

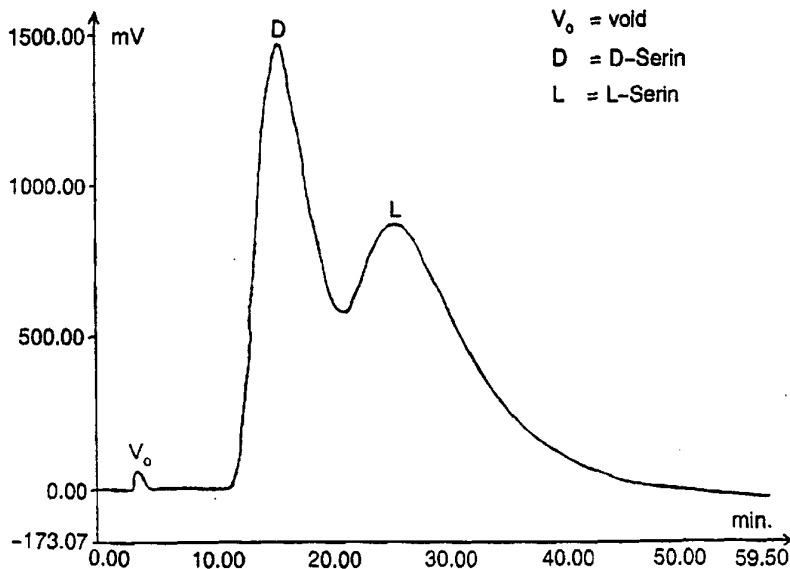


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

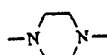
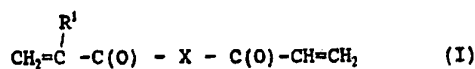
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| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>C07D 295/185, 241/02, 233/02, 233/04, 229/00</b>   | <b>A1</b>  | <b>(11) International Publication Number:</b> <b>WO 95/01347</b><br><b>(43) International Publication Date:</b> 12 January 1995 (12.01.95) |
| <b>(21) International Application Number:</b> PCT/FI94/00293<br><b>(22) International Filing Date:</b> 28 June 1994 (28.06.94)<br><b>(30) Priority Data:</b><br>9302247-3                      30 June 1993 (30.06.93)                      SE<br><b>(71) Applicant (for all designated States except US):</b> CULTOR OY [FI/FI]; Kyllikinportti 2, FIN-00240 Helsinki (FI).<br><b>(72) Inventor; and</b><br><b>(75) Inventor/Applicant (for US only):</b> KORHONEN, Pirjo [FI/FI]; Harju 2 E 40, FIN-02460 Kantvik (FI).<br><b>(74) Agent:</b> OY JALO ANTI-WUORINEN AB; Iso Roobertinkatu 4-6 A, FIN-00120 Helsinki (FI). | <b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i> |  |

**(54) Title:** NOVEL CROSS-LINKING AGENTS AND USE THEREOF**(57) Abstract**

The present invention relates to novel cross-linking agents of the type (I), wherein X has the meaning of (a), (b), (c), (d), which rings (a) to (d) can carry 1 to n substituents, wherein n equals the number of protonated carbon atoms in the ring, the substituents being selected from the group consisting of -OH, -SH, -CN, -C(O)NH<sub>2</sub>, C<sub>1</sub>-C<sub>3</sub>-alkyl and hydroxy-C<sub>1</sub>-C<sub>3</sub>-alkyl, or in the rings (a) to (c), one or more of the -CH<sub>2</sub>-ring members can be replaced by a carbonyl group -C(O)-, and R<sup>1</sup> is selected from the group consisting of C<sub>1</sub>-C<sub>3</sub>-alkyl or hydroxy, and use thereof for the preparation of polymers in water or in organic solvents. The high water solubility of the cross-linking agents compared to other cross-linking agents, makes them suitable for the preparation of strongly cross-linked water compatible polymers, usable as a chromatographic separation material for the separation of amino acid enantiomers, peptides, proteins and viruses. The novel cross-linking agent is e.g. synthesized in two steps by acylating the imino functions in the heterocyclic starting compound in two steps.



V<sub>0</sub> = void  
 D = D-Serin  
 L = L-Serin



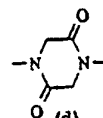
(a)



(b)



(c)



(d)

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## Novel cross-linking agents and use thereof

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Water soluble cross-linking agents are of great industrial interest for the preparation of mechanically stable water compatible polymers. In order to obtain a high mechanical stability in a hydrophilic polymer<sup>1</sup>, a high degree of cross-linking is necessary when polymerizing, for example, in water. This in turn requires a high water solubility of the molecule functioning as cross-linker.

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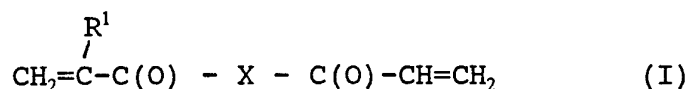
In various (bio)chemical applications there is a need for hydrophilic, inert, porous and mechanically stable polymer matrices. Within the field of separation techniques based on chromatography, hydrophilic polymer matrices, e.g. Sephadex and Sepharose (both products from Pharmacia) are well suited for polar water soluble compounds. Another conventional separation technique for water soluble molecules, electrophoresis, is common for the separation of peptides and proteins. In electrophoresis N,N'-methylene bisacrylamide is often used as a cross-linking agent, in many cases the cross-linking agent 1,4-bisacryloylpiperazine, which is closely related to this invention, has given better results<sup>3</sup>.

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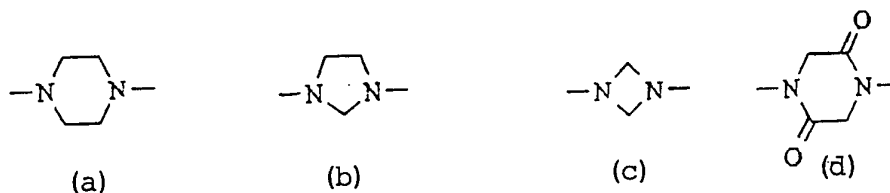
The application of hydrophilic mechanically stable polymers has given improved results in the so-called solid phase synthesis of peptides<sup>4</sup>.

This invention relates to new cross-linking agents of the formula I

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wherein X has the meaning of



10 which rings (a) to (d) can carry 1 to n substituents, wherein n equals the number of protonated carbon atoms in the ring, the substituents being selected from the group consisting of -OH, -SH, -CN, C<sub>1</sub>-C<sub>3</sub>-alkyl and hydroxy-C<sub>1</sub>-C<sub>3</sub>-alkyl, or in the rings (a) to (c), one or more of the -CH<sub>2</sub>-ring members can be replaced by a carbonyl group -C(O)-, and R<sup>1</sup> is selected from the group consisting of C<sub>1</sub>-C<sub>3</sub>-alkyl or hydroxy.

