P/00/001 28/5/91
Section 29

AUSTRALIA
Patents Act 1990

623675

PATENT REQUEST: STANDARD PATENT/PATENT OF ADDITION

We, being the person(s) identified below as the Applicant, request the grant of a patent to the person identified below as the Nominated Person, for an invention described in the accompanying standard complete specification.

Full application details follow.

[71] Applicant: **KABI PHARMACIA AB (formerly KABI VITRUM AB)**
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[70] Nominated Person: **KABI PHARMACIA AB (formerly KABI VITRUM AB)**
Address: **751-82 UPPSALA, SWEDEN.**

[54] Invention Title: **O-GLYCOSYLATED IGF-1**

[72] Name(s) of actual inventor(s): **SKNOTTNER-LUNDIN, ANNA**
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BASIC CONVENTION APPLICATION(S) DETAILS

[31] Application Number	[33] Country	Country Code	[32] Date of Application
8879826.2	GREAT BRITAIN	GB	20.8.88

Drawing number recommended to accompany the abstract FIG. 1

By our Patent Attorneys,
WATERMARK PATENT & TRADEMARK ATTORNEYS

for Darryl B. Mischlewski

16 January, 1992.
(Date)

Registered Patent Attorney

P/00/008b 12/11/91
Section 29 (1)
Regulation 3.1 (2)

AUSTRALIA

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NOTICE OF ENTITLEMENT
(To be filed before acceptance)

We, **KABI PHARMACIA AB (formerly KABI VITRUM AB)**
of, **751-82 UPPSALA, SWEDEN,**

being the applicant in respect of Application No. 40518/89 state the following:-

The Person nominated for the grant of the patent:
has entitlement from the actual inventors: by assignment.

The person nominated for the grant of the patent:
is the applicant of the application listed in the declaration under Article 8 of the PCT.

The basic application listed on the request form:
is the first application made in a Convention country in respect of the invention

By our Patent Attorneys,
WATERMARK PATENT & TRADEMARK ATTORNEYS

.... 16 January, 1992. ....

Darryl B. Mischlewski
Registered Patent Attorney

(12) PATENT ABRIDGMENT (11) Document No. AU-B-40518/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 623675

(54) Title
O-GLYCOSYLATED IGF-1

International Patent Classification(s)
(51)[5] C12N 015/18 A61K 037/26 A61K 037/36 C07K 007/10
C12N 015/81 C12P 021/02

(21) Application No. : 40518/89 (22) Application Date : 17.08.89

(87) PCT Publication Number : WO90/02198

(30) Priority Data

(31) Number (32) Date (33) Country
8819826 20.08.88 GB UNITED KINGDOM

(43) Publication Date : 23.03.90

(44) Publication Date of Accepted Application : 21.05.92

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(57) Claim

1. O-glycosylated IGF-1 essentially free from unglycosylated IGF-1.

4. O-glycosylated IGF-1, having 2 or more mannose residues attached to the Thr $_{29}$ amino acid of the IGF-1 polypeptide chain.

5. A method of obtaining O-glycosylated IGF-1 by the expression of IGF-1 in yeast cells, and isolating O-glycosylated IGF-1 from the medium.

OPI DATE 23/03/90 APPLN. ID 40518 / 89

AOJP DATE 26/04/90 PCT NUMBER PCT/EP89/00972

PCT **623675**

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 21/02, C12N 15/81 A61K 37/36	A1	(11) International Publication Number: WO 90/02198 (43) International Publication Date: 8 March 1990 (08.03.90)

(21) International Application Number: PCT/EP89/00972

(22) International Filing Date: 17 August 1989 (17.08.89)

(30) Priority data:
8819826.2 20 August 1988 (20.08.88) GB

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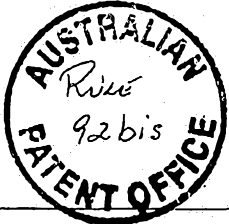

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: O-GLYCOSYLATED IGF-1

(57) Abstract

An O-glycosylated analogue of human IGF-1 which may be expressed, together with unglycosylated IGF-1, by yeast cells. A pharmaceutical composition containing glycosylated IGF-1, and a method of obtaining O-glycosylated IGF-1.

O-GLYCOSYLATED IGF-1

Insulin-like growth factor (IGF-1) is a 70 amino acid single polypeptide chain factor which displays relatively-high homology with proinsulin. It is known to have a mediating effect on the action of growth hormone and also to display insulin-like properties. When produced in nature by the human body it is unglycosylated.

IGF-1 has potential uses as a pharmaceutical agent both in the treatment of pituitary dwarfism and also diabetes. In the latter case it would be useful either as a replacement for insulin or as an adjunct to insulin. Although insulin is the traditional treatment for diabetes, type-2 diabetic have developed a resistance to insulin which means that even if very high doses of insulin are administered, the patients can still suffer from hyperglycaemia. Further, excessive doses of insulin can lead to undesirable side effects such as kidney complaints, obesity, and a disturbed water balance.

Although unglycosylated IGF-1 could be used as a replacement or adjunct for insulin in an attempt to overcome some of these problems, a high proportion of unglycosylated IGF-1 tends to be sequestrated by specific binding proteins circulating in the bloodstream and so

relatively high doses of the IGF-1 need to be administered for the desired pharmaceutical effect to be attained.

We have now discovered that expression of IGF-1 in yeast cells results in the production, along with the normal unglycosylated form, of an O-glycosylated analogue of IGF-1, for example an analogue which carries two mannose residues on the Thr_{29} amino acid of the polypeptide chain. Tests have shown that O-glycosylated IGF-1 has a reduced affinity for the binding proteins and that a desired reduction in blood sugar level can be achieved by a reduced dose of O-glycosylated IGF-1 as compared with the normal unglycosylated protein. This observed affinity to the binding protein will have a profound effect on the profile and dose dependency. The other effects, although not so pronounced are of importance in the total clinical effect.

The expression "O-glycosylated IGF-1" embraces O-glycosylated molecules which comprise fragments of the whole IGF-1 polypeptide sequence, provided that those fragments display qualitatively the growth-hormone mediating effect and/or insulin-like properties of IGF-1.

