(54) Title: NOVEL AFFINITY LIGANDS AND THEIR USE

$$R_1 - (CH_2)_p - Y \underset{N \diagdown X}{\overset{X}{\diagup}} Z - (CH_2)_n - Q - R_6 \quad \overset{R_4 \diagup R_5}{}$$

(a)

A
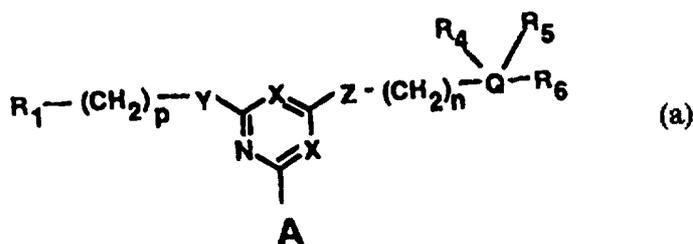
(57) Abstract

The present invention relates to novel affinity ligand-matrix conjugates comprising a ligand with general formula (a), which ligand is attached to a support matrix in position (A), optionally through a spacer arm interposed between the matrix and ligand. The invention furthermore relates to these novel affinity ligand-matrix conjugates and the preparation and use thereof in the purification of proteinaceous materials such as e.g. immunoglobulins, insulins, Factor VII, or human Growth Hormone or analogues, derivates and fragments thereof and precursors.

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Novel Affinity Ligands And Their Use

The present invention relates to novel affinity ligands, their preparation
and attachment to matrices which may consist of solid, semi-solid, particulate
5   or colloidal materials, or soluble polymers. The invention furthermore relates to
these novel affinity ligand-matrix conjugates and the preparation and use
thereof in the purification of proteinaceous materials such as e.g.
immunoglobulins, insulins, Factor VII, or Human Growth Hormone or
analogues, derivatives and fragments thereof and precursors.
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BACKGROUND OF THE INVENTION

Modern protein purification principles very much rely upon
15 chromatographic separation techniques such as gel permeation
chromatography (GPC), ionexchange chromatography (IEC), hydrophobic
interaction chromatography (HIC), reversed phase high pressure
chromatography (RP-HPLC), and affinity chromatography (AC). These
techniques are easily adapted to laboratory scale purification of peptides and
20 proteins meant for research and scientific experiments, resulting in pure and
biologically active substances. In most cases little or no interest is paid to
process economy, process validation, or cleaning in place procedures, as the
material will only rarely be used for clinical experiments and because labour
costs far exceed the costs of equipment and matrices.
25 However, large scale industrial downstream processing must take into
consideration factors such as economy, robustness of matrices and cleaning in
place with *e.g.* NaOH, urea, or ethanol. Today the demand for inexpensive and
robust matrices stable in 1M NaOH, 7M urea, or 80% v/v ethanol is met by a
number of commercial suppliers within the field of GPC, IEC, HIC, and RP-
30 HPLC. A combination of these principles has for many years resulted in almost
pure protein bulk substances although use of extreme buffers and many
purification steps have resulted in poor recoveries, increased costs and

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questionable stability of the bulk preparations.

It has long been realised that the principle of affinity chromatography could also be applied to large scale operations. Unfortunately, adsorbents created with natural biological ligands such as monoclonal or polyclonal
5   antibodies tend to be expensive to produce because the ligands themselves often require extensive purification, are biologically and chemically labile and tend to be difficult to immobilise with retention of their biological activity. Therefore, there has been a long term need to replace the expensive chemically and biologically labile monoclonal or polyclonal antibodies with less expensive
10 and more robust ligands mimicking the specificity of antibodies.

Affinity chromatography occupies a unique place in separation technology as the protein to be purified adsorbs selectively and reversibly to the complementary binding substance such as an antibody molecule. Purification factors of several thousandfold are often observed with high
15 recoveries, in contrast to the conventional purification methods offering factors from 5-50 times. The high purification factors obtained in affinity chromatography dramatically reduces the number of purification steps in the downstream process. Further, the very low non specific binding observed in affinity chromatography, makes it possible to purify a given protein from
20 complex biological mixtures, to separate incorrectly folded forms from native molecules, and to recover the protein specifically from even large volumes of tissue extracts or fermentation cultures.

The affinity sorbent comprises a solid, usually permeable, support matrix, to which a suitable ligand is covalently attached, contained in a
25 conventional chromatographic column. A crude sample, containing the complementary biopolymer is passed over the support matrix, under conditions which promote specific binding interactions with the immobilised ligand. The column is washed with buffer to remove unretarded molecules followed by an elution step in which the protein is eluted in its pure form. A typical affinity
30 adsorbent is based on a solid support, a spacer arm and a ligand. The solid support can be made of bead-formed agarose with an open pore structure. The spacer arm may encourage protein binding by making the ligand more

accessible. The length and nature of the spacer arm can be determined by a person skilled in the art. The ligand should exhibit specific and reversible binding to the protein to be purified even after immobilization. In addition to antibodies, a number of compounds including enzymatic co-factors, amino

5   acids, peptides, proteins, concanavalin A, Lectin, thiols, and dyes have been used as affinity ligands.

Affinity chromatography has been used in many applications. A comprehensive list is given in e.g. "Affinity Chromatography A Practical Approach" from IRL Press, 1985, and "Affinity Chromatography, Principles and Methods" from

10   Pharmacia Fine Chemicals 1979.

Conventional substrate or substrate analogue affinity ligands, especially dyes, have been used for large scale purification of specific enzymes or groups of enzymes. (Scawen M.D. and Atkinson T. 1987, Reactive Dyes in Protein and Enzyme Technology, Ed. Clonis Y.D. *et al*; Macmillan Press, pp. 51-85).

15   Dye affinity chromatography has over the years gained much interest because of the relative low price of such matrices, their robustness and their ability to withstand NaOH, urea and ethanol. Some of the more widely used ligands in this type of affinity chromatography have been a variety of reactive triazine-based textile dyes immobilised to agarose and other supports. The use

20   of affinity chromatography on immobilized dyes has been reviewed (Lowe C.R. and Pearson J.C. 1984, Methods in Enzymology 104, pp. 97-113). Selective interactions with the NAD$^+$-binding site of horse liver alcohol dehydrogenase were shown with dye analogues of Cibacron Blue F3G-A (Lowe C.R. *et al* 1986; Journal of Chromatography 376, pp. 121-130). The selective

25   purification approach was further illustrated with the computer aided design of a novel affinity adsorbent mimicking the phenyl-arginine dipeptide substrate for the purification of porcine pancreatic kallikrein (Burton N.P. and Lowe C.R. 1992, Journal of Molecular Recognition 5, pp. 55-58).

US Patent No. 4,562,252 discloses a particular ligand structure

30   consisting of two m-aminophenyl boronic acid groups attached to a triazine ring for glycoprotein separation.

However, despite the rapid progress in affinity technology over the past

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few years, the need is still to develop a technology by which a specific mimetic ligand can be identified for a protein in order to produce an inexpensive and stable affinity column capable of repeated large scale purification of the said protein, e.g. in the separation and purification of

5   proteinaceous materials, such as immunoglobulins, insulins, Factor VII, or human Growth Hormone or analogues, derivatives and fragments thereof and precursors, whether derived from natural or recombinant sources.

DETAILED DESCRIPTION OF THE INVENTION

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The present invention relates to novel affinity ligands, their preparation and attachment to matrices, and the use of these novel affinity ligand-matrices in the purification of proteinaceous materials.

The current invention is based on the notion that the selectivity of

15 hydrophobic ligands may be increased by increasing the complexity and spatial geometry of the hydrophobic component, and the incorporation of various functional groups capable of partaking in electrostatic and hydrogen bonding interactions thereby promoting selective interactions with protein binding sites. This work led to the discovery of a generic group of novel affinity ligands,

20 which have been unexpectedly found to be generally applicable to the isolation and purification of proteins by affinity chromatography.

In contrast to the above mentioned selective approach where enzyme substrates, analogues thereof or substrate mimetics were used as ligands, the ligands defined in this application are directed towards any surface of the

25 protein molecule, making the principle applicable for any protein. The ligands are designed by computer modelling techniques and/or by screening of mimetic ligand libraries. Further, the current invention has the advantage that the structure of the protein binding site architecture is not required for design and development of the ligand, and consequently the materials and techniques

30 described herein have a significantly greater utility.

A feature of the present invention is the provision of a general tool for protein resolution, isolation and purification. A family of subtly different

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chemical structures has been synthesised, which have the ability to interact with different proteins. A particularly effective ligand structure for a given protein is identified by screening a range of ligands provided by the invention for suitable binding properties.