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Especially contemplated within the invention is a compound of the formula above, wherein X is unsubstituted piperazine and R<sup>1</sup> is methyl, i.e. 1-acryloyl-4-methacryloylpiperazine, or 1-[1-oxo-2-methyl-2-propenyl]-4-[1-oxo-2-propenyl]-piperazine, sum formula C<sub>11</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>. 1-acryloyl-4-methacryloylpiperazine has an unusually high water solubility (2 kg/liter at room temperature) and is thus very useful for the preparation of strongly cross-linked water compatible polymers. 1-acryloyl-4-methacryloylpiperazine has a water solubility which is 667 times higher (per weight) than the often used cross-linking agent N,N'-methylenebisacrylamide (solubility 3.0 g/liter at room temperature<sup>2</sup>. It can also be mentioned that 1-acryloyl-4-methacryloylpiperazine has a high solubility in organic solvents such as e.g. chloroform (2 kg/liter at room temperature), wherefore this cross-linking agent also can be used for the preparation of polymers in organic solvents.

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35 The invention also relates to the preparation of the novel cross-linking agents and their use for the preparation of

polymers, especially of polymers suitable for the separation of amino acids, peptides, proteins and viruses.

5 The aim of the invention was to obtain an improved, water soluble cross-linking agent for use in the preparation of water compatible mechanically stable polymers provided with a special kind of molecular imprint<sup>5</sup>. This special kind of molecular imprinting technique relates to a chromatographic separation method for amino acids, based on ligand-exchange chromatography<sup>6</sup> ("the Davankov method") in combination with  
10 the molecular imprinting technique<sup>7</sup>. Water soluble cross-linking agents which can be used in the preparation of polymers containing molecular imprints must have a high degree of water solubility in order to provide for a high degree of cross-linking and consequently a high mechanical  
15 stability for chromatographic applications. In addition, the structure of the cross-linking agent must be flexible enough to secure the accessibility to the active sites in the ready polymer which are provided with molecular imprints<sup>8</sup>.  
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In the WO-publication 93/13034 the combined ligand-exchange chromatography and molecular imprinting technique is described. According to this technique, a diastereomeric metal ion complex between an amino acid based monomer unit and a  
25 selected amino acid is prepared, the complexed amino acid based monomer unit in the metal ion complex is polymerized in the presence of a cross-linking agent (template polymerization) and the selected amino acid is removed from the polymerized metal ion complex to form a molecular imprint  
30 of the selected amino acid in the polymeric material. This polymeric material can thereafter be used for chromatographic (e.g. HPLC) separation of the selected or a structurally similar amino acid from a mixture of other amino acids, inclusive from a racemic mixture of the selected  
35

amino acid.

The synthesis of the compounds of the formula I being unsymmetrically substituted with an acryloyl and a substituted acryloyl group is a much more demanding task than the synthesis of corresponding symmetrical bisacryloyl substituted compounds, e.g. 1,4-bisacryloylpiperazine, especially if the intermediate and the end product are to be obtained with a high degree of purity and in a high yield without using chromatographic methods during the further processing.

The synthesis of the compounds of the formula I is carried out by first acylating one of the imino functions in a compound having the formula (II) H - X - H, wherein X has the meaning given above, with an acid of the formula  $\text{CH}_2=\text{C}(\text{R}^1)\text{COOH}$ , or especially its chloride, which gives a 1-substituted derivative. The said  $\text{R}^1$ -substituted acryloyl group can also be introduced by reacting a different type of activated acid, for example an active ester between the acid and p-nitrophenol or 1-hydroxybenztriazole, with the heterocyclic compound. In the second and last step, the second imino function is acylated with acrylic acid, or preferably with acryloylchloride, to give the compound of the formula I. Also the acryloyl group can be introduced by using a different type of activated acrylic acid, for example an active ester between acrylic acid and p-nitrophenol or 1-hydroxybenztriazole, for the reaction with the intermediate.

Both reaction steps are preferably carried out under optimal reaction conditions. For the preparation of the 1-substituted derivative in the first step, an excess of 20 to 40 % of acylating chloride is used compared to the heterocyclic compound II, e.g. piperazine, and the pH of

the reaction solution is adjusted and kept between 2.75 and 2.90 and the temperature between 0 and - 5°C when adding the chloride to the reaction solution. The working up of the intermediate, e.g. the 1-methacryloylpiperazine, takes place with simple extractions with dichloromethane and ethylacetate or the like. The intermediate, especially the 1-methacryloylpiperazine, can in this manner be isolated with high yields (appr. 75 to 80 % of the theor. maximum) and with a very high degree of purity (99 % according to HPLC-analysis).

The second reaction step is carried out in a water/organic solvent two-phase system. The organic solvent can be dichloromethane, chloroform, hexane, toluene, benzene or any other inert solvent which is immiscible with water. When preparing the end product, one equivalent of all reactants is used (e.g. 1-methacryloylpiperazine, acryloylchloride, NaOH). The reaction temperature is kept under 0 °C during the addition of acryloylchloride to the reaction solution. The working up of the end product takes place using simple extractions. The end product can in this manner be isolated with high yields (80 to 95 % of the theor. maximum) and with a very high degree of purity (99% according to HPLC-analysis).

According to the invention, it is also possible to first acylate the starting compound of formula II with acrylic acid or a derivative thereof, and in the second step to introduce the R<sup>1</sup>-substituted acryloyl group, in a manner analogous to the two step process described above.

The novel cross-linking agents can be polymerized i.a. using the bulk polymerization method, wherein the monomers are first dissolved in a suitable solvent whereby the monomers, during the polymerization, precipitate as a poly-

meric material in the solvent. Suitable solvents in the bulk polymerization are for example water, methanol, ethanol, 1-propanol, dichloromethane, chloroform, formic acid, acetic acid, N,N-dimethylformamide, dimethylsulfoxide and acetonitril. The solvent is also called a porogen as it forms pores in the polymeric material. The cross-linking agent can be copolymerized with various functional monomers, i.a. with acryloyl-, methacryloyl-, vinyl-, acrylate- and methacrylate-based monomers.<sup>9</sup>

The polymerization can be initiated with thermal or photolytic homolysis of azobisnitriles, e.g. 2,2'-azobis(2-methylpropionitrile) [AIBN]. Polymerizations with this cross-linking agent can also be initiated with the peroxodisulfate/N,N,N',N'-tetramethylethylenediamine system commonly used in aqueous mediums.

Bead-shaped polymer particles using the novel cross-linking agent can also be prepared using a so-called reversed suspension polymerization method. Such beads can be obtained in varying sizes depending on a variety of factors, such as choice of detergent(s), volumetric relationship between water and organic phase, shape of polymerization vessel, stirrer speed, etc. The beads, usually of a size of 45 to 80  $\mu\text{m}$ , have a high mechanical stability and they withstand pressures up to 200 bar without being deformed.

