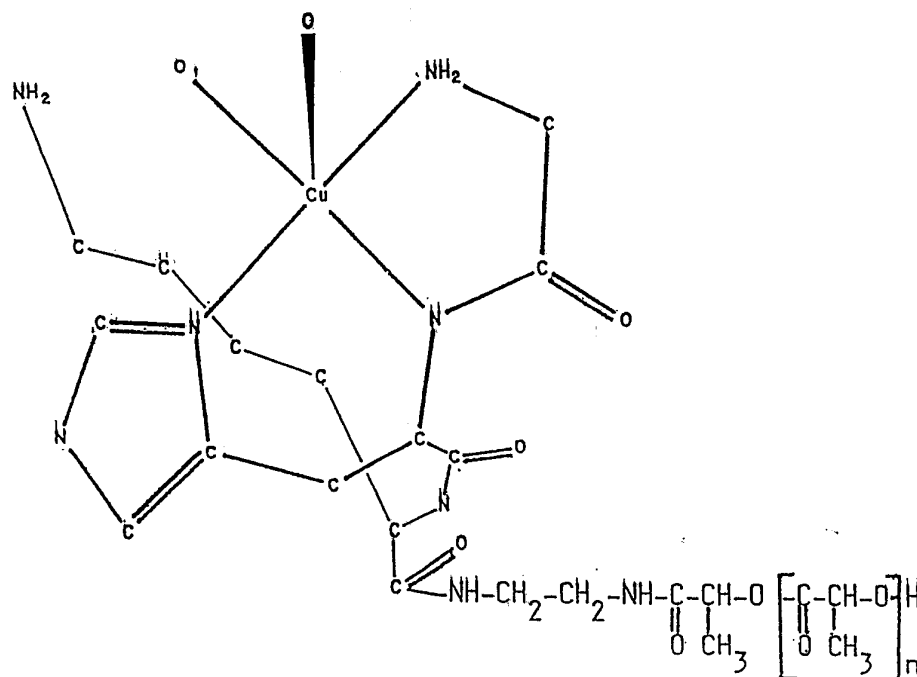


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(54) Title: A GROWTH FACTOR IN CONNECTION WITH ARTIFICIAL TRANSPLANTS



(57) Abstract

This invention utilizes the biological activity of the copper complex of the tripeptide glycyl-L-histidyl-L-lysine. This peptide is covalently bound to artificial transplants where it has a chemoattractive effect and also acts when it is released by hydrolysis of the transplants. The peptide increases fibroblastic collagen synthesis thereby enabling a more rapid replacement of the transplants with human tissue.

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Title:

1

A growth factor in connection with artificial transplants.

Technical field:

This invention utilizes the biological activity of the copper complex of the tripeptide glycyl-L-histidyl-L-lysine. This peptide is covalently bound to artificial transplants where it has a chemoattractive effect and also acts when it is released by hydrolysis of the transplants. The peptide increases fibroblastic collagen synthesis thereby enabling a more rapid replacement of the transplants with human tissue.

Background:

The evolution of multicellular organisms is dependent upon the ability of cells to communicate with each other and with their environment. One method the cells use for this communication is to release peptides that induce a specific activity in the receiving cell.

Such a peptide with biological activity is the human plasma growth factor copper-binding tripeptide glycyl-L-histidyl-L-lysine (GHK-Cu²⁺).

L Pickart and S Lovejoy describe the properties of the peptide GHK-Cu²⁺ in Methods in Enzymology, Vol 147 (1987) pp 314-328, Academic Press. It plays a physiological role in the healing of wounds by stimulation the complex course of events necessary for the formation of new tissues such as angiogenesis and axon and dendrite growth in neurons. The peptide has also a chemoattractive effect on cells necessary for wound-healing such as macrophages, monocytes, mast cells and capillary endothelial cells.

Collagen is a fibrous protein that constitutes a

quarter of the total amount of protein in the human body. It is the major fibrous element of skin, bone, tendons, cartilage, ligaments and blood vessels. Collagen is synthesized by fibroblasts, a type of cell localized in the area surrounding other cells and tissues.

In a publication in FEBS Letters 238 (1988) 343-346, F-X Maquart et al present data showing that GHK-Cu^{2+} stimulates collagen synthesis in cultures of fibroblasts. This stimulation is observed at a peptide concentration as low as 10^{-12}M and reaches a maximum at 10^{-9}M where the increase in collagen synthesis is about 80 %.