5   By way of example, affinity ligands of high selectivity and specificity which are currently available for the separation and purification of immunoglobulins are often proteinaceous materials derived from either bacterial or recombinant sources and include materials such as Protein A, Protein G and Protein L. Immobilisation of these, and similar, proteins often results in a

10 significant loss of biological activity. Continual and repeated use of immobilised proteins as affinity media leads to a further diminution of biological activity. Furthermore, the inherent nature of these biological macromolecules imposes strict limitations with respect to the use of buffer salts, organic solvents and pH levels in affinity chromatography and related techniques.

15   Novel affinity ligands provided by this invention can be used in place of protein A and Protein G and are significantly more flexible in their use, are more robust, less expensive to produce and offer equivalent levels of purification.

        Another example is the use of novel affinity matrices provided by this

20 invention in biotechnology.

        The present invention relates to affinity ligand matrix conjugates comprising a ligand with the general formula (a):

(a) $$R_1-(CH_2)_p-Y \diagdown X \diagup Z-(CH_2)_n-Q-R_6$$
with R_4, R_5 attached, ring containing N, X, and A
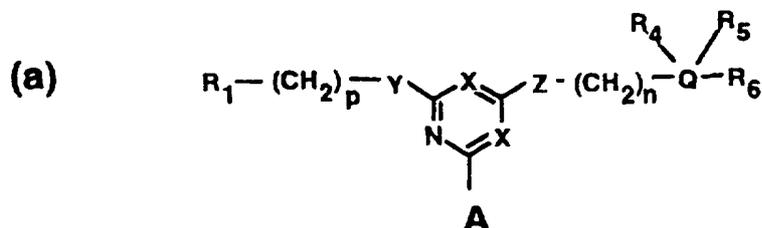
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wherein

        R_1 represents a hydrogen atom, an alkyl group containing from 1 to 6 carbon atoms, a hydroxyalkyl group containing from 1 to 6 carbon atoms, a cyclohexyl group, an amino group, a phenyl group, naphthyl group,1-

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phenylpyrazole, indazole, benzthiazole group, benzoxazole group, or a benzimidazole group, each of which benzene, naphthalene, phenylpyrazole, indazole, benzthiazole, benzoxazole or benzimidazole ring is optionally substituted with one or more substituents independently selected from the

5   group consisting of alkyl groups containing from 1 to 6 carbon atoms, alkoxy groups containing from 1 to 6 carbon atoms, acyloxy or acylamino groups containing from 1 to 6 carbon atoms, amino groups, hydroxyl groups, carboxylic acid groups, sulphonic acid groups, carbamoyl groups, sulphamoyl groups, alkylsulphonyl groups containing from 1 to 6 carbon atoms or halogen

10   atoms;

Y represents an oxygen atom, a sulphur atom or a group $N\text{-}R_2$;

Z represents an oxygen atom, a sulphur atom or a group $N\text{-}R_3$;

R_2 and R_3 each independently represent a hydrogen atom, an alkyl group containing from 1 to 6 carbon atoms; a hydroxyalkyl group containing from 1

15   to 6 carbon atoms, a benzyl group or a β-phenylethyl group;

R_4, R_5 and R_6 each independently represent a hydrogen atom, a hydroxyl group, an alkyl group containing from 1 to 6 carbon atoms, an alkoxy group containing from 1 to 6 carbon atoms, an amino group, an acyloxy or acylamino group containing from 1 to 6 carbon atoms, a carboxylic acid group, a

20   sulphonic acid group, a carbamoyl or sulphamoyl group, an alkylsulphonyl group containing from 1 to 6 carbon atoms or a halogen atom;

one of the symbols X represents a nitrogen atom and the other symbol X represents a nitrogen atom or a carbon atom carrying a chlorine atom or a cyano group;

25   Q represents a benzene; naphthalene, benzthiazole, benzoxazole 1-phenylpyrazole, indazole or benzimidazole ring;

n is an integer between 0 and 6;

p is an integer between 0 and 20;

and

30   which ligand is attached to a support matrix in position A, optionally through a spacer arm interposed between the matrix and ligand.

The optional spacer arm is preferably represented by the general formula

(b)

$$-T-[-L-V-]_m- \qquad \text{(b)}$$
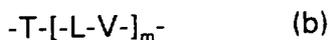
5　　wherein T represents an oxygen atom, a sulphur atom or a group $N-R_7$;

wherein R_7 represents a hydrogen atom or an alkyl group containing from 1 to

6 carbon atoms;

　　　　V represents an an oxygen atom, a sulphur atom, a -COO- group, a

CONH group or an NHCO group or a $-PO_3H-$ group , an NH-arylene-SO_2-CH_2-

10　CH_2 group or an $N-R_8$ group; wherein R_8 represents a hydrogen atom or an alkyl

group containing from 1 to 6 carbon atoms;

　　　　L represents an optionally substituted hydrocarbon linkage containing

from 2 to 20 carbon atoms; and

m is 0 or 1.

15　　　The support matrix may be any compound or material, particulate or non

particulate, soluble or insoluble, porous or non porous which may be used in

conjunction with affinity ligands to form a affinity ligand matrix conjugate and

which provides a convenient means of separating the affinity ligands from

solutes in a contacting solution.

20　　　The present invention provides novel affinity ligand-matrix conjugates,

which affinity ligand-matrix conjugates may be used in the separation and

purification of proteinaceous materials, such as immunoglobulins, insulins,

Factor VII, or human Growth Hormone or analogues, derivatives and

fragments thereof and precursors, whether derived from natural or recombinant

25　sources.

　　　　In a preferred embodiment, the invention provides novel affinity ligand

matrix conjugates which are represented by the General Formula (I):

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$$R_1-(CH_2)_p-Y\underset{\underset{\displaystyle T-[-L-V-]_m-M}{\overset{\displaystyle N\diagdown X}{\big|}}}{\overset{\displaystyle}{\diagup X}}Z-(CH_2)_n-Q-R_6 \qquad \text{(I)}$$

$$\overset{R_4\diagdown \diagup R_5}{}$$

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wherein

R_1, Y, Z, R_2, R_3, R_4, R_5, R_6, X, Q, n and p have the meanings specified above,

T represents an oxygen atom, a sulphur atom or a group $N-R_7$;

V represents an oxygen atom, a sulphur atom, a -COO- group, a CONH group or an NHCO group or a $-PO_3H-$ group , an NH-arylene-SO_2-CH_2-CH_2 group or an $N-R_8$ group;

R_7 and R_8 each independently represents a hydrogen atom or an alkyl group containing from 1 to 6 carbon atoms;

L represents an optionally substituted hydrocarbon linkage containing from 2 to 20 carbon atoms;

m is 0 or 1; and

M represents the residue of a support matrix.

The term "alkyl group containing from 1 to 6 carbon atoms" as used herein, alone or in combination, refers to a straight or branched, satura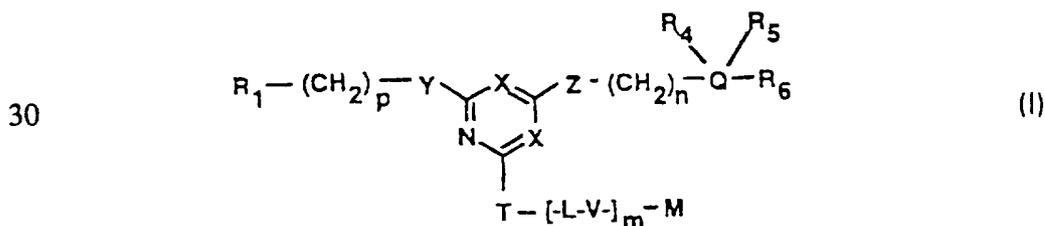ted hydrocarbon chain having 1 to 6 carbon atoms such as e.g. methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl, tert-butyl, n-pentyl, 2-methylbutyl, 3-methylbutyl, n-hexyl, 4-methylpentyl, neopentyl, n-hexyl and 2,2-dimethylpropyl.

The term "hydroxyalkyl group containing from 1 to 6 carbon atoms" as used herein, alone or in combination, refers to a straight or branched, saturated hydrocarbon chain having 1 to 6 carbon atoms substituted with one or more hydroxy groups, preferably one hydroxy group, such as e.g. hydroxymethyl, 2-hydroxyethyl, 3-hydroxypropyl, 2-hydroxypropyl, 4-hydroxybutyl, 5-hydroxypentyl, 6-hydroxyhexyl.