The polymers thus obtained using the novel cross-linking agents can be used in a manner described in WO-publication 93/13034, the contents of which is included herein for reference, for the separation of amino acids from a mixture of amino acids, such as for the separation of chiral amino acids from a racemic mixture. According to the said process, the mixture of amino acids to be separated is contacted with a polymer material which is composed of cross-



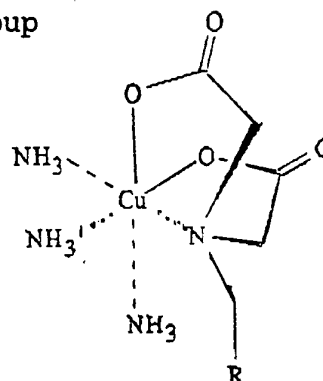
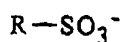
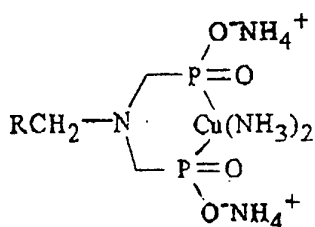
linked, amino acid-based monomer units, said polymer material containing a molecular imprint of the selected amino acid, in which molecular imprint there is also bound a divalent metallic ion and the amino-acid based monomer unit. The amino acid-based monomer is according to one embodiment N-methacrylaminomethyl-L-proline or N-methacrylaminomethyl-D-proline, which is especially suitable for the preparation of a polymer suitable for the resolution of racemic amino acids. The divalent metal is selected from copper(II), manganese(II), iron(II), cobalt(II), zinc(II), cadmium(II) and nickel(II), preferably copper(II).

Due to their high water solubility and chemical inertness, the new cross-linking agents, especially 1-acryloyl-4-methacryloylpiperazine, are suitable also for the template polymerization of biological molecules, for example peptides, proteins and viruses. Indeed, the new cross-linking agent provides a polymeric material having a chemical character compatible with peptides and proteins, and its high mechanical strength and durability makes it suitable for use in chromatography. It is assumed that the novel cross-linking agents possess a suitable degree of flexibility resulting in a polymeric material having feasible mass transfer properties enabling the diffusion of the large template into and out of the active sites during the chromatographic separation process. The porosity and the flexibility of the the polymeric material can be adjusted by the amount and nature of porogen and/or the existence of an extra small inert comonomer during the template polymerization, or by modifying the degree of cross-linking. Due to their beneficial physico-chemical properties, the novel cross-linking agents, and in particular 1-acryloyl-4-methacryloyl-piperazine, can be polymerized under different pH-values and temperatures, which is of importance in the template polymerization of proteins and peptides.

In the template polymerization of biological molecules the chelating monomer can be any monomer containing iminopoly-carboxylate, sulfonate or iminopolyphosphonate groups capable of coordination with the divalent metal ion, capable of retaining the chelating metal in the resin and possessing a favourable polymerization ratio to the new cross-linking agent. For example monosubstituted (meth)acrylamide based monomers are compatible with the new cross-linking agents, as are the following exemplary monomers, wherein R is a polymerizable vinyl group

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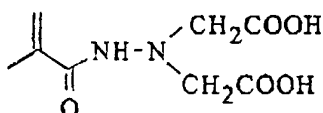
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A common chelating functional group is the iminodiacetate group, e.g. the following new monomer

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1,1-dicarboxymethyl-2-methacryloyl-hydrazine.

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The monomers may be prepared using methods which are analogous to methods already described in literature. (Houben Weil, 8, 4. Aufl. Georg Thieme verlag, 1952, Stuttgart, p. 676; Houben Weil, 10/2, 4. Aufl. Georg Thieme Verlag, 1967, Stuttgart, p. 13; W.T. Read, J. Am. Soc. 36 (1914)

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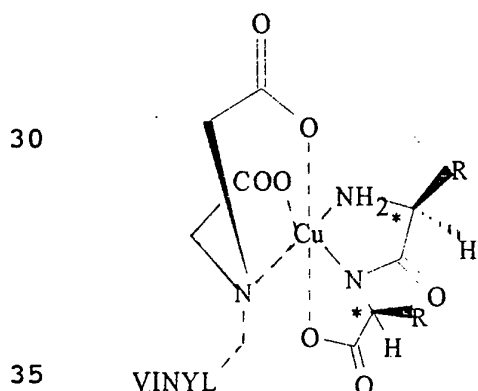
1747-1765).

As chelating metals the metals mentioned above may be used. Cu(II) is preferred due to the thermodynamic stability of the mixed Cu(II)-complexes. The net charge on the metal ion must remain positive, or the metal will be stripped from the column. Of the functional groups co-ordinating with the metal ion, the iminopolyphosphonate and iminopolycarboxylate groups hold metal ions better than the sulfonate groups.

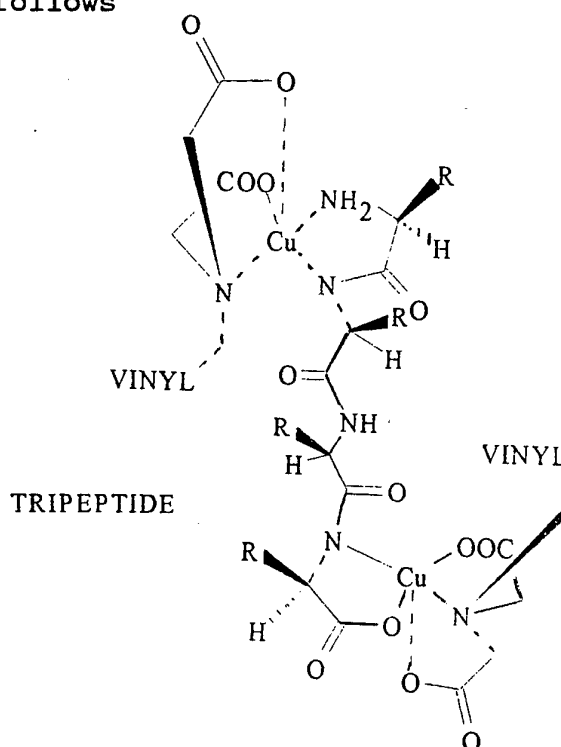
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The size of peptides to be used as templates varies from two amino acids to even some hundred amino acid units. In addition to the free end amine group and the free end carboxylate group, also functional groups in the amino acid side chains are capable of co-ordinating to metal ions, such as in histidine and cysteine. Such functional groups make it possible to use metal chelation in the creation of isomer and substrate specific cavities by template polymerization in aqueous solution. As peptides tend to co-ordinate Cu(II)-ions through the end amino group and a neighboring amide function, it is especially contemplated within the invention to form a Cu(II)-complex between an iminodi-acetic based monomer and a simple water soluble dipeptide or tripeptide, e.g. as follows

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DIPEPTIDE



The metal should naturally be selected in accordance to the number and types of groups present in the peptides capable of co-ordination with the metal.