In European Patent Application 0190736, GHK-Cu^{2+} with a modified C-terminal carboxyl group is used as an ointment for faster healing of wounds.

GHK-Cu^{2+} possesses a significant superoxide dismutase-like activity with a rate constant of about 25 % of the activity of enzymatic Cu,Zn-superoxide dismutase on a molar basis. When wounds and damaged tissue are present, cells from the immune system invade the injured area and large quantities of toxic oxygen radicals are released to kill invading bacteria. These radicals also destroy intact tissue which starts a vicious circle where more radicals are released, thus delaying healing. GHK-Cu^{2+} superoxide dismutase activity detoxifies the tissue destroying superoxide anions.

Aggregation of blood platelets is the first stage of thrombosis. GHK-Cu^{2+} inhibits this aggregation and it also inhibits the hormone thromboxane which causes thrombosis.

The structure of the tripeptide GHK-Cu^{2+} is shown in Fig 1. The affinity of the peptide for copper is very high with a pK for the dissociation constant of about 16. For biological activity the ϵ -amino group

on the side-chain of the lysine must be free.

The technical problem:

The use of artificial materials in surgery is gradually increasing. In orthopedic surgery, transplants are used for soft parts such as muscles, tendons and ligaments. In the case of ligaments, the preferred material used has been poly propylen bands which are unfortunately not degradable, and results have been unfavorable in the long run. When degradable material is used to replace ligaments, it is of utmost importance that this material is biocompatible. Polymers of lactic acid and glycolic acid are degraded to non-toxic products that living tissues tolerate. In the human body they are hydrolysed to their monomers. Lactic acid is metabolized to carbondioxide and water in the citric acid cycle while glycolic acid is excreted with the urine or is oxidized to pyruvate and is also metabolized in the citric acid cycle resulting in the same products as for lactic acid. These harmless products are very advantageous when compared with the hydrolysis products of many other polymers.

Polymers of lactic and glycolic acid have been used for long time as resorbable suture material. When this material is used for artificial ligaments the main problem is that they hydrolyse so rapidly that the body is not allowed enough time to replace them with its own tissue where collagen constitutes the main part. The rapid hydrolysis could be compensated for if one could increase the rate of collagen synthesis.

The solution:

The invention presented here is that material used for transplants incorporates the copper-complex of the tripeptide glycyl-L-histidyl-L-lysine GHK-Cu^{2+} . As may

be seen in Fig 1, the C-terminal carboxyl group is not involved in the copper binding and for that reason is suitable for the covalent binding of the peptide to transplants.

With well-known peptide synthesis, the free carboxyl group of the peptide is coupled to a polymer containing free primary amino groups or to a polymer which can be modified to contain free amino groups. Since the peptide has maximal activity at a concentration as low as 10^{-9} M where it almost doubles the fibroblasts synthesis of collagen, it is fully sufficient that one peptide molecule is coupled to a polymer molecule having a molecular weight of 500 000.

Thought of chemoattraction and when the peptide GHK-Cu^{2+} is released by hydrolysis of the transplant in the tissues, very specific biological processes are started involving many closely coordinated reactions that must be in balance in order to allow healing to occur. This includes an increase in collagen synthesis, an increase of tissue-protective superoxide dismutase activity, and a chemoattractive effect on among others mast cells and capillary endothelial cells which accumulate at the transplant site, there stimulating new formation of blood vessels and the flow nourishment to the area.

Best mode of carrying out the invention:

Example 1.

The free carboxyl group of poly-L-lactic acid are activated with carbodiimide and then allowed to react with an excess of a diamine, $\text{NH}_2(\text{CH}_2)_n\text{NH}_2$ where $n = 2-6$ depending upon the length of the desired spacer-arm. The carbodiimide will be dicyclohexylcarbodiimide if the reaction is carried out in organic solution, or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide if the

reaction is performed in a water solution.

The tripeptide NH_2 -glycyl-L-histidyl-L-lysine-COOH is blocked on the amino group of glycine, the imidazole group of histidine and the ϵ -amino group of lysine with 9-fluorenylmethyl chloroformate (FMOC-Cl). The free carboxyl group of lysine is activated with carbodiimide as described above and allowed to react with the free amino group of the derivative of poly-L-lactic acid $[\text{poly-L-lactic acid-C-NH}(\text{CH}_2)_n\text{-NH}_2]$.