The term " alkoxy group containing from 1 to 6 carbon atoms" as used herein, alone or in combination, refers to a straight or branched monovalent substituent comprising an alkyl group containing from 1 to 6 carbon atoms linked through an ether oxygen having its free valence bond from the ether oxygen and having 1 to 6 carbon atoms e.g. methoxy, ethoxy, propoxy, isopropoxy, butoxy, pentoxy.

Term "halogen" means fluorine, chlorine, bromine or iodine.

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The term "acyloxy or acylamino containing from 1 to 6 carbon atoms " as used herein refers to a monovalent substituent comprising an alkyl group containing from 1 to 5 carbon atoms linked through a carbonyloxy or oxycarbonyl group such as a methylcarbonyloxy, ethylcarbonyloxy,

5   methyloxycarbonyl or ethyloxycarbonyl group or linked through a carbonylamino or aminocarbonyl group such as a methylcarbonylamino, ethylcarbonylamino, methylaminocarbonyl or ethylaminocarbonyl group.

The term "alkylsulfonyl containing from 1 to 6 carbon atoms" as used herein refers to a monovalent substituent comprising a alkyl group containing

10   from 1 to 6 carbon atoms linked through a sulfonyl group such as e.g. methylsulfonyl, ethylsulfonyl, n-propylsulfonyl, isopropylsulfonyl, n-butylsulfonyl, sec-butylsulfonyl, isobutylsulfonyl, tert-butylsulfonyl, n-pentylsulfonyl, 2-methylbutylsulfonyl, 3-methylbutylsulfonyl, n-hexylsulfonyl, 4-methylpentylsulfonyl, neopentylsulfonyl, n-hexylsulfonyl and 2,2-

15   dimethylpropylsulfonyl.

The term "one or more substituents independently selected from" shall more preferably refer to from 1-3 substituents. The term shall further preferably refer to 1-2 substituents and most preferably refer to one substituent.

20   In the present specification, whenever the term insulin is used in a plural or a generic sense, it is intended to encompass both naturally occurring insulins and insulin analogues and derivatives thereof and precursors. By the term insulin is thus also meant insulin from any species, e.g. human insulin. By the term "insulin analogue" as used herein is meant human insulin with one or

25   several amino acid substitutions, one or several amino acid deletions, one or several amino acid additions or combinations hereof. The term "insulin derivative" means insulin chemically modified in one or several residues. By "insulin precursor" as used herein is meant any molecule which by enzymatic or chemical conversion results in formation of insulin, insulin fragments, e.g.

30   des-Thr(B30)-insulin, insulin analogues, or insulin derivatives.

The term "optionally substituted hydrocarbon linkage containing from 2 to 20 carbon atoms" as used herein refers to one or more linear or branched

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alkyl chains, optionally substituted with for example hydroxy or alkoxy groups
containing from 1 to 6 carbonatoms, and optionally linked together by amino,
ether, thioether, ester, amide or sulphonamide bonds providing a chain
containing from 2 to 20 carbon atoms. The construction is preferably flexible.
5 The construction of such optionally substituted hydrocarbon linkages is for
example described in Lowe, C. R. and Dean, P.D.G, 1974, Affinity
Chromatography, John Wiley & Sons, London, which hereby are incorporated
by reference.

In a preferred embodiment, these conjugates are represented by the
10 General Formula (I):

$$R_1 - (CH_2)_p - Y \underset{N}{\overset{X}{\diagdown}} \overset{X}{\diagup} Z - (CH_2)_n - Q - R_6 \qquad (I)$$

$$\overset{R_4 \diagdown \diagup R_5}{}$$

$$T - [-L-V-]_m - M$$
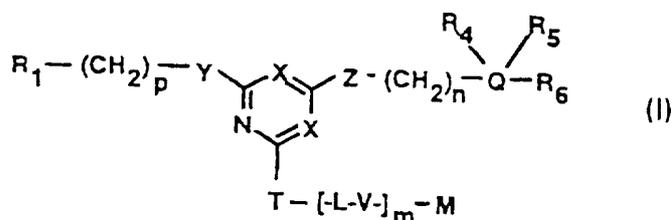
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wherein

R_1 represents a hydrogen atom, an alkyl group containing from 1 to 6
carbon atoms, a hydroxyalkyl group containing from 1 to 6 carbon atoms, a
20 cyclohexyl group, an amino group, a phenyl group or a naphthyl group, which
may be substituted on the benzene or naphthalene ring by alkyl groups
containing from 1 to 6 carbon atoms, alkoxy groups containing from 1 to 6
carbon atoms, acyloxy or acylamino groups containing from 1 to 6 carbon
atoms, amino groups, hydroxyl groups, carboxylic acid groups, sulphonic acid
25 groups, carbamoyl groups, sulphamoyl groups, alkylsulphonyl groups or
halogen atoms;

T represents an oxygen atom, a sulphur atom or a group $N-R_7$;

Y represents an oxygen atom, a sulphur atom or a group $N-R_2$;

Z represents an oxygen atom, a sulphur atom or a group $N-R_3$;

30   R_2 and R_3 each independently represent a hydrogen atom, an alkyl group
containing from 1 to 6 carbon atoms; a hydroxyalkyl group containing from 1
to 6 carbon atoms, a benzyl group or a β-phenylethyl group;

R_4, R_5 and R_6 each independently represent a hydrogen atom, a hydroxyl group, an alkyl group containing from 1 to 6 carbon atoms, an alkoxy group containing from 1 to 6 carbon atoms, an amino group, an acyloxy or acylamino group containing from 1 to 6 carbon atoms, a carboxylic acid group, a

5   sulphonic acid group, a carbamoyl or sulphamoyl group, an alkylsulphonyl group or a halogen atom;

one of the symbols X represents a nitrogen atom and the other symbol X represents a nitrogen atom or a carbon atom carrying a chlorine atom or a cyano group;

10   V represents an oxygen atom, a sulphur atom, a -COO- group, a CONH group or an NHCO group or a -PO_3H- group , an NH-arylene-SO_2-CH_2-CH_2 group or an N-R_8 group;

R_7 and R_8 each independently represents a hydrogen atom or an alkyl group containing from 1 to 6 carbon atoms;

15   L represents an optionally substituted hydrocarbon linkage containing from 2 to 20 carbon atoms;

Q represents a benzene or naphthalene ring;

n is an integer between 0 and 6;

p is an integer between 0 and 20;

20   m is 0 or 1; and

M represents the residue of a support matrix which may be any compound or material, particulate or non particulate, soluble or insoluble, porous or non-porous which may be used in conjunction with affinity ligands to form a novel affinity ligand-matrix conjugate of General Formula (I) and which

25   provides a convenient means of separating the affinity ligands from solutes in a contacting solution.

It will be appreciated that this invention relates, inter alia, to the use of compounds which are pyridines, diazines or triazines carrying a T-[L-V]$_{0-1}$-M substituent, or the precursor thereof, and other substituents linked to the ring

30   via a heteroatom. Such substituents may include any non-interfering group comprising 0 to 10 or 20 C atoms.

In a preferred embodiment of the invention, R_1 represents a phenyl or

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naphthyl group each of which is optionally substituted on the benzene or naphthalene ring with one or more independently selected from the group consisting of hydroxyl groups or carboxylic acid groups.

In another preferred embodiment of the invention, R_2 represents a hydrogen atom.

In another preferred embodiment of the invention, R_3 represents a hydrogen atom.

In another preferred embodiment of the invention, R_4 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group, or an amino group.

In another preferred embodiment of the invention, R_5 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group, or an amino group.

In another preferred embodiment of the invention, R_6 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group, or an amino group.

In another preferred embodiment of the invention, R_7 represents a hydrogen atom.

In another preferred embodiment of the invention, T represents an oxygen atom or an NH group.

In another preferred embodiment of the invention, Y represents N-R_2 wherein R_2 is as defined above.

In another preferred embodiment of the invention, Z represents N-R_3 wherein R_3 is as defined above.

In another preferred embodiment of the invention, both X represents a nitrogen atom.

In another preferred embodiment of the invention, Q represents a benzene or naphthalene ring. ·

In another preferred embodiment of the invention, n represents 0 or 2.

In another preferred embodiment of the invention, p represents 0 or 2.

In another preferred embodiment of the invention, m represents 0 or 1.

In another preferred embodiment of the invention, L represents an ethyl, propyl, hydroxypropyl, butyl, pentyl, hexyl, octyl or decyl group and V and m are as defined above.

In another preferred embodiment of the invention, V represents an

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oxygen atom, a -COO- group, a -PO$_3$H- group, or an N-R$_8$ group; and more preferred an oxygen atom or an NH group and L and m are as defined above.