According to one aspect of the invention we therefore provide O-glycosylated IGF-1 essentially free from

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unglycosylated IGF-1.

According to another aspect of the invention we provide O-glycosylated IGF-1 in which the glycosylation is at the Thr $_{29}$ amino acid of the polypeptide chain.

According to yet another aspect of the invention, we provide O-glycosylated IGF-1 in which the glycosylation comprises two or more mannose residues attached to the same threonine residue of the IGF-1 polypeptide chain.

According to a further aspect of the invention, we provide an O-glycosylated analogue of IGF-1 in which two or more mannose residues are attached to the Thr $_{29}$ amino acid of the polypeptide chain.

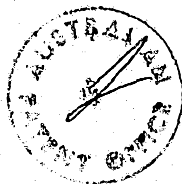
According to a still further aspect of the invention, we provide a method of obtaining O-glycosylated IGF-1 by the expression of IGF-1 in yeast cells, and isolating O-glycosylated IGF-1 from the medium. Preferably, the yeast is Saccharomyces cerevisiae.

The gene for IGF-1 is preferably linked to DNA coding for a secretory signal sequence, for example that of the gene for α-mating factor, so that mature authentic IGF-1 which has been O-glycosylated is secreted from the cytoplasm of the yeast cells.

The α -mating factor is a 13-amino acid peptide secreted by yeast cells of the α -mating type to promote efficient conjugation of a cells to form a α -diploid cells. Sequence data of the α -mating factor structural gene shows that the α-mating factor is initially synthesized as a 165 amino acid precursor containing an 85 amino acid leader peptide and four coding regions each interrupted by an 8 amino acid spacer peptide. The 85 amino acid leader polypeptide contain a 19 amino acid signal sequence involved in targeting the precursor to the endoplasmatic membrane.

We further provide a pharmaceutical composition containing O-glycosylated IGF-1 but substantially no unglycosylated IGF-1, and a pharmaceutically-acceptable carrier, diluent, or excipient. The composition may also include insulin.

According to another feature of the invention, we provide a method of preparing a pharmaceutical composition comprising mixing O-glycosylated IGF-1 and unglycosylated IGF-1.



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The plasmid utilised for the expression of IGF-1 in yeast may be p539/12, the construction of which is described in the following Example. Other yeast expression plasmids would also be suitable.

By way of example only, a process for the construction of a plasmid carrying the gene for IGF-1, transformation of Saccharomyces cerevisiae with that plasmid, and expression of mature human O-glycosylated IGF-1 from the transformed yeast cells will now be described in detail, with reference to the accompanying figures, in which:

Figure 1 illustrates the construction of IGF-1 expression plasmid p539/12;
Figure 1a is a restriction map of plasmid p539/12;
Figure 2 shows the polypeptide structure of the fusion between the α-mating factor leader sequence and IGF-1;
Figure 3 shows the separation of the two forms of IGF-2 by HI-HPLC;
Figure 4 shows the structure of IGF-1 with the tryptic fragments separated by transverse lines;

Figure 5 is a tryptic map showing both forms of IGF-1; and

Figure 6 shows the structure of the two mannose residues bound to Thr_{29} in the O-glycosylated form of IGF-1.

EXAMPLE

The IGF-1 gene was expressed in Saccharomyces cerevisiae, using an α-mating factor leader peptide-IGF-1 expression plasmid, p539/12.

Plasmid and yeast mutant strain:

Plasmid p539/12 was constructed by Dr. J.F. Ernst at Biogen S.A. Ch-1227, Geneva, Switzerland. Its construction is as follows. Plasmid pJDB219/G was constructed from JDB219 (Beggs, J.D., In: A. Benzon Symposium 16, editors: D. von Wettstein, J. Fries, M. Kielland-Brand & A. Stenderup, Munksgaard, Copenhagen, 383-389, 1981) by inserting a 1.7 kb SalI fragment carrying the APH gene Tn903 (Ern st, J. F. and R. C. Chan, J. Bacteriol. 163, 8-14, 1985) into the single SalI site of JDB219 (stippled box in Figure 1). The SMC expression unit from plasmid p364/1, encompassing the ACT promoter, the MF α 1 leader sequence and the SMC gene were transferred as a 1.1 kb BglII-BamHI fragment into the single BamHI site of pJDB219/G. The resulting plasmid, p510/14, was linearized by partial digestion

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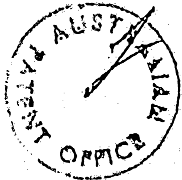
with HindIII and a 1.1 kb HindIII fragment carrying the yeast URA3 gene, which was derived from YIp30 (Botstein, D. et al., Gene 8, 17-24, 1979) was inserted into p510/14 to create p539/12 which carries the markers Kan^R $G418^R$ Ura3+ Leu^2+ . An outline of its genetic elements are shown in Figure 1a. The production of IGF-1 was carried out in the Saccharomyces cerevisiae mutant strain YE 449 (Mat α leu 2, ura 3-52, prbl-1122, pep 4-3, cir 4°).

The plasmid is an improved version of other IGF-1 expression plasmids, reported earlier (Ernst J.F. (1986) DNA 5, 483-491). The total amino acid sequence (155 amino acids) for the α -mating factor leader peptide-IGF-1 hybrid protein is shown in Figure 2. The α - mating factor leader peptide comprises the first 85 residues and IGF-1 the remaining 70 residues. Several possible O-linked glycosylation sites are present in the IGF-1 sequence. These are indicated. The newly synthesized 155 amino acid long α -mating factor leader peptide-IGF-1 hybrid protein was secreted out from the cell into the medium. During this process the hybrid protein was cleaved by an endogenous yeast peptidase (KEX2), generating the authentic IGF-1 molecule with its correct N-terminal amino acid (Gly). However, in addition to the authentic form of human IGF-1 a new analogue of human IGF-1 was also synthesized and secreted.

The yeast fermentation medium contained approximately 50% of authentic human IGF-1 and 50% of the analogue. These two IGF-1 forms were isolated from the medium using traditional biochemical separation techniques. The final separation of the IGF-1 analogue from authentic human IGF-1, was achieved by hydrophobic interaction chromatography (HI-HPLC) using a TSK-Phenyl-5PW matrix (Figure 3). The IGF-1 analogue eluted earlier than authentic IGF-1, indicating that it is slightly more hydrophilic.

