭による開示、使用、展示等に言及する文献

「P」国際出願日前で、かつ優先権の主張の基礎となる出願の 日の後に公表された文献

「T」国際出願日又は優先日の後に公表された文献であって出 願と矛盾するものではなく、発明の原理又は理論の理解 のために引用するもの

「X」特に関連のある文献であって、当該文献のみで発明の新 規性又は進歩性がないと考えられるもの

「Y」特に関連のある文献であって、当該文献と他の1以上の 文献との、当業者にとって自明である組合せによって進 歩性がないと考えられるもの

「&」同一パテントファミリーの文献

IV. 認 証

国際調査を完了した日 24. 06. 91	国際調査報告の発送日 08.07.91
国際調査機関 日本国特許庁 (ISA/JP)	権限のある職員 特許庁審査官 前 田 憲 彦 ㊞ 4,H 8,3,1,8