After removal of the protective FMOC-groups with piperidine and the addition of copper (II) acetate the desired product is obtained, the structure of which is shown in Fig 2. In this figure the diamine $\text{NH}_2(\text{CH}_2)_2\text{NH}_2$ has been used. The same methods of synthesis are also valid for poly-DL-lactic acid, poly-D-lactic acid, polyglycolic acid and their co-polymers.

Above, FMOC has been used as the blocking group. Another blocking agent is the tert-butoxycarbonyl group (t-BOC-). Deblocking after the synthesis is in this case performed with dilute acid (25% trifluoroacetic acid).

The peptide glycyl-L-histidyl-L-lysine is commercially available.

Other examples

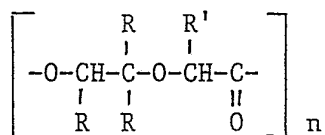
Another way for synthesis is by usual peptide synthesis coupling the amino blocked L-lysine, L-histidine and glycine one after the other to the amino derivative of the polymer.

Lactic acid and glycolic acid are α -hydroxycarboxylic acids. A number of other α -hydroxycarboxylic acids are useful for homo- or co-polymerization. Examples are α -hydroxybutyric acid, α -hydroxysisobutyric acid, α -hydroxyvaleric acid, α -hydroxyisovaleric acid.

To increase the amount of peptider per polymer molecule, one can treat the poly- α -hydroxycarboxylic acid with mild hydrolysis to increase the available number of carboxyl groups.

Among β -hydroxycarboxylic acid D- β -hydroxybutyric acid has been used as a polymer for transplants (British Patent 1034123).

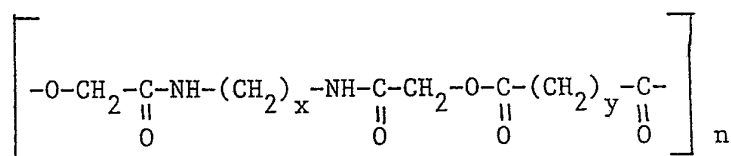
Polymer based on p-dioxanon has also been used as transplants. This has the formula



where R and R' represent hydrogen, methyl- or ethyl-group and n the degree of polymerization.

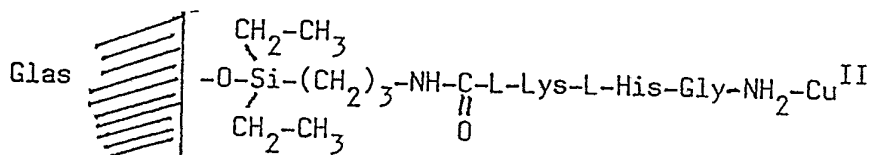
Synthetic biodegradable polyester amides have been described by T H Barrows et al at 3M Center, St Paul, Minnesota, USA.

These polymers consist of units with the formula



All of these polymers can be coupled covalently to $\text{GHK}-\text{Cu}^{2+}$ with the same methods described above.

Another method is to covalently couple $\text{GHK}-\text{Cu}^{2+}$ to other biocompatible materials, which may or may not be resorbed, that contain an amino group or a site where an amino group can be introduced. For example glass or silicic acid can be silylated with 3-amino-propyl-triethoxysilane. In this case a great number of $\text{GHK}-\text{Cu}^{2+}$ could be bound to the glass.



When metal transplants are to be made the surface can first be covered with a protein, for instance serumalbumin, and the amino groups bridge-bound with glutardialdehyde. After introduction of new amino groups, the GHK-Cu^{2+} can be bound to the protein.

Fig 1 shows the three-dimensional structure of GHK-Cu^{2+} . The side-chains of lysine and histidine and the amino terminal of glycine are necessary for the copper-binding and for the biological activity. One of the hydrogens of the α -carbon in the glycine can be exchanged with another radical without disturbing the conformation of the copper-complex. Proposals for new tripeptides can, for example, be Ala-His-Lys, Val-His-Lys or Leu-His-Lys. One advantage of this is that we can use the D-form of the amino-terminal amino acid which renders the peptide more resistant to proteolytic hydrolysis.