In another preferred embodiment of the invention, m represents 1 and L and V are as defined above.

5   The term "integer between x and y" may include the values x (including zero) and y.

The invention also provides methods for the manufacture of the novel affinity ligand-matrix conjugates according to the invention which comprises reacting, in any order, a halogenoheterocyclic compound of General Formula 10 (II):

$$ \underset{\text{W}}{\overset{\text{W}\diagdown\text{X}\diagup\text{W}}{\underset{\text{N}\diagup\text{X}}{\|}}} \qquad \text{(II)} $$

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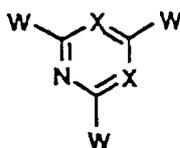
wherein the symbols X have the meaning hereinbefore specified and W represents a halogen atom with

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(i) a compound of General Formula (III):

R$_1$-(CH$_2$)$_p$-Y-H   (III)

25   wherein the symbols R$_1$, Y and p have the meanings hereinbefore specified and H is hydrogen,

(ii) a compound of General Formula (IV)

30

$$R_4 \qquad R_3$$
$$\diagdown \diagup$$
$$\text{H-Z-(CH}_2)_n\text{---Q---R}_6 \qquad \text{(IV)}$$

wherein the symbols R_4, R_5, R_6, Q, Z and n have the meanings hereinbefore specified, and

(iii) with either an optionally derivatised support matrix of General Formula V

$$\text{H-T-[-L-V-]}_m\text{-M} \qquad \text{(V)}$$

wherein the symbols L, M, V, T, and m have the meanings hereinbefore specified

or, with a linking unit of General Formula (VI)

$$\text{H-T-L-V-H} \qquad \text{(VI)}$$

wherein the symbols H, L, V and T have the meanings hereinbefore specified to give a compound of General Formula (VII):

$$R_1\text{---(CH}_2)_p\text{---Y} \diagdown \diagup X \diagdown Z\text{-(CH}_2)_n\text{---Q---R}_6$$

wherein R_1, R_4, R_5, R_6, Q, L, T, V, X, Y, Z, m, n and p have the meanings

hereinbefore specified; the compound of General Formula (VII) is then reacted further with a support matrix whose residue is represented by M using activating and coupling procedures well known to those skilled in the art.

As examples of halogenoheterocyclic compounds of General Formula (II)
5 there may be mentioned 5-chloro-2,4,6-trifluoropyrimidine, 5-cyano-2,4,6-trichloropyrimidine, cyanuric fluoride, cyanuric bromide and, above all, cyanuric chloride.

As examples of compounds of General Formula (III) there may be mentioned amines such as ammonia, methylamine, ethylamine, propylamine,
10 isopropylamine, diisopropylamine, isobutylamine, amylamine, hexylamine, ethanolamine, diethanolamine, aniline, N-methylaniline, N-ethylaniline, N-isopropylaniline, 1,4-diaminobutane, 1,6-diaminohexane, N-tert-butylaniline, p-toluidine, p-butylaniline, 2,4-dimethylaniline, p-anisidine, p-ethoxyaniline, p-aminoacetanilide, p-aminophenol, p-chloroaniline, orthanilic acid, metanilic acid,
15 sulphanilic acid, 4-methylaniline-2-sulphonic acid, 4-methoxyaniline-2-sulphonic acid, aniline-2,5-disulphonic acid, N-methylmetanilic acid, o-, m- and p-aminobenzoic acid, p-aminobenzamide, p-aminobenzenesulphonamide, 1-amino-2-, 3-, 4-, 5-, 6-, 7- and 8-naphthol, 2-amino-3-, 4-, 5-, 6-, 7- and 8-naphthol, 5-, 6- and 7-amino-1-naphthol-3-sulphonic acid, N-benzylaniline,
20 benzylamine, 4-methylbenzylamine, 4-hydroxybenzylamine, 4-methoxybenzylamine, 4-acetoxybenzylamine, 4-acetylaminobenzylamine, N-methylbenzylamine, β-phenylethylamine, N-butyl-benzylamine, N-benzyl-β-phenylethylamine, N-(β-hydroxyethyl)-benzylamine, N-tert-butyl-benzylamine, N-benzyltyramine and tyramine; phenols such as phenol, o-, m- and p-cresol,
25 catechol, resorcinol, hydroquinone, p-chlorophenol, 1-naphthol and 2-naphthol, 1-naphthol-4-sulphonic acid, 2-naphthol-6-sulphonic acid and 2-hydroxy-3-naphthoic acid; thiols such as ethylthiol, thioglycollic acid, thiophenol and thio-p-cresol, and aromatic heterocycles such as 5-amino-1-phenylpyrazole, 6-aminoindazole, 2-aminobenzimidazole, 2-aminobenzthiazole, and 2-amino-5-
30 chlorobenzoxazole.

As examples of compounds of General Formula (IV) there may be mentioned amines such as aniline, N-methylaniline, N-ethylaniline, N-

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isopropylaniline, N-tert-butylaniline, p-toluidine, p-butylaniline, 2,4-
dimethylaniline, p-anisidine, p-ethoxyaniline, p-aminoacetanilide, p-
aminophenol, p-chloroaniline, orthanilic acid, metanilic acid, sulphanilic acid, 4-
methylaniline-2-sulphonic acid, 4-methoxyaniline-2-sulphonic acid, aniline-2,5-

5   disulphonic acid, N-methylmetanilic acid, o-, m- and p-aminobenzoic acid, 1-
amino-2-, 3-, 4-, 5-, 6-, 7- and 8-naphthol, 2-amino-3-, 4-, 5-, 6-, 7- and 8-
naphthol, 5-, 6- and 7-amino-1-naphthol-3-sulphonic acid, p-aminobenzamide,
p-aminobenzenesulphonamide, N-benzylaniline, benzylamine, 4-
methylbenzylamine, 4-hydroxybenzylamine, 4-methoxybenzylamine, 4-

10 acetoxybenzylamine, 4-acetylaminobenzylamine, N-methylbenzylamine, β-
phenylethylamine, N-butyl-benzylamine, N-benzyl-β-phenylethylamine, N-(β-
hydroxyethyl)-benzylamine, N-tert-butyl-benzylamine, N-benzyltyramine and
tyramine; phenols such as phenol, o-, m- and p-cresol, catechol, resorcinol,
hydroquinone, p-chlorophenol, 1- and 2-naphthol, 1-naphthol-4-sulphonic acid,

15 2-naphthol-6-sulphonic acid and 2-hydroxy-3-naphthoic acid; thiols such as
thiophenol and thio-p-cresol, and aromatic heterocycles such as 5-amino-1-
phenylpyrazole, 6-aminoindazole, 2-aminobenzimidazole, 2-aminobenzthiazole,
and 2-amino-5-chlorobenzoxazole.

        As examples of support matrices whose residue is represented by M,
20 there may be mentioned insoluble support matrices such as a naturally
occurring polymer, for example a polypeptide or protein such as cross linked
albumin or a polysaccharide such as agarose, alginate, carrageenan, chitin,
cellulose, dextran or starch; synthetic polymers such as polyacrylamide,
polystyrene, polyacrolein, polyvinyl alcohol, polymethylacrylate,

25 perfluorocarbon; inorganic compounds such as silica, glass, kieselguhr,
alumina, iron oxide or other metal oxides or co-polymers consisting of any
combination of two or more of a naturally occurring polymer, synthetic
polymer or inorganic compounds. Also included within the definition of support
matrices whose residue is represented by M are soluble support matrices

30 comprising polymers such as dextran, polyethylene glycol, polyvinyl alcohol or
hydrolysed starch which provide affinity-ligand matrix conjugates for use in
liquid partitioning; or support matrices comprising compounds such as

17

perfluorodecalin which provide affinity-ligand matrix conjugates for use in the formation of affinity emulsions. For the avoidance of doubt, a support matrix is defined herein as any compound or material whether particulate or non-particulate, soluble or insoluble, porous or non-porous which may be used to

5   form a novel affinity ligand-matrix conjugate according to the invention and which provides a convenient means of separating the affinity ligand from solutes in a contacting solution.

Also included within the definition of support matrices whose residue is represented by M are support matrices such as agarose, cellulose, dextran,

10   starch, alginate, carrageenan, synthetic polymers, silica, glass and metal oxides which have been, or are, modified by treatment with an activating agent prior to, or during, attachment of the ligand.

In a preferred embodiment of the invention M represents optionally activated agarose, silica, cellulose, glass, toyopearl, hydroxyethylmethacrylate,

15   polyacrylamide, styrenedivinylbenzene, Hyper D, perfluorocarbons.