It was found by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) that authentic IGF-1 had a slightly lower molecular weight than the analogue and the higher molecular weight (approx. 400) daltons exhibited by the analogue suggests that this form has molecules bound to the polypeptide chain, most likely carbohydrates added during the secretion process.

The amino acid composition of the IGF-1 analogue was determined and found to be identical to that of the authentic molecule. Hence, the slightly more hydrophilic property of the IGF-1 analogue, as deduced from the HI-HPLC experiment, is not due to a change in the polypeptide backbone, but rather some other structural modification.



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Yeast cells are known to contain glycoproteins, and the possibility that the new analogue was a glycosylated form of IGF-1 was investigated.

ConA has been widely used in the study of glycoproteins due to its high affinity for oligosaccharide chains containing three or more mannose residues. Its carbohydrate specificity has been determined (α-D-Man $>$ α-D-Glc $>$ α-DGlcNAc).

ConA blotting was carried out after sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the IGF-1 analogue and authentic human IGF-1 (Figure 4). ConA binding was observed to the IGF-1 analogue but not to the authentic form, indicating that it is a glycoprotein.

The carbohydrate moiety bound to the IGF-1 polypeptide background was identified by gas chromatography mass-spectrometry (GC/MC) after alkali hydrolysis (4M TFA, 120 ^{o}C, 15') and subsequent reduction (NaBH) and acetylation. A peak (7.7 minutes) was found after gas chromatography when the IGF-1 analogue was analyzed. No such peak was observed when authentic IGF-1 was analyzed (not shown). The material eluting at 7.7 minutes was further subjected to mass-spectrometry analysis. A mass-

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spectrum identical to a mannose reference mass-spectrum, was found demonstrating unambiguously that the IGF-1 analogue contains mannose. Quantitation of the mannose content by gas chromatography against a calibrated mannose standard showed that the mannose content was approximately 2.1% (w/w). No mannose or other carbohydrates were found in the authentic IGF-1 form.

Tryptic map analysis

G1cNAc (N-acetyl glucosamine) has been shown always to be the first carbohydrate residue in N-linked glycosylation. Since no G1cNAc was found in the IGF-1 analogue, the glycosylation has to be of the O-linked type, which can occur only to serine or threonine residues. IGF-1 contains 5 serine residues (Ser_{33} ; Ser_{34} ; Ser_{51} , and Ser_{69}), and 3 threonine residues (Thr_{4} , Thr_{29} ; and Thr_{41}). (See Figure 2).

To identify the glycosylated serine or threonine residue(s), the IGH-1 analogue was digested with trypsin (the polypeptide chain cleaves after Arg and Lys residues) to generate shorter tryptic fragments. These were subsequently separated by RP-HPLC and their mobility compared to their corresponding counterparts generated by trypsin digestion of authentic IGF-1. Any change in mobility relative to the standard (authentic IGF-1) would

reveal the glycosylated tryptic fragment.

Figure 4 shows the tryptic map of authentic IGF-1. Thirteen unique fragments were separated, the different fragments being shown separated by transverse lines. The identity of these fragments were determined by amino acid analysis. These fragments fit with the seven tryptic cleavage points (Arg, Lys) in IGF-1 plus the three chymotryptic-like cleavages that have also taken place. When the tryptic map of IGF-1 analogue was compared to authentic human IGF-1 a slight difference in elution time for tryptic fragments 22-36 was observed (Figure 5). No other differences could be detected. This shows clearly that the mannosylation site is within tryptic fragments 22-36. Based on its sequence (-Gly-Phe-Tyr-Phe-Asn-Lys-Pro-Thr-Gly-Tyr-Ser-Ser-Ser-Arg-) four possible O-linked glycosylation sites are present, e.g. Thr_{29}; Ser_{33}; Ser_{34}; Ser_{35}.

The tryptic fragments 22-36 was isolated from the IGF-1 analogue as well as from authentic IGF-1. These fragments were analyzed by protein sequence analysis. Thr present in the tryptic fragment from the IGF-1 analogue was the only amino acid that migrated anomalously demonstrating that it had been modified. No other differences in the entire sequence 22-36 was found between the two tryptic fragments. No modification was

observed in the three consecutive serine residues Ser $_{33}$; Ser $_{34}$; or Ser $_{35}$; showing that these had not been glycosylated. Hence, these results demonstrate unambiguously that Thr $_{29}$ in the IGF-1 analogue is the only amino acid which binds mannose. No other serine or threonine has been O-glycosylated.

To determine accurately the number of mannose residues bound to Thr $_{29}$, the tryptic fragment 22-36 isolated from the IGF-1 analogue was analyzed by mass-spectrometry. This showed a molecular weight difference between the mannosylated and the non-mannosylated fragment of 324. Assuming a molecular weight for peptide bound mannose of 162 a stoichiometry of 2 mannose residues per tryptic fragment can be calculated. Consequently, each IGF-1 molecule contains two mannose residues bound to Thr $_{29}$. The structure of the two mannose residues bound to Thr $_{29}$ in the IGF-1 analogue molecule was determined by H-NMR spectroscopy and is shown in Figure 15.

BIOLOGICAL ACTIVITY

It was clearly shown after incubation with radiolabelled forms of IGF-1 that the O-glycosylated IGF-1 bound less well to the high molecular weight binding protein (150 K) in both normal human serum and normal rat serum but no difference in the serum profiles was obtained when the

serum from hypophysectomized rats was used. The area under the curves of the high and low molecular weight peaks indicated that only between 50 and 60% of the O-glycosylated IGF-1 was bound to the large molecular weight form compared to the authentic IGF-1. A small increase in binding to the low molecular weight form was observed, but the increase was not large enough to account for the change in ratio between binding to the high and low carriers, respectively.