5 Favourable water soluble peptides are small di-, tri- and oligopeptides with not too hydrophobic amino acid side chains. Suitable peptides are those formed from e.g. glycine, alanine, serine and threonine.

10 The use of viruses as templates is based on the fact that viruses have proteins on their surfaces, and proteins that contain histidines and cysteines are capable of co-ordination e.g. with Cu(II) ions. The same holds true for proteins in general, such as enzymes. In fact the presence  
15 of a single histidine residue on the surface of a protein is sufficient for the molecule recognition by specific Cu(II) chelation interaction during the template polymerization. If the protein surface contains several histidine or other chelating groups on its surface, it is recommended  
20 to use e.g. Zn(II), Co(II), Ni(II), Cd(II) or Ca(II) which form thermodynamically less stable complexes with amino acid residues.

The following examples illustrate the invention without  
25 restricting the same.

In the example 1 C-D, three polymers were prepared based on the cross-linking agents 1-acryloyl-4-methacryloylpiperazine, 1,4-bisacryloylpiperazine (1,4-bis[1-oxo-2-propenyl]-piperazine) and N,N'-methylenebisacrylamide, in order  
30 to prepare a polymer which can effectively split amino acid racemates under chromatographic (HPLC) conditions. These three polymers are compared as to their capability of separating D- and L-serine, hardness and thus applicability  
35 under chromatographic conditions. The comparison of Example

1 C-D shows that 1-acryloyl-4-methacryloylpiperazine gives a highly superior polymer both as regards the racemic separation capability and as regards physical characteristics. The polymer prepared with the commonly used cross-linking agent N,N'-methylenebisacrylamide was much too porous to be tested under HPLC conditions. In addition, the results indicate that the very presence of an acryloyl group and a methacryloyl group in the same piperazine derivative is of great importance for the beneficial properties in the resulting polymer, as a polymer made with the structurally similar 1,4-bisacryloylpiperazine in accordance with the results in the Example 1 B shows poorer characteristics.

#### 15 EXAMPLE 1

##### A. Preparation of 1-methacryloylpiperazine (1-oxo-2-methyl-2-propenyl-piperazine)

20 17.28 g (0.2 moles) of piperazine is dissolved in 400 ml of distilled water in a vessel provided with a pH-meter, thermometer, dripping funnel and a mechanical stirrer. 240 ml of 2 M HCl is added and the pH is adjusted to 2.80 with 3 M sodium acetate. While vigorously stirring, 27 ml (0.28 moles) of methacryloylchloride is added in small portions while simultaneously adjusting the pH with 3.0 M sodium acetate and cooling with an acetone/ice bath. During this process, the pH is adjusted to 2.8 - 2.9 and the temperature of the reaction solution is maintained between -5 and 0 °C. After the methacryloylchloride addition, the solution is slowly brought to room temperature. Thereafter the reaction solution is extracted with 3 x 200 ml dichloromethane. While cooling with water/ice and while vigorously stirring, the reaction solution is saturated with Na<sub>2</sub>CO<sub>3</sub> and the product is extracted thereafter with 4 x 200 ml of

ethylacetate. The ethylacetate phases are dried with  $\text{Na}_2\text{CO}_3$ . After evaporation and drying in an exsiccator 23.1 g (0.15 moles) of 1-methacryloylpiperazine is obtained as an oil, corresponding to 75 % of the theor. maximum amount.

5

$R_f = 0.23$  (silicagel in tetrahydrofuran/N-hexane, 3/1).

$k' = 1.68$ , 99% (HPLC. Solid phase:

Spherisorb  $\text{C}_8$ , 5  $\mu\text{m}$ . Mobile phase: acetonitrile/0,2 %  $\text{H}_3\text{PO}_4$ , pH 6.9, 1/4).

10

$^1\text{H}$ - NMR ( $\text{CDCl}_3/\text{TMS}$ , 200 MHz):  $\delta = 1.96$  [s, 3H,  $-\text{CH}_3$ ], 2.84 [m, 4H, (ring 2 x  $(\text{CH}_2)$ ), 3.56 (broad, 4 H, ring 2 x  $(\text{CH}_2)$ ), 5.0 [s, 1H, ( $=\text{CHH}$ )], 5.18 [s, 1H, ( $=\text{CHH}$ )].

15

$^{13}\text{C}$ - NMR ( $\text{CDCl}_3/\text{TMS}$ , 200 MHz):  $\delta = 20.49$  [ $-\text{CH}_3$ ], 46.31 [ring carbon], 115.20 [ $=\text{CH}_2$ ], 140.56 [ $>\text{C}=\text{}$ ], 171.19 [ $>\text{C}=\text{O}$ ].

MS (70 eV):  $m/e = M^+$  154 (80 %), 85, (80 %,  $M^+ -\text{C}_4\text{H}_5\text{O}$ ), 112 (15 %,  $M^+ -\text{C}_3\text{H}_6$ ).

20

IR: 3500  $\text{cm}^{-1}$  H-H stretching (m) sec.amine, 3095  $\text{cm}^{-1}$  unsat. C-H stretching (m), 2918 & 2862  $\text{cm}^{-1}$  saturated C-H stretching (m), 1648  $\text{cm}^{-1}$  (s) tert.amide carbonyl.

25

B. Preparation of 1-acryloyl-4-methacryloylpiperazine (1-[1-oxo-2-methyl-2-propenyl],4-[1-oxo-2-propenyl]piperazine)

18.6 g (0.12 moles) of 1-methacryloylpiperazine is dissolved in 110 ml of dichloromethane in a vessel provided with a mechanical stirrer, thermometer and a dripping funnel.

30

While vigorously stirring and cooling in an acetone/ice bath ( $-20$  °C) 9.95 ml (0.12 moles) of acryloylchloride in 10 ml of dichloromethane and 4.8 g (0.12 moles) of NaOH in 15 ml of water are added simultaneously in portions. The temperature of the reaction solution is maintained under 0

35

°C. The reaction solution is slowly allowed to reach room

temperature. The water and the dichloromethane phases are separated and the dichloromethane phase is washed with 2 x 50 ml of saturated NaHCO<sub>3</sub>-solution and 50 ml of a saturated NaCl-solution. A pinch of hydroquinone (autopolymerisation inhibitor) is added to the reaction solution which thereafter is dried with sodium sulfate. After evaporation and drying in an exsiccator 22.5 g (0.11 moles) of 1-acryloyl-4-methacryloylpiperazine is obtained as an oil, corresponding to 90 % of the theoretically maximum amount.

$R_f = 0.62$  (silica gel in tetrahydrofuran/N-hexane, 3/1).  
 $k' = 2.68$ , 99% (HPLC. Fast phase: Spherisorb C<sub>8</sub>, 5  $\mu$ m. Mobile phase: acetonitrile/0.2 % H<sub>3</sub>PO<sub>4</sub> pH 6.9, 1/4.).