Preferably M represents optionally tresyl activated, sulphonylchloride activated, tosyl activated, vinylsulphone activated or epoxy activated agarose.

Preferred affinity ligand matrix conjugates according to the invention are

20   i)
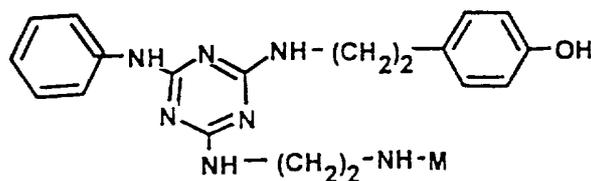
25

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ii)

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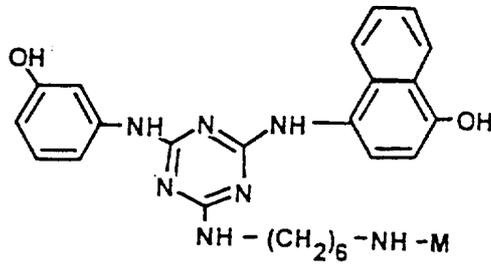
iii)

10

15

iv)

20

25 v)

30

vi)

vii)
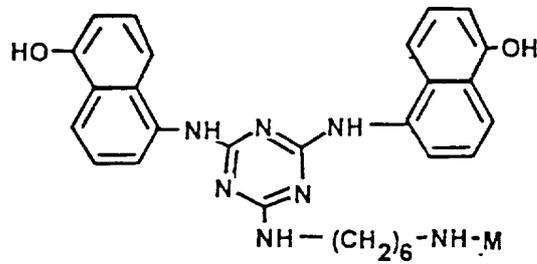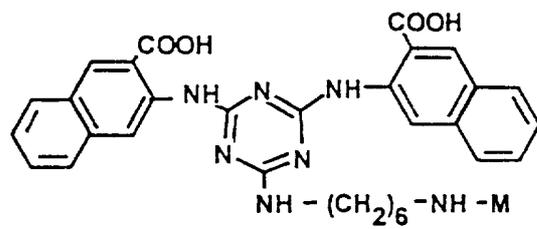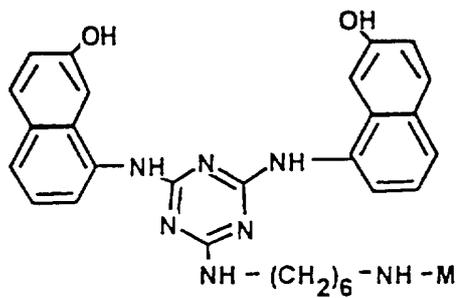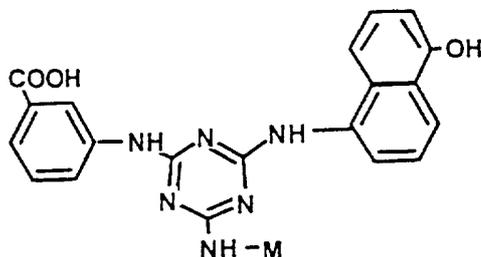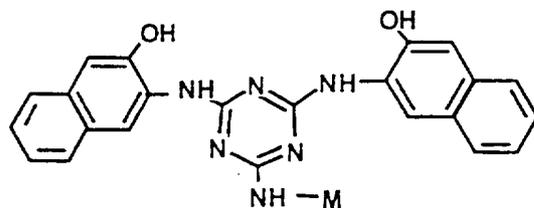
viii)
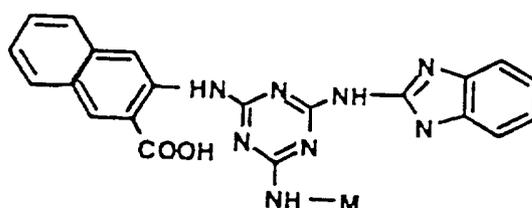
wherein M is as defined above.

There exists a considerable number of activating agents which have found use for the general purpose of attaching ligands to support matrices. These compounds and their method of use are well known to those skilled in the art and, since the nub of the present invention lies in the nature of the ligand attached to the matrix and not in the mode of attachment, any of these activating agents will serve in the preparation of the new matrix-ligand conjugates of the invention. As non-limiting examples of such activating agents

20

there may be mentioned such diverse compounds as cyanogen bromide, cyanuric chloride, epichlorohydrin, divinyl sulphone, p-toluenesulphonyl chloride, 1,1'-carbonyldiimidazole, sodium meta-periodate, 2-fluoro-1-methylpyridiniumtoluene-4-sulphonate, glycidoxypropyltrimethoxysilane and

5   2,2,2-trifluoroethanesulphonyl chloride. As indicated above, the procedures by which such activating steps are carried out are well known to those skilled in the art.

        Similarly, a wide variety of condensing agents may be used to attach the compounds of General Formulae (VI) to support matrices such as agarose,

10 cellulose, dextran, starch, alginate, carrageenan, silica or glass. Again these compounds, and their method of use are well known to those skilled in the art and, again, since the nub of the present invention lies in the nature of the ligand and not in the mode of attachment, any of these condensing agents will serve in the preparation of the new matrix-ligand conjugates of the invention.

15 As non-limiting examples of such condensing agents, there may be mentioned N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, dicyclohexyl carbodiimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide.

        As examples of linking units of General Formula (VI) which may be used to produce compounds of General Formula (VII) there may be mentioned

20 diamines such as ethylene diamine, N,N'-dimethylethylene diamine, N-ethylethylene diamine, N-(β-hydroxyethyl)-ethylene diamine, propylene diamine, N-methylpropylene diamine, N-(β-hydroxyethyl)-propylene diamine, 1,4-diaminobutane, 1,5-diaminopentane, 1,6-diaminohexane, 1,7-diaminoheptane, 1,8-diaminooctane, 1,9-diaminononane, 1,10-diaminodecane, 1,12-

25 diaminododecane, piperazine, 3-hydroxy-1,5-diaminopentane, m- and p-phenylene diamine, m- and p-aminobenzylamine; amino alcohols such as ethanolamine, N-methylethanolamine, N-propylethanolamine, diethanolamine, 3-hydroxypropylamine, 2,3-dihydroxypropylamine, isopropanolamine, 5-aminopentan-1-ol and 6-aminohexan-1-ol; aminophenols such as o-, m- and p-

30 aminophenol, aminocarboxylic acids such as glycine, N-methylglycine, 3- and 4-aminobutyric acid, 3-aminoisobutyric acid, 5-aminovaleric acid, 6-aminocaproic acid, 7-aminoheptanoic acid, m- and p-aminobenzoic acid;

aminophosphonic acids such as m-aminobenzenephosphonic acid and p-
aminobenzylphosphonic acid; and aminoarylene vinylsulphone precursors such
as aniline-3-β-sulphatoethylsulphone and aniline-4-β-sulphatoethylsulphone.

    The reaction of halogenoheterocyclic compounds of General Formula (II)

5 with compounds of General Formulae (III), (IV) and (V) or (VI) may be carried
out in an organic solvent which is not miscible with water; or in an organic
solvent which is miscible with water, or in a mixture of water and a water
miscible organic solvent. Examples of suitable organic solvents which are not
miscible with water are toluene, xylene or chlorobenzene; Examples of suitable

10 organic solvents which are miscible with water are acetone, methyl ethyl
ketone or dioxan. The first reaction of the halogenoheterocyclic compound may
be carried out at temperatures between 0°C and 25°C, ideally between 0°C
and 5°C; the second reaction may be carried out at temperatures between
20°C and 50°C, ideally between 30°C and 45°C and the third reaction at

15 temperatures between 20°C and 100°C. During such reactions, the inorganic
acid such as hydrochloric acid or hydrofluoric acid which is produced is
neutralised by the use of an acid binding agent such as sodium hydroxide,
sodium carbonate, sodium bicarbonate, calcium hydroxide or calcium
carbonate.