In the practical use of the invention reinforcing material can of course be used. This material can be degradable or not degradable.

Although the invention has been described here with reference to certain examples, it should be observed the invention is by no means restricted to such specific characteristics since closely related variations and modifications are obvious for the specialist in the field.

Claims:

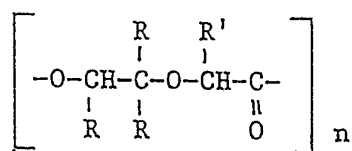
1. A method of increasing natural collagen synthesis at the site of an artificial transplant by immobilizing a biologically active agent on the transplant, characterized by the fact that this agent is a growth factor for collagen and that it is the copper-complex of the tripeptide glycyl-L-histidyl-L-lysine which is immobilized because the carboxyl-terminal

carboxyl group of lysine is covalently bound to the transplant.

2. A method according to claim 1 characterized by the fact that the transplant consists of poly-L-lactic acid, polyglycolic acid or copolymer of this, where the carboxyl group of the polymer has first been bound by a peptide bond to a diamine, preferably 1,6,-hexandiamine, and where the free amino group of the product is bound by a peptide bond to the carboxyl group of the lysine in the copper complex of the tripeptide glycyl-L-histidy-L-lysine (GHK-Cu²⁺).

3. A method according to claim 2 characterized by the fact that the transplants are homo- or copolymers of the L-hydroxy-derivatives of glycolic acid, lactic acid, butyric acid, isobutyric acid, valeric acid or isovaleric acid.

4. A method according to claim 2 characterized by the fact that the transplant is a polymer based on p-dioxanone consisting of units with the formula

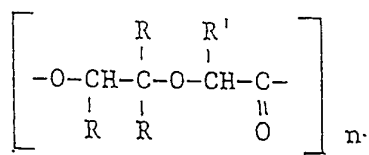


where R and R' indicate hydrogen, methyl or ethyl group and n the degree of polymerization.

AMENDED CLAIMS

[received by the International Bureau
on 5 July 1991 (05.07.91);
original claims 1 and 3 amended; other claims unchanged (1 page)]

1. A method of increasing natural collagen synthesis at the site of an artificial transplant by immobilizing a biologically active agent on the transplant, characterized by the fact that this agent is a growth factor for collagen and that it is the copper-complex of the tripeptide glycyl-L-histidyl-L-lysine which is immobilized because the carboxyl-terminal carboxyl group of lysine is covalently bound to the transplant.
2. A method according to claim 1 characterized by the fact that the transplant consists of poly-L-lactic acid, polyglycolic acid or copolymer of this, where the carboxyl group of the polymer has first been bound by a peptide bond to a diamine, preferably 1,6,-hexandiamine, and where the free amino group of the product is bound by a peptide bond to the carboxyl group of the lysine in the copper complex of the tripeptide glycyl-L-histidyl-L-lysine (GHK-Cu²⁺).
3. A method according to claim 2 characterized by the fact that the transplants are homo- or copolymers of glycolic acid, lactic acid, the α -hydroxy-derivatives of butyric acid, isobutyric acid, valeric acid or isovaleric acid.
4. A method according to claim 2 characterized by the fact that the transplant is a polymer based on p-dioxanone consisting of units with the formula



where R and R' indicate hydrogen, methyl or ethyl group and n the degree of polymerization.