<sup>1</sup>H-NMR (CDCl<sub>3</sub>/TMS, 200 MHz):  $\delta = 1.97$  (s, 3 H, -CH<sub>3</sub>), 3.62 (broad s, 8H, ring protons), 5.07 (s, 1H, CH<sub>3</sub>C(CO)CH<sub>2</sub>H<sub>x</sub>) 5.25 (s, 1H, CH<sub>3</sub>C(CO)CH<sub>a</sub>H<sub>x</sub>), 5.75 (dd, 1H, <sup>3</sup>J<sub>trans</sub> 10.36 Hz, <sup>3</sup>J<sub>cis</sub> 2.00 Hz), 6.31 (dd, 1H, J<sub>gem</sub> 16.79 Hz, <sup>3</sup>J<sub>cis</sub> 2,00 Hz), 6.60 (dd, J<sub>gem</sub> 16.79 Hz, <sup>3</sup>J<sub>trans</sub> 10.36 Hz).

<sup>13</sup>C- NMR (CDCl<sub>3</sub>/TMS, 200 MHz):  $\delta = 20.39$  (CH<sub>3</sub>),  $\equiv 41-43$  (ring C), 116.080 [(CH<sub>3</sub>) (CO) C = CH<sub>2</sub>], 127.20 [(H) (CO) > C =], 128.42 [(H) (CO) C = CH<sub>2</sub>], 139.957 [(CH<sub>3</sub>) (CO) > C =], 165.59 [>C=O], 171.33 [>C=O].

MS (70 eV): m/e = M + 208 (40%), 193 (20 %, M + -15), 153 (20 %, M + - C<sub>3</sub>H<sub>3</sub>O), 139 (50 %, M + - C<sub>4</sub>H<sub>5</sub>O), 85 (100 %, M+ - C<sub>7</sub>H<sub>8</sub>O<sub>2</sub>), 69 (90 %), 55 (60 %).

IR: 3095 cm<sup>-1</sup> unsaturated C-H stretching (m), 2918 & 2862 cm<sup>-1</sup> saturated C-H stretching (m), 1648 cm<sup>-1</sup> (s) tert.amide carbonyl.

C. Preparation of a polymer using 1-acryloyl-4-methacrylo-

## ylpiperazine

2.47 g (0.0064 moles) of a (N-[methacrylamidomethyl]-L-pro-  
linate)(L-serinate)-cupper[II] x 2 H<sub>2</sub>O complex and 7.70 g  
5 (0.037 moles) of 1-acryloyl-4-methacryloylpiperazine is  
dissolved in 7.0 ml of water. 700  $\mu$ l of ammonium peroxy-  
disulfate solution (1.0 g/10 ml water) is added, and the  
solution is purged with nitrogen gas for two minutes. The  
polymerization is initiated with 200  $\mu$ l of Temed  
10 (N,N,N',N'-tetramethylethylenediamine). The polymerisation  
is continued at room temperature in a glass vessel (30 ml)  
which is provided with a tight fitting plastic lid. The  
obtained hard polymer is ground, screened in methanol  
through a 25  $\mu$ m screening cloth (Retsch) and sedimented  
15 finally in methanol to eliminate undersized particles. The  
thus obtained material (10-25  $\mu$ m) is washed with 1M ammo-  
nia, water, 0.1 M CuSO<sub>4</sub> and finally with water. After pack-  
ing the material under a pressure of 200 bar in a HPLC  
column (4.6 x 100 mm) using water as the mobile phase, the  
20 column containing the polymer is equilibrated in 0.1 M NH<sub>3</sub>  
with 0.1 mM CuSO<sub>4</sub> in a Kontron HPLC-apparatus. A mixture of  
D and L-serine (100  $\mu$ g D-serine + 100  $\mu$ g L-serine) is  
injected in 20  $\mu$ l of mobile phase. Flow: 0.40 ml/min.  
Counter pressure: 15 bar. Detection at 254 nm, separation  
25 factor 1.8. Eluation profile, see Figure 1.

## D. Preparation of polymer with 1,4-bisacryloylpiperazine

2.47 g (0.0064 moles) of a (N-[methacrylamidomethyl]-L-pro-  
linate)(L-serinate)-cupper[II] x 2 H<sub>2</sub>O complex and 7.18 g  
30 (0.037 moles) 1,4-bisacryloylpiperazine is dissolved in 7.0  
ml of water. 700  $\mu$ l of ammonium peroxodisulfate solution  
(1.0/10 ml water) is added, and the solution is purged with  
nitrogen gas for two minutes. The polymerization is ini-  
35 tiated with 200  $\mu$ l of Temed. The polymerization is con-



tinued at room temperature in a glass vessel (30 ml) which is provided with a tight fitting plastic lid. The obtained less hard polymer is ground, screened in methanol through a 25  $\mu\text{m}$  screening cloth (Retsch) and sedimented finally in methanol to eliminate undersized particles. The thus obtained material (10-25  $\mu\text{m}$ ) is washed with 1M ammonia, water, 0.1 M  $\text{CuSO}_4$  and finally with water. After packing the material under a pressure of 75 bar in a HPLC column (4.6 x 100 mm) using water as the mobile phase, the column with the polymer in 0.1 M  $\text{NH}_3$  is equilibrated with 0.1 mM  $\text{CuSO}_4$  in a Kontron HPLC-apparatus. A mixture of D and L-serine (100  $\mu\text{g}$  D-serine + 100  $\mu\text{g}$  L-serine) is injected in 20  $\mu\text{l}$  of mobile phase. Flow: 0.40 ml/min. Counter pressure: 50 bar. Detection at 254 nm, separation factor 1.0. Elution profile, see Figure 2.

#### E. Preparation of polymer with N,N'-methylenebisacrylamide

2.47 g (0.0064 moles) of a (N-[methacrylamidomethyl]-L-proline)(L-serinate)-copper[II] x 2  $\text{H}_2\text{O}$  complex and 5.70 g (0.037 moles) of N,N'-methylenebisacrylamide is dissolved in 160 ml of water. 0.125 g of ammonium peroxodisulfate is added, and the solution is purged with nitrogen gas for two minutes. The polymerization is initiated with 40  $\mu\text{l}$  of Temed. The polymerization is continued at room temperature in a glass vessel which is provided with a tight fitting plastic lid. The obtained soft polymer is washed with 1 M ammonia, water, 0.1 M  $\text{CuSO}_4$  and finally with water. The polymer is too soft to be packed in a HPLC-column.

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35

## EXAMPLE 2

## 2.1 Preparation of chelating monomer 1,1-dicarboxymethyl-2-methacryloyl hydrazine

5

A. To 1.5 moles of hydrazine hydrate (Sigma H 0883) heated on a water bath, 1.07 moles of methacrylic acid dimethylester (Sigma M 1283) is added dropwise while refluxing, and the reaction mixture is heated for two days until the reaction has ended. When cooling with a cooling mixture the liquid solidifies to colourless crystal bundles. The slurry is shaken twice with anhydrous ether, the ether is poured away and the rest is heated on a water bath at about 60 °C, whereby a solution is formed and the rest of the ether is evaporated. When cooling with a cooling mixture the hydrazide is solidified to crystals which can be recrystallized from chloroform-ether. As the product methacryloylhydrazine is obtained.