In freshly prepared rat hepatocytes the two forms of IGF-1 had a dose-dependent effect on the transport into the cells of the non-metabolizable amino acid α-AIB. The results indicate that the glycosylated form was slightly more effective at the high concentration used (1 mole/1) than was the authentic IGF-1. The results on gluconeogenesis in hepatocytes after incubation with either of the IGF-1s or insulin showed that no effect was observed when insulin or authentic IGF-1 were added which is what would be expected. However, by addition of the O-glycosylated IGF-1 an unexpected increase in the amount of glucose found in the medium was observed. This effect seems to be due to the mannose bound to the IGF-1 since the addition of the equivalent concentration of mannose alone resulted in the same effect. The effects were slightly lower than that observed with glucagon.

An acute insulin-like activity measured as the hypoglycemia induced in hypophysectomized rats, could be demonstrated for both O-glycosylated and authentic IGF-1. A dose of 10 ug/rat, induced a pronounced hypoglycemia with both peptides. The nadir was observed at 30-45 minutes and the glucose levels had returned to initial after approximately 2 hours. The effect of the O-glycosylated IGF-1 was slightly greater than that of the authentic IGF-1 at similar doses, the maximal decrease in blood glucose being -27% at 45 min. for the O-glycosylated form, compared to -14.4% at 30 minutes for authentic IGF-1.

The results of the _in vivo_ membrane transport showed that the maximal effect was reached after approximately 2 hours, where a plateau was observed. No significant difference between the peptides was observed, although there was a tendency for the O-glycosylated IGF-1 to be slightly more effective.

Preliminary calculations of the half-lives ($T_{1/2}$) of the O-glycosylated (mannosylated) and authentic IGF-1 were done. This resulted in $T_{1/2}$ of the α-phase of 3 minutes and 4 minutes, respectively, in normal rats, whereas the half-lives of the α-phase in hypophysectomized rats were slightly longer, i.e. 8 and 11 minutes for O-glycosylated and authentic IGF-1,

respectively. The half-lives of the α-phases, however, were in normal rats 3.5 hours and 5.3 hours, respectively, and in hypophysectomized rats 3.3 hours and 3.5 hours, respectively.

In summary the O-glycosylated IGF-1 has been compared to the authentic IGF-1 in several different biological assays in vitro and in vivo. No significant difference in their specific activity according to placental receptor assay or radioimmunoassay was observed indicating that the mannosylated amino acid is not involved in the binding to either the IGF-1 receptor or to the site binding to the antibody.

This is also evident from the experiment showing different effects in vitro. Both IGF-1 forms have shown similar effects on membrane transport (amino acids) in hepatocytes. If binding to these receptors had been disturbed, a difference between the two forms would have been observed.

One unexpected finding, however, was observed in vitro in the hepatocytes, namely the fact that O-glycosylated IGF-1 increased the amount of glucose in the medium. This effect was also found by the addition of only mannose. It is well known that mannose can enter the gluconeogenetic pathway at the step of fructose-6-

phosphate after conversion by phosphomannoisomerase. The slightly increased activity of O-glycosylated IGF-1 on the membrane transport, might be explained by the possibility that the cells use the mannose for generating energy.

It was recently demonstrated that the receptor for a peptide homologue insulin-like growth factor II (IGF-II) was apparently equivalent to the mannose-6-phosphate receptor, which is involved in lysosomal targeting. It has further been shown that addition of mannose-6-phosphate increased binding of IGF-II to its receptor two-fold, indicating that modulation of the receptor might occur.

The difference in binding to the high molecular weight binding protein between the two forms of IGF-1 is a very important observation. In the circulation only a very small amount of free endogenous IGF-1 is found, ($<$1%) and the majority of the peptide is bound to at least two different carrier proteins, a high molecular weight form, which is regulated by growth hormone and is lacking in Laron dwarfs, GHDs growth-hormone deficient dwarfs and hypophysectomized rats, and a small molecular weight form, which is in part regulated by insulin. The amount of this latter carrier protein is increased in diabetic patients.

Since it has been suggested that the free IGF-1 is responsible for the insulin-like effects (e.g. hypoglycemia *in vivo*) it might be important to increase the free fraction of IGF-1 for the treatment of hyperglycaemia. Preliminary studies have indicated that IGF-1 would be a possible candidate to use, e.g. in patients displaying insulin resistance.

Furthermore, the results from the *in vivo* studies on acute insulin-like activity also indicate that the O-glycosylated IGF-1 has a more pronounced effect than authentic IGF-1 in lowering the blood glucose, perhaps due to the difference in hydrophobicity of the two peptides or to a difference in kinetics.

Preliminary results of pharmacokinetics indicate that there is no difference between the O-glycosylated and the authentic IGF-1 when given to hypophysectomized rats. However, the results in normal rats indicate that the O-glycosylated form of IGF-1 may have a slightly shorter apparent half-life of the α-phase. (3.5 hours compared to 5.3 hours for the authentic IGF-1.) This might be due to a difference in binding to the carrier proteins.

CLAIMS:

1. O-glycosylated IGF-1 essentially free from unglycosylated IGF-1.

2. O-glycosylated IGF-1 according to Claim 1, in which the glycosylation is at the Thr_{29} amino acid of the polypeptide chain.

3. O-glycosylated IGF-1 according to Claim 1, in which the glycosylation comprises 2 or more mannose residues attached to the same threonine residue of the IGF-1 polypeptide chain.

4. O-glycosylated IGF-1, having 2 or more mannose residues attached to the Thr_{29} amino acid of the IGF-1 polypeptide chain.

5. A method of obtaining O-glycosylated IGF-1 by the expression of IGF-1 in yeast cells, and isolating O-glycosylated IGF-1 from the medium.

6. A method according to Claim 5, in which the yeast is *Saccharomyces cerevisiae*.

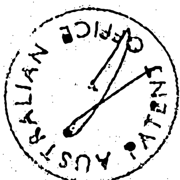
7. A method according to Claim 6, in which DNA coding for IGF-1 is operatively linked to DNA coding for a yeast secretory signal sequence, so that mature authentic IGF-1 which has been O-glycosylated is secreted from the cytoplasm of the yeast cells.

8. A method according to Claim 7, wherein the secretory signal sequence is the signal sequence of the gene for alpha mating factor.

9. A pharmaceutical composition containing O-glycosylated IGF-1, but substantially no unglycosylated IGF-1, and a pharmaceutically acceptable carrier, diluent or excipient.