20   Additionally, compounds of General Formula (VII) may be reacted with a
reactive polymerisable monomer to form a polymerisable compound of General
Formula (VIII):

25

$$R_1 - (CH_2)_p - Y \diagdown \diagup X \diagdown \diagup Z - (CH_2)_n - Q - R_6$$

(VIII)

$$T - [-L-V-]_m - R_{10} - C = CH_2$$

30 wherein R_1, R_4, R_5, R_6, Q, L, T, V, X, Y, Z, m, n and p have the meanings
hereinbefore specified; R_9 represents a hydrogen atom or an alkyl group
containing from 1 to 6 carbon atoms; R_{10} represents a carbonyl group, a

methylene group, a -NH-CH$_2$- group or a -S-CH$_2$- group. Examples of reactive
polymerisable monomers include acryloyl chloride, methacryloyl chloride, allyl
bromide, allylamine or 3,4-epoxybutene. Polymerisable compounds of General
Formula (VIII) may be polymerised, optionally in the presence of other
5   polymerisable monomers, to form affinity ligand matrix conjugates of General
Formula (I). Such polymerisation procedures are well known to those skilled in
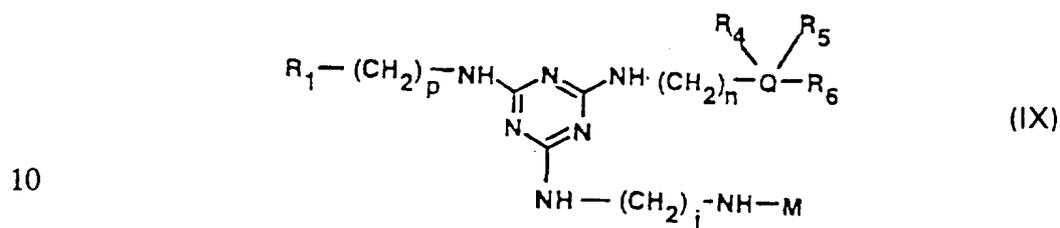the art.

The invention further covers the use of all such affinity ligand-support
matrices in the separation, isolation, purification, quantification, identification
10   and characterisation of proteinaceous materials, such as immunoglobulins,
insulins, Factor VII, or Human Growth Hormone or analogues, derivatives and
fragments thereof and precursors.

Immunoglobulins are a family of proteins, often abbreviated as I$_g$, which
share a common structure. Immunoglobulins are also known collectively as
15   antibodies and either word may be used to describe this group of proteins.
Immunoglobulins exist in a number of different forms, for example, the most
significant antibody types being I$_g$A, I$_g$D, I$_g$E, I$_g$G, I$_g$M and I$_g$Y and various sub-
classes thereof. Immunoglobulins may occur in body fluids, such as plasma,
ascities, saliva, milk or egg yolk or may be produced using genetic engineering
20   methodologies. Immunoglobulins may be altered by a variety of techniques to
confer desirable properties upon them. Such procedures are well known to
those skilled in the art and the resulting modified antibodies are also subject to
the Claims of this invention. As non-limiting Examples of antibody modification
techniques, antibody fragments, labelled antibodies, antibody conjugates or
25   antibody-fusion proteins may be obtained through chemical modification, by
treatment with one or more enzymes or by a combination of both techniques.
There exists a considerable number of chemical modification reagents and
enzymes which have found use in antibody modification and these compounds
and their use are well known to those skilled in the art. A further way of
30   obtaining modified or novel antibodies is to produce them using genetic
engineering methodologies. Such methodologies and their use are well known
to those skilled in the art and may be used to produce, for example, antibody

23

fragments or antibody-fusion products. Modified or novel antibodies derived by genetic engineering methodologies are also subject to the Claims of this invention.

A valuable group of affinity ligand-support matrices is represented by the

5 General Formula (IX):

$$R_1 - (CH_2)_p - NH \underset{N}{\overset{N}{\bigtriangleup}} NH \cdot (CH_2)_n - Q - R_6 \quad \overset{R_4 \quad R_5}{\diagup}$$

(IX)

10

$$NH - (CH_2)_j - NH - M$$

wherein R_1, R_4, R_5, R_6, M, Q, n and p have the meanings hereinbefore specified and j is an integer between 2 and 20.

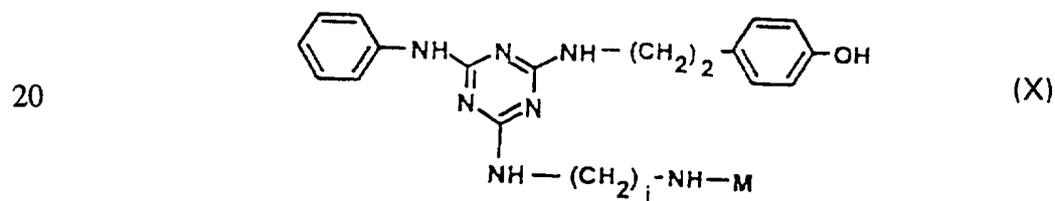15   An especially valuable group of affinity ligand-support matrices is represented by the general Formula (X):

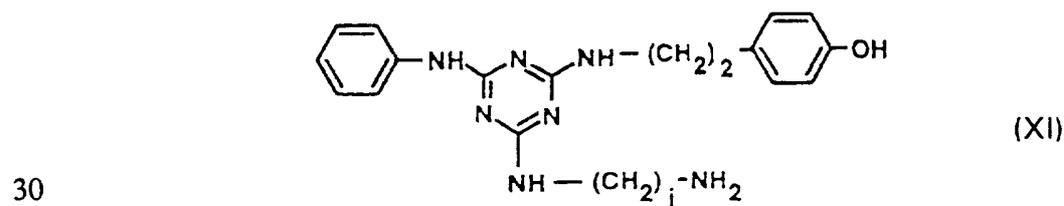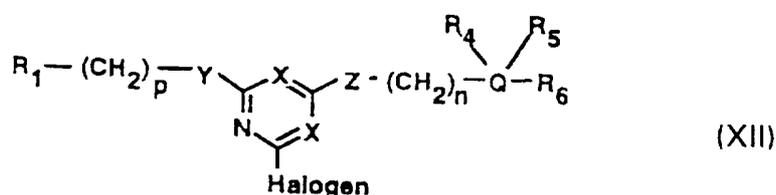20   (X)

$$NH - (CH_2)_j - NH - M$$

wherein j and M have the meanings hereinbefore specified.

25   Typically, reaction of compounds of General Formula (XI)

(XI)

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$$NH - (CH_2)_j - NH_2$$

24

with 3-propoxy-(1,2-epoxy)-agarose at temperatures between 10°C and 30°C in the presence of an acid binding agent produces novel affinity ligand-matrix conjugates which are of outstanding value in the purification of proteinaceous materials. These new affinity ligand-matrix conjugates possess high affinity for

5 the immunoglobulin group of proteins. This unique property makes them of exceptional value in the separation, isolation, purification quantification, identification and characterisation of proteins of this class.

In another embodiment the invention relates to novel affinity ligands of General Formula (XII):

10

$$R_1 - (CH_2)_p - Y \diagdown \underset{N \diagdown X}{\overset{X}{\diagup}} Z - (CH_2)_n - Q - R_6 \text{, with } R_4 \diagdown \diagup R_5 \text{ on } Q$$

Halogen

(XII)

15

wherein R_1, R_4, R_5, R_6, Q, X, Y, Z, n and p have the meanings specified above and Halogen represents a fluorine, chlorine, bromine or iodine atom.

Furthermore, the invention relates to a method of attaching novel affinity

20 ligands of General Formula (XII) as defined above to a matrix of General Formula (V) as defined above by reacting the novel affinity ligands with the matrix at temperatures between -20°C and 121°C, optionally in the presence of an acid binding agent.

In another embodiment the invention relates to novel affinity ligands of

25 General Formula (XIII):

$$R_1 - (CH_2)_p - Y \diagdown \underset{N \diagdown X}{\overset{X}{\diagup}} Z - (CH_2)_n - Q - R_6 \text{, with } R_4 \diagdown \diagup R_5 \text{ on } Q$$

NH — $(CH_2)_j$ -NH_2

(XIII)

30

25

wherein R_1, R_4, R_5, R_6, Q, X, Y, Z, m, n and p have the meanings specified above and j is an integer between 2 and 20.

Furthermore the invention relates to a method of preparing above novel affinity ligands of General Formula (XIII) by reacting a compound of above

5   General Formula (XII) with an alkylene diamine of General Formula H_2N-$(CH_2)_j$-NH_2 at temperatures between 0°C and 100°C in the presence of an acid binding agent.

In another embodiment the invention relates to novel affinity ligands of General Formula (XIV):

10

$$R_1-(CH_2)_p-Y\underset{\underset{\displaystyle NH-(CH_2)_j-(CO)_q-OH}{N\diagdown X}}{\overset{X}{\diagup}}Z-(CH_2)_n-Q-R_6 \quad \underset{R_4\diagdown\diagup R_5}{}$$

(XIV)

15

wherein R_1, R_4, R_5, Q, X, Y, Z, m, n and p have the meanings specified above,

20   q is 0 or 1 and j is an integer between 2 and 20.