20

B. 189 g (2 moles) of monochloroacetic acid in 200 ml of water is neutralized by adding 138 g (1 mole) of potassium carbonate in small portions and then 100 g (1 mole) of methacryloylhydrazine is poured into this solution. A second portion of 138 g (1 mole) of potassium carbonate is added gradually, with a steady evolution of CO<sub>2</sub> and the temperature rises to 70 °C. The solution is heated for as long as the gas evolution continues. At the end of the reaction, the hydrazino-diacetic acid is precipitated by making the solution acid with hydrochloric acid. The hydrazine acid is recrystallized from water. 1,1-dicarboxymethyl-2-methacryloylhydrazine is obtained as the product.

35

2.2 Preparation of a (1,1-dicarboxymethyl-2-methacryloyl-hydrazine)-C(II)-(peptide)-complex

5 a) A simple water soluble dipeptide without chelating side chains as template:  $\text{NH}_2\text{-ala-ser-COO}^-$ , Sigma A 3503

10 1.55 g (7.3 mmole) of 1,1-dicarboxymethyl-2-methacryloyl-hydrazine, 1.17 g (7.3 mmole) of  $\text{CuSO}_4$  and 1.28 g (7.3 mmole)  $\text{NH}_2\text{-ala-ser-COO}^-$  is dissolved in 20 ml distilled water. The pH is adjusted by means of 1 M NaOH to pH 8. The solution is evaporated at reduced pressure and dried overnight in a desiccator.

15 b) A larger water soluble peptide containing no chelating side chains:  $\text{NH}_2\text{-ala-gly-gly-COO}^-$ , Sigma A 1378

20 3.15 g (14.6 mmole) of 1,1-dicarboxymethyl-2-methacryloyl-hydrazine, 2.34 g (14.6 mmole) of  $\text{CuSO}_4$  and 1.48 g (7.3 mmole)  $\text{NH}_2\text{-ala-gly-gly-COO}^-$  is dissolved in 20 ml distilled water. The pH is adjusted by means of 1 M NaOH to pH 8. The solution is evaporated at reduced pressure and dried overnight in a desiccator.

25 c) A larger water soluble peptide with several chelating side chains:  $\text{NH}_2\text{-gly-gly-his-COO}^-$ , Sigma G 5772

30 4.73 g (21.9 mmole) of 1,1-dicarboxymethyl-2-methacryloyl-hydrazine, 3.93 g (21.9 mmole) of  $\text{ZnSO}_4\cdot\text{H}_2\text{O}$  and 1.96 g (7.3 mmole)  $\text{NH}_2\text{-gly-gly-his-COO}^-$  is dissolved in 20 ml distilled water. The pH is adjusted by means of 1 M NaOH to pH 8. The solution is evaporated at reduced pressure and dried overnight in a desiccator.

## 2.3. Preparation of polymer

NH<sub>2</sub>-ala-ser-COO<sup>-</sup> as template

5 7.3 mmole of (1,1-dicarboxymethyl-2-methacryloyl-hydrazine)-Cu(II)-(NH<sub>2</sub>-ala-ser-COO<sup>-</sup>)-complex is dissolved together  
with 8.26 g (39.7 mmole) of 1-acryloyl-4-methacryloylpipe-  
10 razine in 13 ml water in a test tube. Through the solution  
nitrogen gas is passed and before fitting the test tube  
with a tight fitting cork, 750 μl of a stock solution of  
ammonium-peroxodisulfate (1,0 g/10 ml) and 200 μl of  
N,N,N',N'-tetramethylethylenediamine (TEMED) are added. The  
polymerization is allowed to proceed at room temperature  
15 over night. The formed polymer is crushed by hand in a  
mortar and is then ground in a mechanical mortar device  
(Retsch, Haan, Germany) for 5 min. The material is sieved  
through a 25 μm sieve. Subsequently the material is allowed  
to sediment in 2 turns in 95 % ethanol, while removing the  
20 supernatant. The sediment is carefully washed with 1/1 95%  
EtOH/distilled H<sub>2</sub>O and 1 M ammonia in order to remove the  
peptide, and is then dried in a desiccator over night.

The same procedure can be carried out using NH<sub>2</sub>-ala-gly-  
gly-COO<sup>-</sup> or NH<sub>2</sub>-gly-gly-his-COO<sup>-</sup> as templates, whereby the  
25 starting complexes are a (1,1-dicarboxymethyl-2-methacry-  
loyl-hydrazine)<sub>2</sub>-Cu(II)<sub>2</sub>-(NH<sub>2</sub>-ala-gly-gly-COO<sup>-</sup>)-complex and  
a (1,1-dicarboxymethyl-2-methacryloyl-hydrazine)<sub>3</sub>-Cu(II)<sub>3</sub>-  
(NH<sub>2</sub>-gly-gly-his-COO<sup>-</sup>)-complex, respectively.

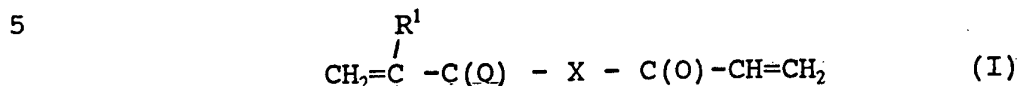
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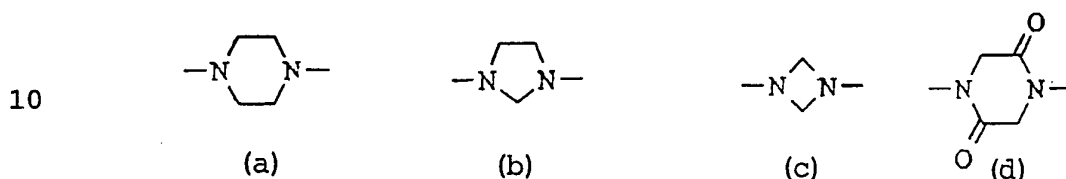
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- 25

## Claims

## 1. Compounds of the formula



wherein X has the meaning of



15 which rings (a) to (d) can carry 1 to n substituents, wherein n equals the number of protonated carbon atoms in the ring, the substituents being selected from the group consisting of -OH, -SH, -CN, C<sub>1</sub>-C<sub>3</sub>-alkyl and hydroxy-C<sub>1</sub>-C<sub>3</sub>-alkyl, or in the rings (a) to (c), one or more of the -CH<sub>2</sub>-ring members can be replaced by a carbonyl group -C(O)-, and R<sup>1</sup> is selected from the group consisting of C<sub>1</sub>-C<sub>3</sub>-alkyl or hydroxy.