10. A pharmaceutical composition according to Claim 9, also including insulin.

11. A pharmaceutical composition comprising both O-glycosylated IGF-1 and unglycosylated IGF-1, wherein the proportion of O-glycosylated IGF-1 is higher than in a mixture of O-glycosylated and unglycosylated IGF-1 as expressed by a culture of yeast cells.



7. A method according to Claim 6, in which DNA coding for IGF-1 is operatively linked to DNA coding for a yeast secretory signal sequence, so that mature authentic IGF-1 which has been O-glycosylated is secreted from the cytoplasm of the yeast cells.

8. A method according to Claim 7, wherein the secretory signal sequence is the signal sequence of the gene for alpha mating factor.

9. A pharmaceutical composition containing O-glycosylated IGF-1, but substantially no unglycosylated IGF-1, and a pharmaceutically acceptable carrier, diluent or excipient.

10. A pharmaceutical composition according to Claim 9, also including insulin.

11. A method of preparing a pharmaceutical composition comprising mixing O-glycosylated IGF-1 and unglycosylated IGF-1.

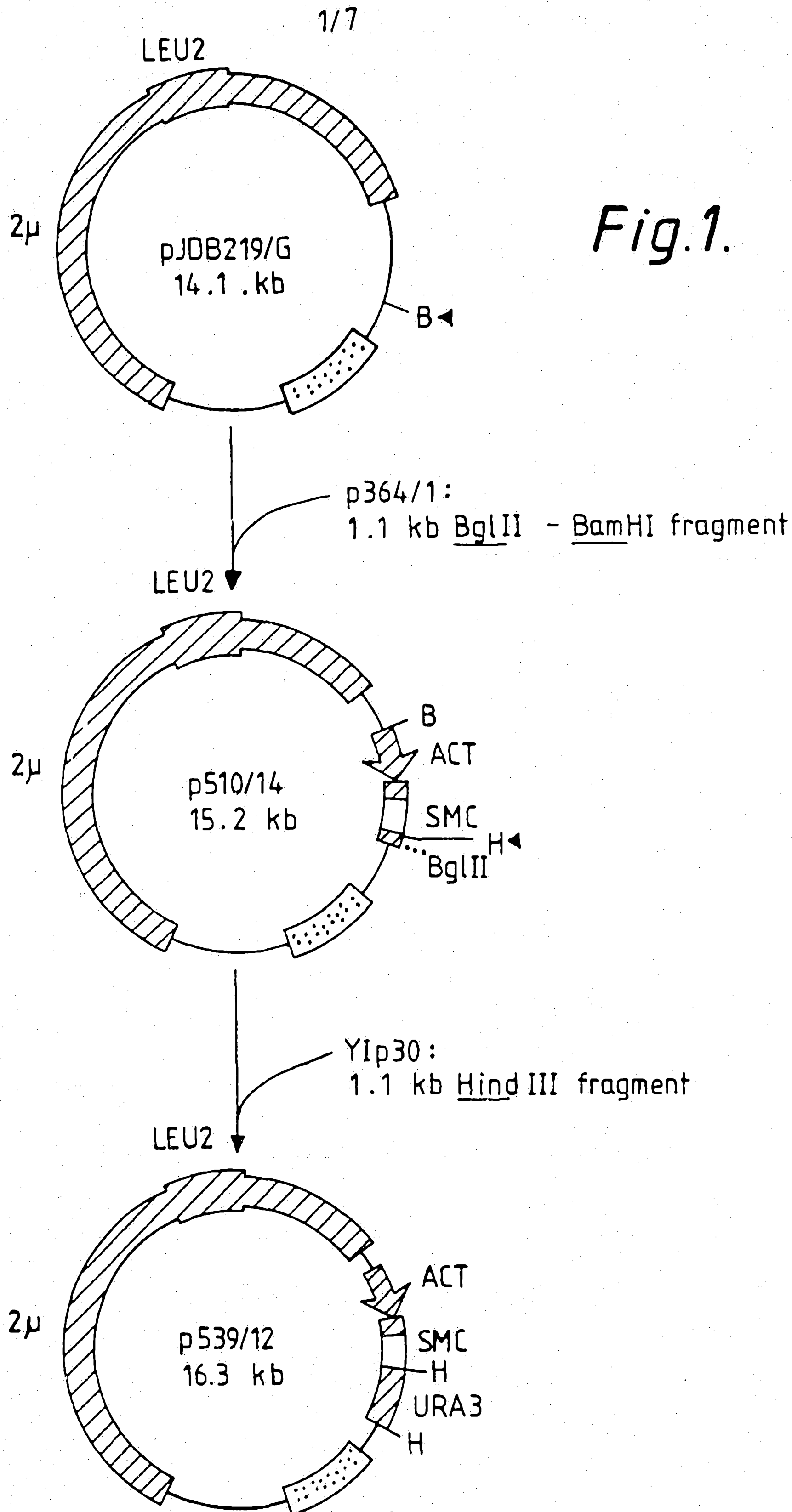
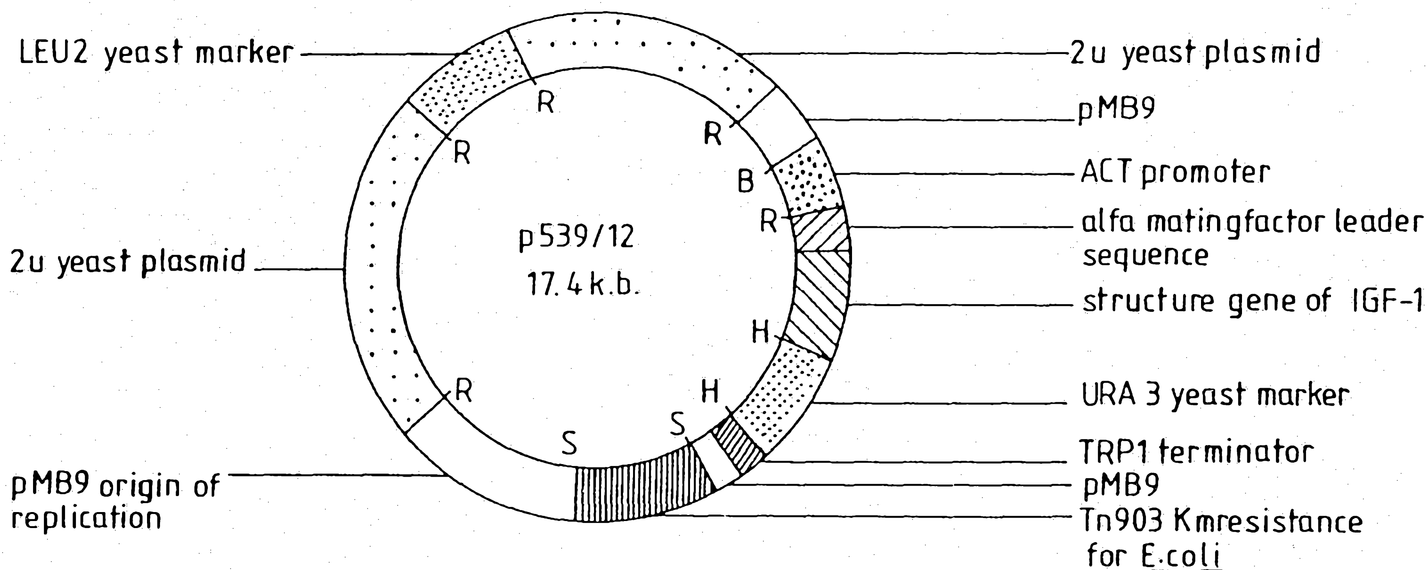