Furhermore the invention relates to a method of preparing novel affinity ligands of above General Formula (XIV) by reacting a compound of above General Formula (XII) with an amino hydroxy compound of General Formula H_2N-$(CH_2)_j$-$(CO)_q$-OH at temperatures between 0°C and 100°C, optionally in the

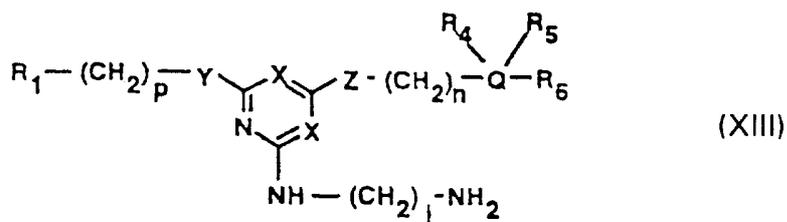25   presence of an acid binding agent.

In another embodiment the invention relates to novel affinity ligands of above General Formula (VIII) wherein R_1, R_4, R_5, R_6, L, Q, T, V, X, Y, Z, m, n and p have the meanings specified above; R_9 represents a hydrogen atom or an alkyl group containing from 1 to 6 carbon atoms; R_{10} represents a carbonyl

30   group, a methylene group, an -NH-CH_2- group or an -S-CH_2- group; preferably L is an ethyl, butyl or hexyl group, preferably T represents a -NH- group, preferably V represents a -NH- group and m is prefably 1.

26

In another embodiment the invention relates to novel affinity ligands of General Formula (XV):

5

$$R_1 - (CH_2)_p - Y \diagdown \diagup N \diagup Z - (CH_2)_n - Q - R_6 \quad \text{with} \quad R_4, R_5 \quad (XV)$$
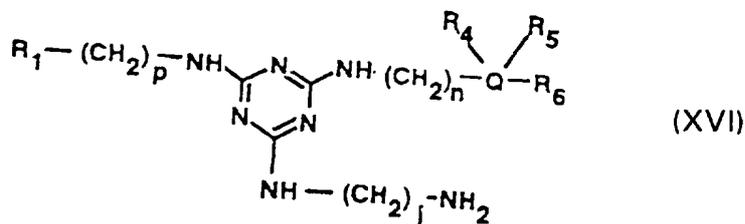
$$Cl$$

10

wherein R_1, R_4, R_5, R_6, Q, n and p have the meanings specified above.

In another embodiment the invention relates to novel affinity ligands of General Formula (XVI):

15

$$R_1 - (CH_2)_p - NH \diagdown N \diagup NH \cdot (CH_2)_n - Q - R_6 \quad \text{with} \quad R_4, R_5$$

$$NH - (CH_2)_j - NH_2 \quad (XVI)$$

20

wherein R_1, R_4, R_5, R_6, Q, n and p have the meanings specified above and j is
25 an integer between 2 and 20.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein R_1 represents a phenyl or naphthyl group each of which is optionally substituted on the benzene or naphthalene ring with one or more independently selected
30 from hydroxyl groups or carboxylic acid groups.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein R_4

represents a hydrogen atom, a hydroxyl group, a carboxylic acid group or an amino group.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein R_5

5 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group or an amino group

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein R_6 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group or an

10 amino group.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein Q represents a benzene or naphthalene ring.

In another preferred embodiment the invention relates to affinity ligands

15 of General Formula (XII), (XIII), (XIV), and (VIII) wherein X represents a nitrogen atom.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), and (VIII) wherein Y represents a -NH-group.

20   In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), and (VIII) wherein Z represents a -NH-group.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein n is 0 or 2.

25   In another preferred embodiment the invention relates to affinity ligands of General Formula (XII), (XIII), (XIV), (VIII), (XV) and (XVI) wherein p is 0 or 2.

In another preferred embodiment the invention relates to affinity ligands of General Formula (XIII), (XIV) and (XVI) wherein j is 2, 4 or 6.
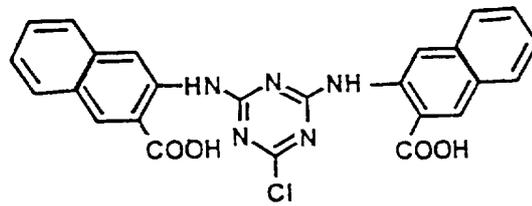
In another embodiment the invention relates to affinity ligands of above

30 General Formula (XI) wherein j is an integer betweem 2 and 20.

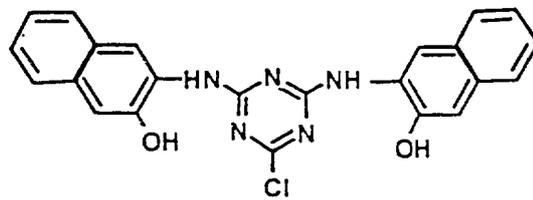Preferred affinity ligands according to the invention are:

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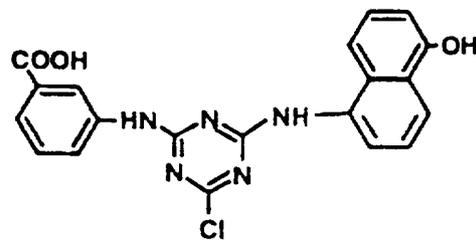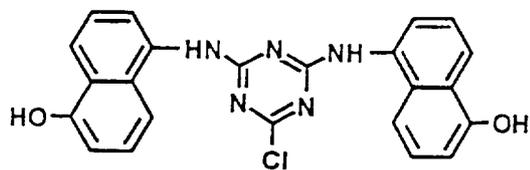
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(1)

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(2)

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(3)

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(4)

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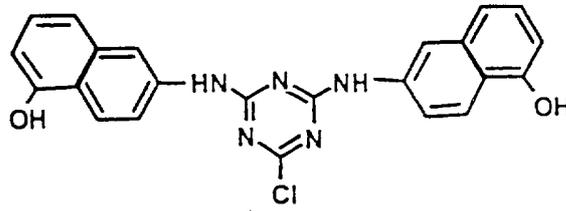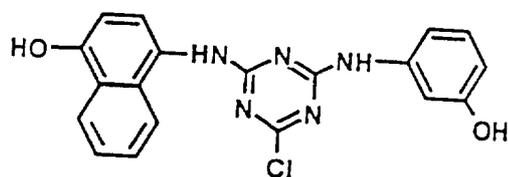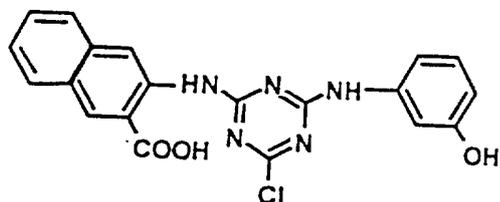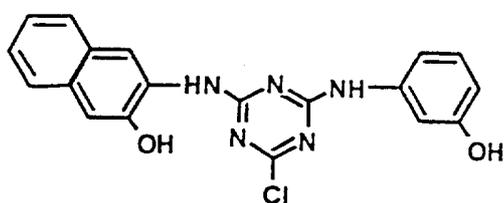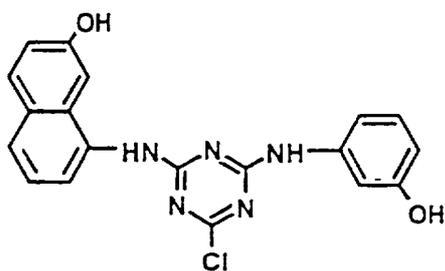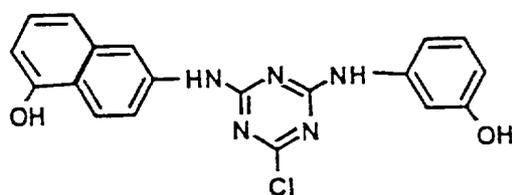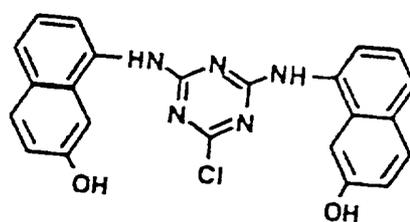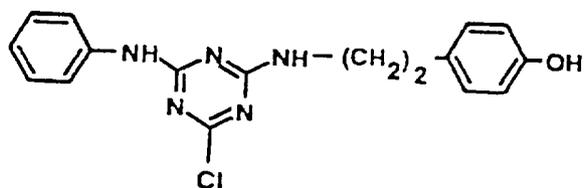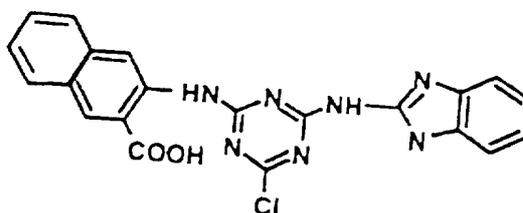
(13)