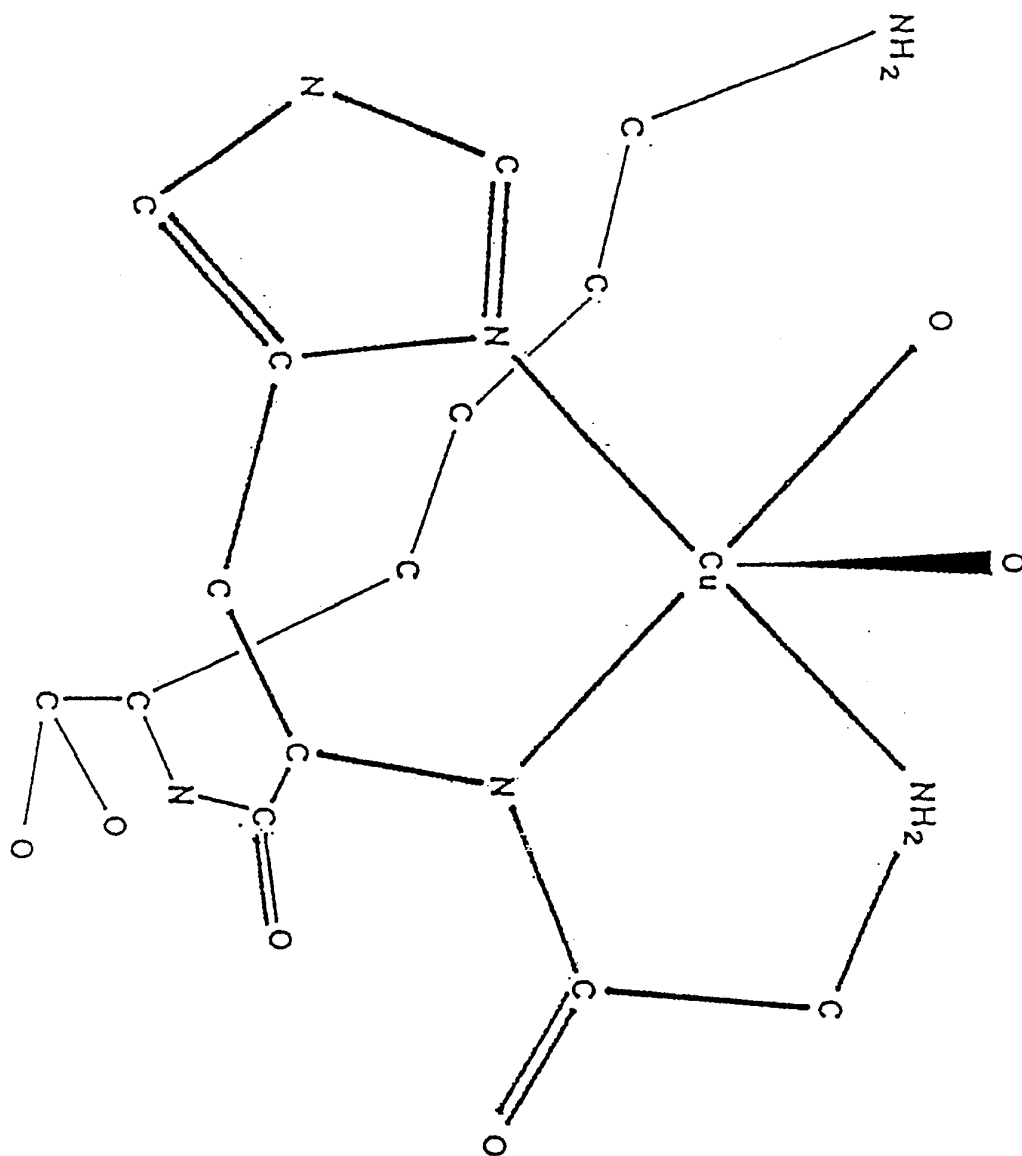
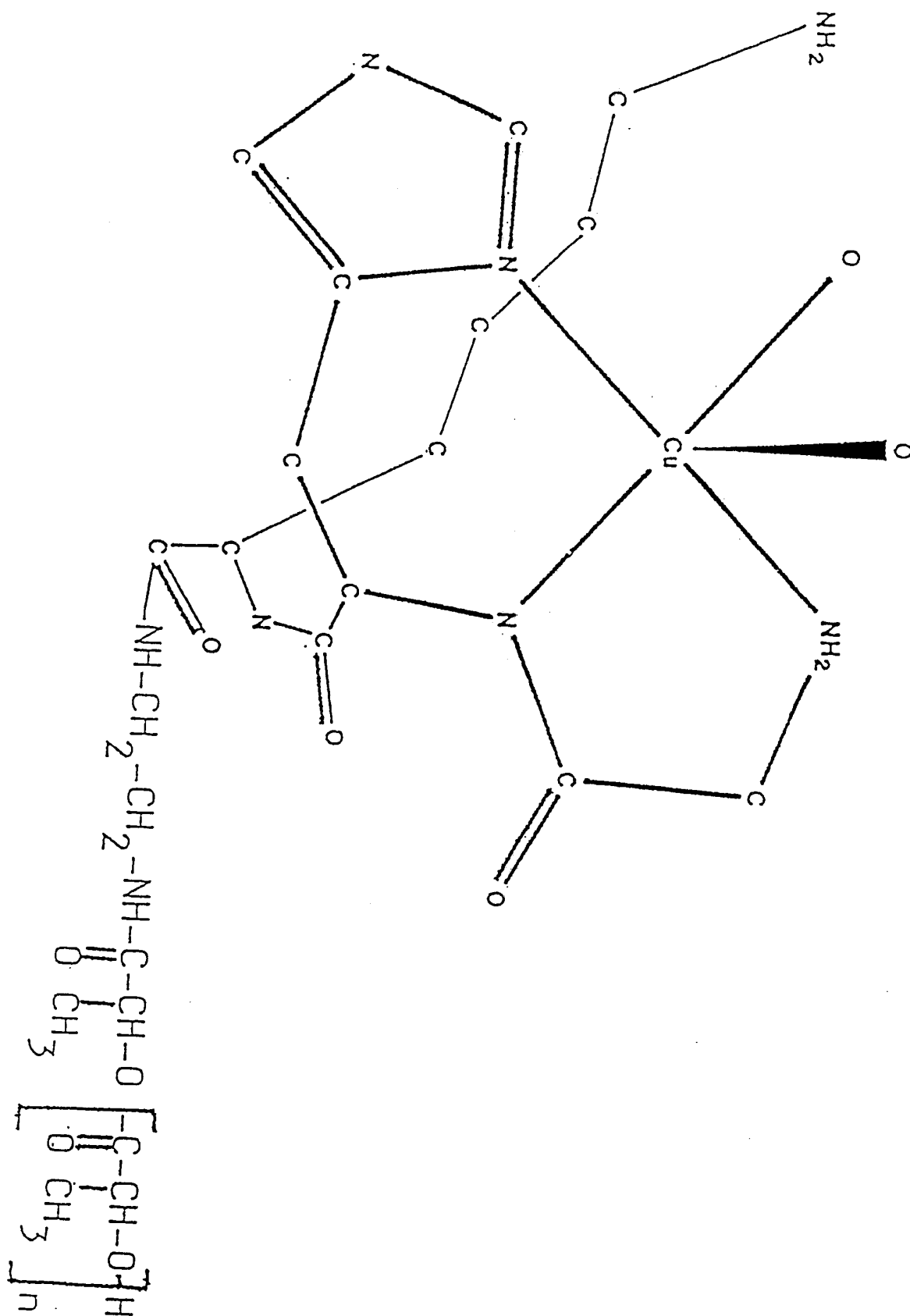