Fig.1.

RESTRICTION MAP OF P539/12

Fig.1a.



R = EcoRI
S = SalI
H = HindIII
B = BamHI

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Fig.2.

Yeast alpha-mating factor leader peptide - IGF-1

met-arg-phe-pro-ser-ile-phe-thr-ala-val-leu-phe-ala-ala-ser-ser-
1 10

ala-leu-ala-ala-pro-val-asn-thr-thr-thr-glu-asp-glu-thr-ala-gln-ile-
20 30

pro-ala-glu-ala-val-ile-gly-tyr-ser-asp-leu-glu-gly-asp-phe-asp-
40

val-ala-val-leu-pro-phe-ser-asn-ser-thr-asn-asn-gly-leu-leu-phe-
50 60

ile-asn-thr-thr-ile-ala-ser-ile-ala-ala-lys-glu-glu-gly-val-ser-leu-
70 80

asp-lys-arg-|-gly-pro-glu-thr-leu-cys-gly-ala-glu-leu-val-asp-ala-
→ IGF-1 90

leu-gln-phe-val-cys-gly-asp-arg-gly-phe-tyr-phe-asn-lys-pro-thr-
100 110

gly-tyr-gly-ser-ser-ser-arg-arg-ala-pro-gln-thr-gly-ile-val-asp-
120 130

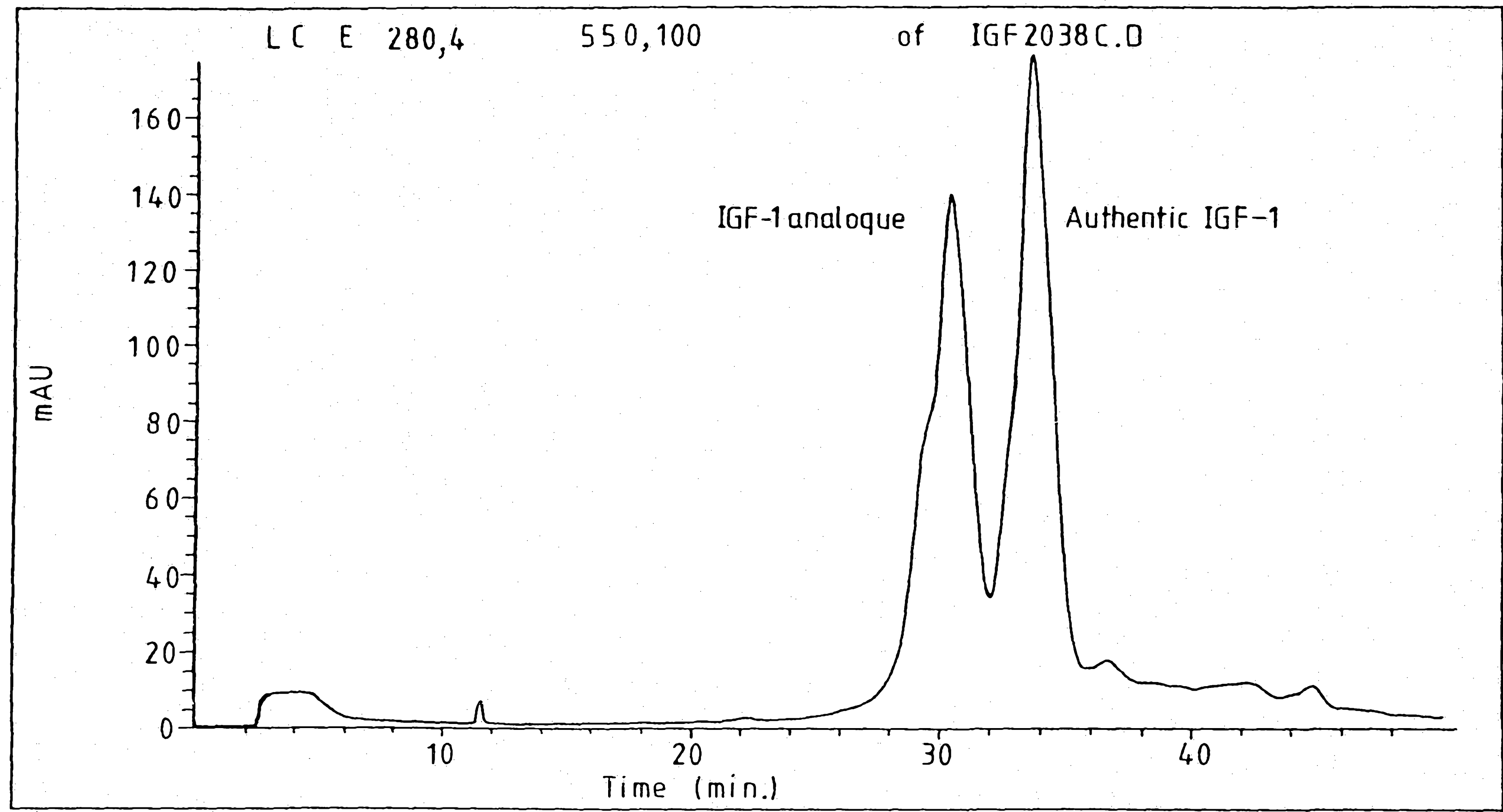
glu-cys-cys-phe-arg-ser-cys-asp-leu-arg-arg-leu-glu-met-tyr-
140

cys-ala-pro-leu-lys-pro-ala-lys-ser-ala
150 155

Potential O-linked glycosylation sites are underlined

Fig. 3.