20

2. The compound of claim 1, which is 1-acryloyl-4-methacryloylpiperazine.

25 3. Process for the preparation of the compound according to claim 1, characterized in that a compound of the formula H - X - H (II), wherein X has the meaning indicated in claim 1, is first reacted with an acid of the formula CH<sub>2</sub>=C(R<sup>1</sup>)-COOH or a functional derivative thereof, preferably its chloride, to prepare a compound of the formula

30 CH<sub>2</sub>=C(R<sup>1</sup>)-C(O)-N-X-H, which thereafter is reacted with acrylic acid, or a functional derivative thereof, preferably its chloride, to give the compound of formula I.

35 4. Process for the preparation of the compound according to

claim 1, **characterized** in that a compound of the formula H - X - H (II), wherein X has the meaning indicated in claim 1, is first reacted with acrylic acid or a functional derivative thereof, preferably its chloride, to prepare 1-  
5 acryloylpiperazine, which thereafter is reacted with an acid of the formula  $\text{CH}_2=\text{C}(\text{R}^1)-\text{COOH}$  or a functional derivative thereof, preferably its chloride, to give the compound of formula I.

10 5. Use of the compounds of claim 1 or 2 for the preparation of polymers.

6. Use of the compounds of claim 1 or 2 as a crosslinking agent for the preparation of polymers.

15

7. Use of the compounds of claim 1 or 2 for the preparation of polymers in aqueous media.

20 8. Use of the compounds of claim 1 or 2 for the preparation of polymers in organic solvents.

9. Use according to claim 5 for the preparation of acryl- and methacryl-based polymers.

25 10. Use according to any one of the claims 5 to 9 for the preparation of a polymer for use in separating a selected amino acid from a mixture of amino acids, or for the separation of peptides, proteins and viruses from mixtures containing the same.

30

11. Process for the preparation of a acryl and/or methacryl based polymer, **characterized** in that a acrylic acid and/or a methacrylic acid based monomer is polymerized with a compound according to claim 1 or 2 as a cross-linking agent, in an aqueous medium or in an organic solvent or in  
35

a mixture of these, or in a reverse suspension polymerization method in a medium containing water and an organic solvent, and the polymer obtained is separated.

5 12. Process for the preparation of a polymer for use in separating a selected amino acid from a mixture of amino acids, **characterized** in that a compound according to claim 1 or 2 is polymerized with a monomer which is a diastereomeric metal ion complex between an amino acid based  
10 monomer unit and the selected amino acid, preferably an enantiomer of a chiral amino acid, and the selected amino acid is separated from the prepared polymer.

15 13. Process for the preparation of a polymer for use in separating a selected peptide, protein or virus from a mixture containing said selected species, **characterized** in that a compound according to claim 1 or 2 is polymerized with a monomer which is a diastereomeric metal ion complex between a monomer unit containing sulfonate, iminopolycarboxylate and/or iminopolyphosphonate groups, and the selected species, and the selected species is separated from the  
20 prepared polymer.

25 14. Process according to claim 12 or 13, **characterized** in that the metal is selected from copper(II), manganese(II), iron(II), cobalt(II), zinc(II), cadmium(II) and nickel(II), preferably copper(II).

30 15. Process according to claim 12 and 14, **characterized** in that the monomer is a copper(II) complex between N-methacrylamidomethyl-L-prolinate or N-methacrylamidomethyl-D-prolinate and the selected amino acid.

35 16. Use of a polymer obtained according to the process of any one of the claims 12-15 in HPLC separation techniques.