Fig. 1.

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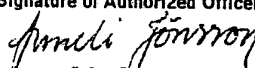
Fig. 2.



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00079

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 IPC5: A 61 K 37/02, A 61 L 27/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	A 61 K; A 61 L	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
SE,DK,FI,NO classes as above		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	WO, A1, 8912441 (PROCYTE CORPORATION) 28 December 1989, see the whole document --	1-4
A	EP, A1, 0358819 (DOW CORNING KABUSHIKI KAISHA) 21 March 1990, see col. 6 lines 27-35 --	1-4
A	WO, A1, 8808714 (PROCYTE CORPORATION) 17 November 1988, see the whole document --	1-4
A	EP, A2, 0190736 (IAMA INCORPORATED) 13 August 1986, see the whole document --	1-4
* 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	
14th May 1991	1991 -05- 1 5	
International Searching Authority	Signature of Authorized Officer	
SWEDISH PATENT OFFICE	 Anneli Jonsson	

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
A	WO, A1, 8803417 (MATERIAL CONSULTANTS OY) 19 May 1988, see the whole document -- -----	1-4

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 91/00079**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on **91-03-23**
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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EP-A1- 0358819	90-03-21	JP-A- 64002643 US-A- 4882162	89-01-06 89-11-21
WO-A1- 8808714	88-11-17	AU-D- 1794988 EP-A- 0313643 JP-T- 2500275 US-A- 4767753	88-12-06 89-05-03 90-02-01 88-08-30
EP-A2- 0190736	86-08-13	JP-A- 61191694 US-A- 4665054 US-A- 4767753 US-A- 4810693 US-A- 4877770	86-08-26 87-05-12 88-08-30 89-03-07 89-10-31
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