Separation by HI-HPLC of authentic and an analoque form of IGF-1



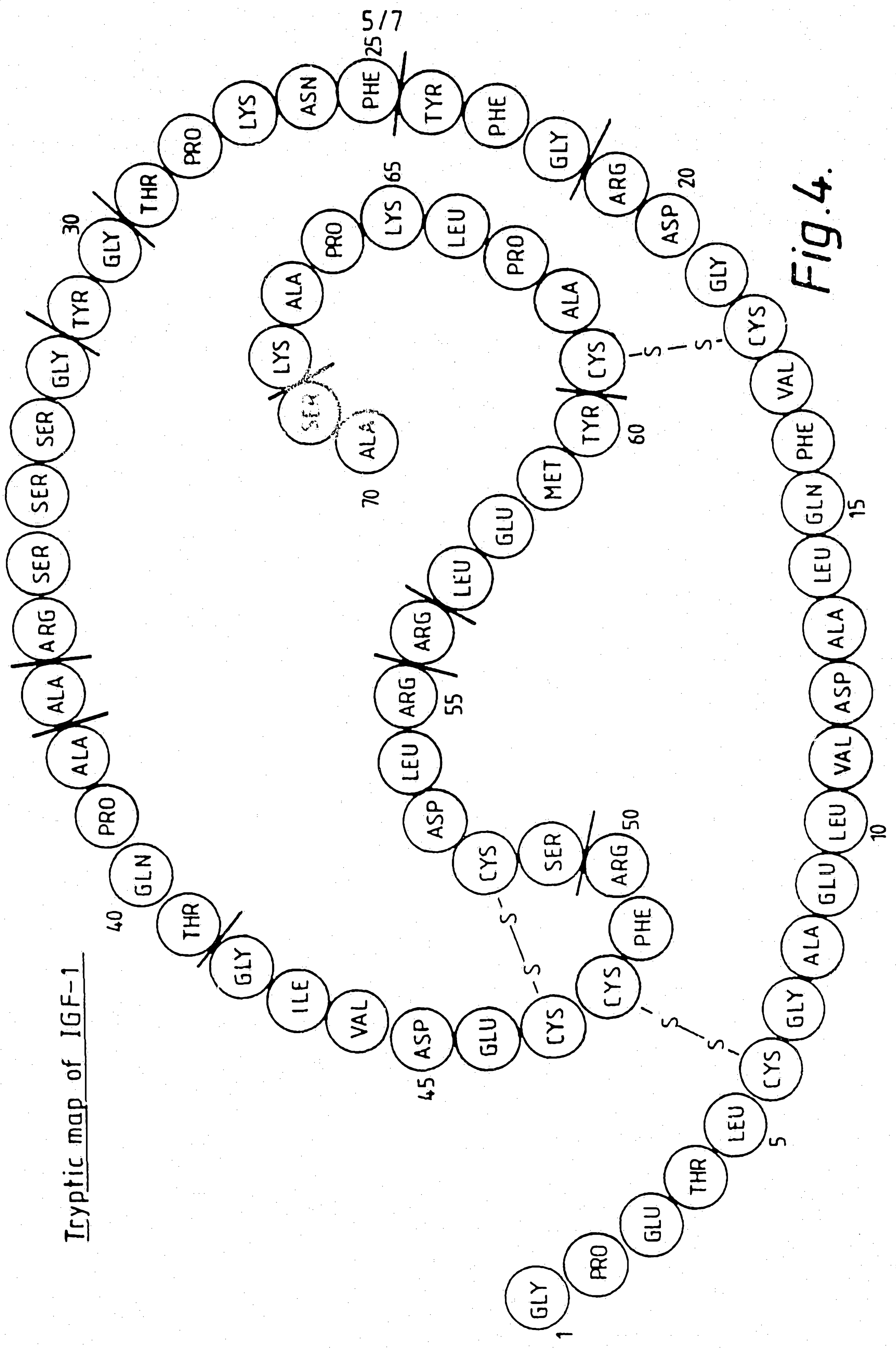


Fig.4.