Figure 1

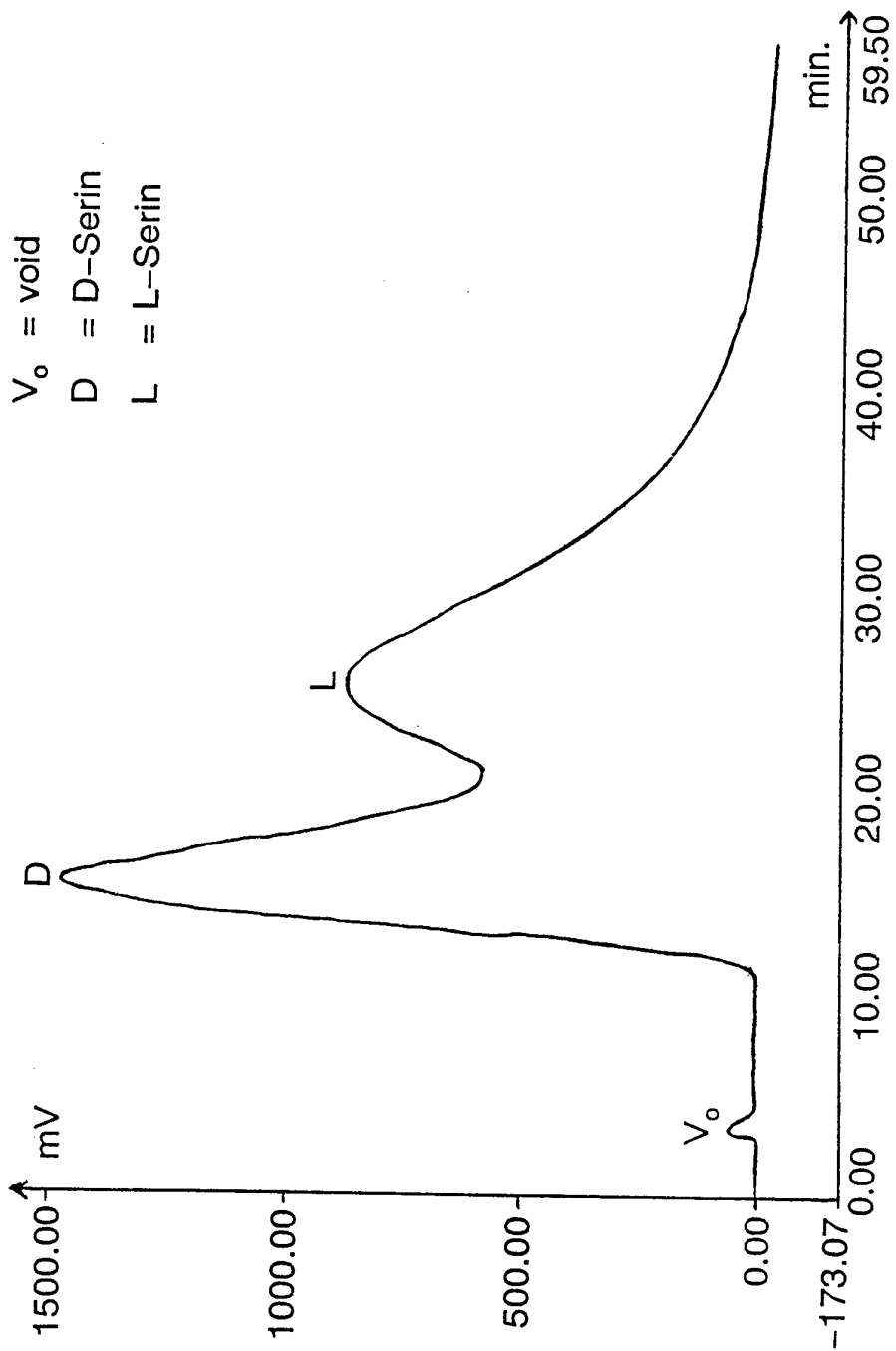
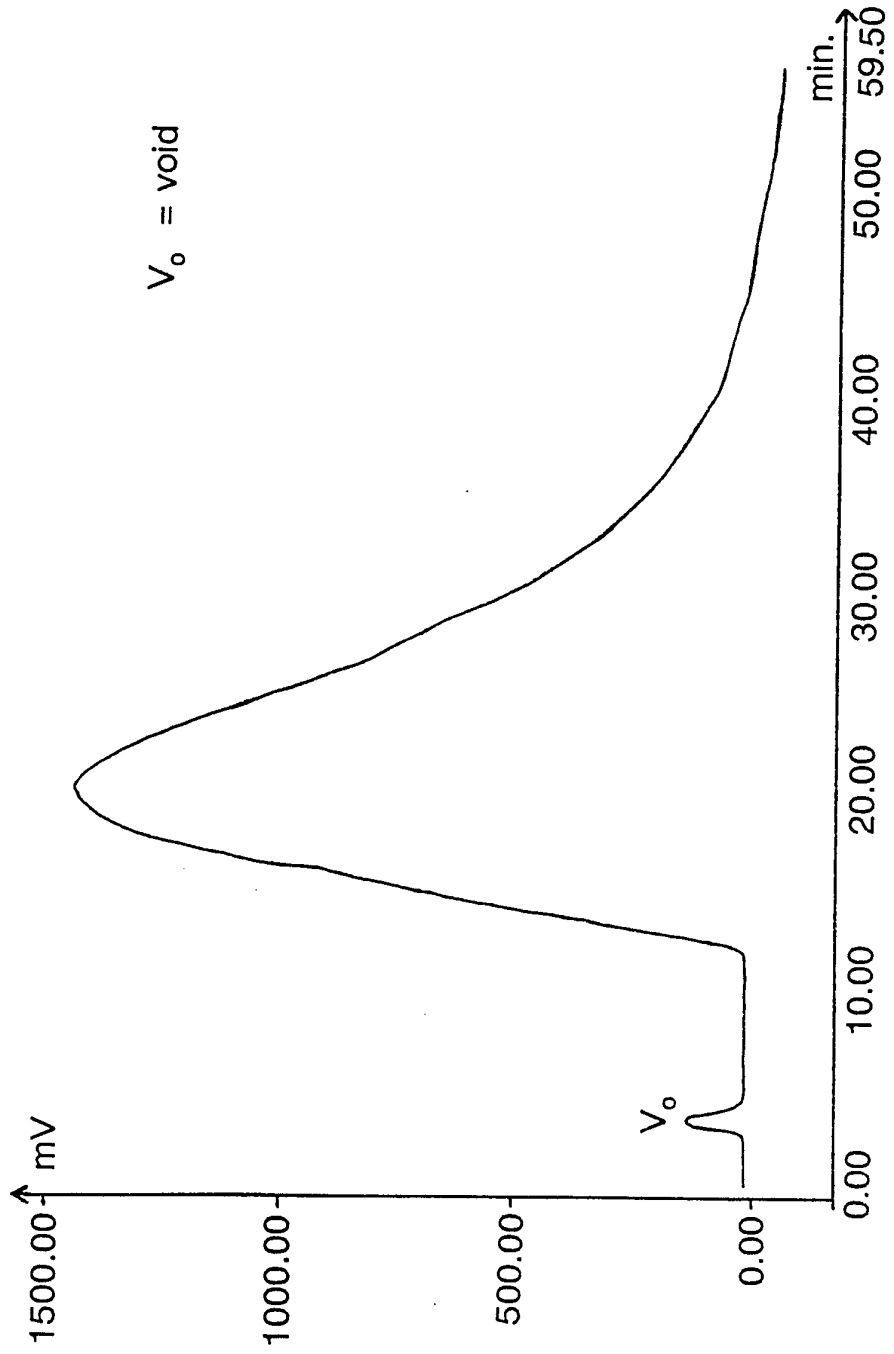


Figure 2



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 94/00293

| A. CLASSIFICATION OF SUBJECT MATTER   |  |  |
|---|--|--|
| IPC : C07D 295/185, C07D 241/02, C07D 233/02, C07D 233/04, C07D 229/00<br>According to International Patent Classification (IPC) or to both national classification and IPC   |  |  |
| B. FIELDS SEARCHED  |  |  |
| Minimum documentation searched (classification system followed by classification symbols)   |  |  |
| IPC : C07D  |  |  |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched   |  |  |
| SE,DK,FI,NO classes as above  |  |  |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  |  |  |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT  |  |  |
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No.  |
| X   | Chemical Abstracts, vol. 68, 1968, (Columbus, Ohio, USA), Shigeho Inaba et al: "Substituted piperazines", page 4815, THE ABSTRACT No 49649M, Japan 11,955('67)                                       | 1-16   |
|   | --   |  |
| X   | Chemical Abstracts, vol. 69, 1968, (Columbus, Ohio, USA), Kinoshita Yoshiro et al: " $\alpha$ , $\beta$ - Unsaturated monocarboxylic piperazides", page 6301 THE ABSTRACT No 67423h, Japan 67 24,912 | 1-16   |
|   | --   |  |
| X   | GB, A, 1218780 (MATSUSHITA ELECTRIC INDUSTRIAL CO. LTD.), 13 January 1971 (13.01.71), see esp. page 3 lines 56-57  | 1-16   |
|   | --   |  |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.   |  |  |
| * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"B" earlier document but published on or after the international filing date<br>"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)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"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<br>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"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<br>"&" document member of the same patent family |
| Date of the actual completion of the international search   |  | Date of mailing of the international search report   |
| 12 October 1994   |  | 17 -10- 1994   |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86  |  | Authorized officer<br>Solveig Gustavsson<br>Telephone No. +46 8 782 25 00  |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 94/00293

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |   |                       |
|---|---|-----------------------|
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
| A   | <p>Analytical Biochemistry, Volume 173, 1988,<br/>Denis F. Hochstrasser et al., "Development of<br/>Polyacrylamide Gels that Improve the Separation of<br/>Proteins and Their Detection by Silver Staining"<br/>page 412 - page 423</p> <p style="text-align: center;">--<br/>-----</p> | 1-16                  |

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

27/08/94

International application No.  
PCT/FI 94/00293

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)     | Publication<br>date  |
|---|---------------------|--------------------------------|----------------------|
| GB-A- 1218780                             | 13/01/71            | DE-A- 1817178<br>FR-A- 1604680 | 21/08/69<br>03/01/72 |
| -----                                     |                     |                                |                      |