Tryptic map of glycosylated and non glycosylated IGF-1

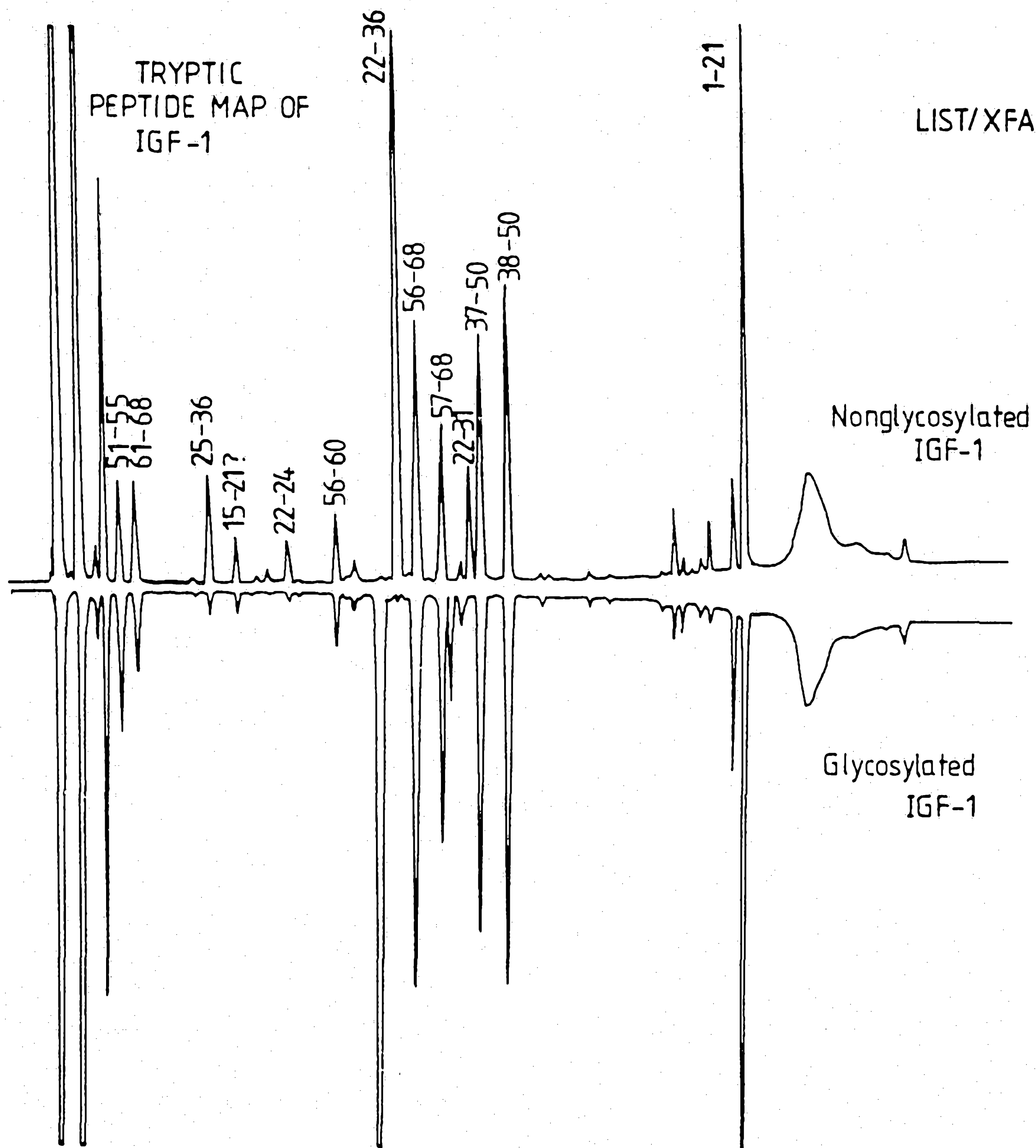


Fig.5.

Structure of the two mannose residues bound to Thr_{29} in yeast derived human IGF-1

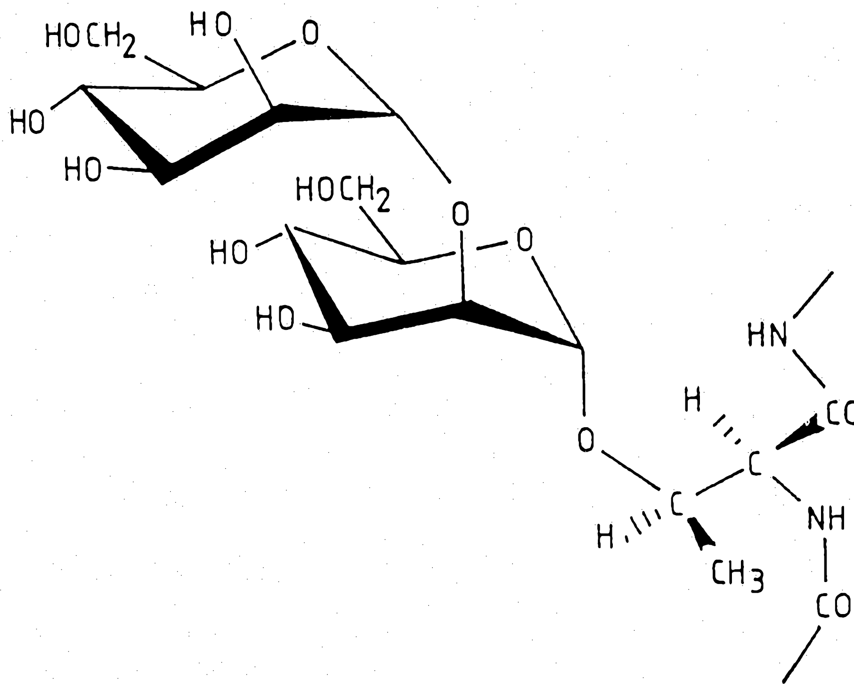


Fig.6.

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[5]: C 12 P 21/02, C 12 N 15/81, A 61 K 37/36

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 P, C 12 N

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]
P,X	FEBS Letters, volume 248, no. 1,2, May 1989, Elsevier Science Publishers B.V (Biomedical Division), (Amsterdam, NL), K. Hård et al.: "O-Mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in Saccharomyces cerevisiae", pages 111-114 see the whole article --	1-11
A	Gene, volume 66, 13 July 1988, Elsevier Science Publishers B.V. (Biomedical Division), (Amsterdam, NL), M.L. Bayne et al.: "Expression, purification and characterization of recombinant human insulin-like growth factir I in yeast", pages 235-244 --	
A	The Journal of Biological Chemistry, volume 263, no. 13, 5 May 1988, The American Society for Biochemistry ./.	

[*] Special categories of cited documents: [10]

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

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"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
19th September 1989	20 NOV. 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

International Application No. PCT/EP 89/00972

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	and Molecular Biology, Inc., (Baltimore, US), M.L. Bayne et al.: "Structure analogs of human insulin-like growth factor I with reduced affinity for serum binding proteins and the type 2 insulin-like growth factor receptor", pages 6233-6239 --	
A	Acta Paediatrica Scandinavia, (Suppl.), volume 325, 1986, (Stockholm, SE), A. Skottner et al.: "Experimental Research on IGF-1", pages 107-111 --	
A	Annual Review on Biochemistry, volume 56, 1987, Annual Reviews inc., (Palo Alto, CA, US), M.A. Kukuruzinska et al.: "Protein glycosylation in yeast", pages 915-944	