Furthermore the invention relates to a method of attaching the novel affinity ligands of General Formulae (VII) as defined above, (XIII) as defined above, (XVI) as defined above and (XI) as defined above to carbohydrate or organic polymer matrices by reacting the carbohydrate or organic polymer matrix with an activating agent followed by reaction of the activated matrix with the novel affinity ligand, optionally in the presence of an acid binding agent. The invention also relates to a method of attaching the novel affinity ligands of General Formulae (XIV) as defined above to carbohydrate or organic polymer matrices by condensation with the matrix. The invention furthermore relates to a method of attaching the novel affinity ligands of General Formulae (VII) as defined above, (XIII) as defined above, (XVI) as defined above and (XI) as defined above to metal oxide, glass or silica matrices, optionally coated with an organic polymer by reacting the optionally coated metal oxide, glass or silica matrix with an activating agent followed by reaction of the activated matrix with the novel affinity ligand, optionally in the presence of an acid binding agent. Another embodiment of the invention relates to a method of attaching the novel affinity ligands of General Formulae (XIV) as defined above to metal oxide, glass or silica matrices, optionally coated with an organic polymer by condensation with the matrix. In another embodiment the invention realates to a method of attaching novel affinity ligands of General Formula (XV) as defined above and (XII) as defined in above to a matrix of General Formula (V) as defined above by reacting the novel affinity ligands with the matrix at temperatures between -20°C and 121°C, optionally in the presence of an acid binding agent. The invention also relates to all the affinity ligand-matrix conjugates, prepared as described in the above methods.

In another embodiment the invention relates to the use of the affinity ligands according to the invention and the affinity ligand-matrix conjugates comprising such ligands according to the invention for the separation, isolation, purification, characterisation, identification or quantification of proteins or

5   proteinaceous materials, such as immunoglobulins or subclasses, fragments, precursors or derivatives thereof, whether derived from natural or recombinant sources e.g. immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA) or subclasses, fragments, precursors or derivatives thereof, whether derived from natural or recombinant sources. In another embodiment the

10   invention relates to the separation, isolation, purification, characterisation, identification or quantification of immunoglobulins by any process whereby the said immunoglobulins are applied to affinity ligand-matrix conjugates according to the invention at a pH in the range 5.0 to 12.0 and subsequently removed, eluted or desorbed by reducing the pH to 4.9 or lower.

15   The invention also relates to a process for the separation or purification of proteinaceous materials comprising carrying out affinity chromatography using as the biospecific ligand a ligand of general formula (a) as defined above.

        Another embodiment of the invention relates to the use of the affinity ligands according to the invention and the affinity ligand-matrix conjugates

20   comprising such ligands according to the invention for the separation, isolation, purification, characterisation, identification or quantification of insulins and analogues, derivatives and fragments thereof and precursors, whether derived from natural or recombinant sources and Factor VII, or human Growth Hormone or analogues, derivatives and fragments thereof and precursors.

25   By way of example, a library comprising more than 60 different ligands were designed. The library was screened with partly purified insulin precursor and selected ligands were immobilised by solid phase synthesis to agarose. The matrices were optimized with respect to coupling technology, length and nature of spacer arm, ligand density etc. resulting in 3 prototypes of dynamic

30   binding capacity of 2-5 mg/ml. A recombinantly derived insulin precursor was purified to more than 95% purity from clarified yeast fermentation broth by a single mimetic affinity purification step under very mild conditions.

An especially preferred use of the affinity ligands according to the invention and the affinity ligand-matrix conjugates comprising such ligands according to the invention is thus the purification of insulins and analogues, derivatives and fragments thereof and precursors, whether derived from natural

5   or recombinant sources. Especially preferred affinity ligand-matrix conjugates for this use comprises a ligand selected among the following:
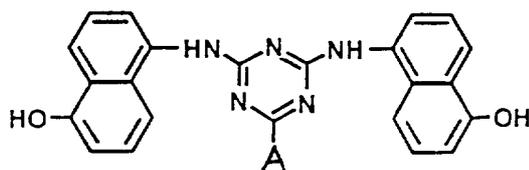
10   (5a)

15
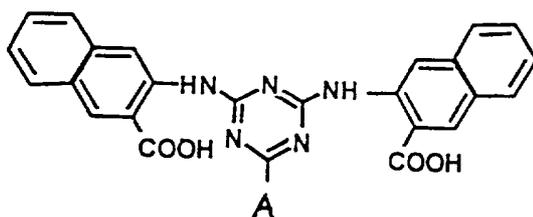
                                                                              (2a)

20
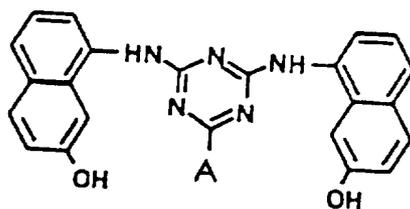
                                                                              (11a)

25

which ligand is attached to a support matrix in position A as specified above, optionally through a spacerarm interposed between the matrix and ligand

30   represented by the general formula (b) as defined above. Preferably, the ligand is 11a, and the support matrix is preferably optionally activated agarose, cellulose, silica or glass.

34

In another embodiment the invention relates to the separation, isolation, purification, characterisation, identification or quantification of insulins or insulin analogues or derivatives thereof and precursors by any process whereby the said insulins, insulin derivatives, analogues, and precursors are applied to

5   affinity ligand-matrix conjugates according to the invention at a pH in the range 4,0 to 9,0 and subsequently removed, eluted or desorbed by reducing the pH to 3,99 or lower or to 9,01 or higher. The elution procedure can e.g. alternatively be carried out by lowering the ionic strength or by addition of co-solvents such as organic solvents.

10

The invention will now be described in further detail with reference to the following examples. The examples are provided for illustrative purposes, and are not to be construed as limiting the scope of the invention in any way.

Example 1

This Example illustrates the synthesis of a typical affinity ligand defined by the reaction of halogenoheterocyclic compounds of General Formula (II)

5 with compounds of General Formula (III) and (IV).

19.8 parts of aniline were dissolved in 50 parts of acetone and the solution run into a suspension formed by pouring a solution of 36.8 parts of cyanuric chloride in parts of 200 parts of acetone into a mixture of 50 parts of ice and 50 parts of water. The mixture was stirred for 2 hours during which

10 time the pH was maintained at between 6 and 7 by the addition of a solution of 16.8 parts of sodium hydrogen carbonate in 300 parts of water. At the end of this time, the precipitate of 2-anilino-4,6-dichloro-1,3,5-triazine was filtered off, washed with water, dried in vauco and crystallised from dichloromethane.

A solution of 2.74 parts of tyramine in a mixture of 50 parts of acetone

15 and 10 parts of water was added to a solution of 4.82 parts of 2-anilino-4,6-dichloro-1,3,5-triazine in 100 parts of acetone. The mixture was heated at 50°C and held at this temperature for 2 hours whilst the pH was maintained between 6 and 7 by the addition of a solution of 1.68 parts of sodium hydrogen carbonate in 30 parts of water. At the end of this time the

20 precipitate of 2-anilino-4-[β-(4'-hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine was filtered off, washed with water and dried in vacuo.

Example 2

25 This Example illustrates the immobilisation of the product of Example 1 onto a solid phase support.

Ten parts of agarose bearing amino groups was transferred into an acetone solvent by solvent exchange using 100 parts of 30% aqueous acetone followed by 100 parts of 70% aqueous acetone and then 100 parts of

30 acetone. One part of 2-anilino-4-[-β-(4'-hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine was dissolved in 20 parts of acetone and warmed to 50°C. This warm acetone solution was added to the acetone suspension of an agarose

derivative bearing amino groups. A solution of 0.42 parts of sodium hydrogen carbonate in 5 parts of water was added to the resultant suspension and the mixture then stirred for 16 hours at 50°C. At the end of this time, the agarose support matrix was washed free of non-bonded 2-anilino-4-[-β-(4'-

5   hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine with acetone. The resulting derivatised affinity support was then transferred back to water by washing first with 70% aqueous acetone, then with 30% aqueous acetone and finally with water.

10   **Example 3**

    This Example demonstrates the use of the novel affinity matrix described in Example 2 as a specific chromatography matrix for immunoglobulins.

    One and one quarter parts of human plasma was diluted with 3.75 parts
15 of aqueous phosphate buffer having a pH of 7 and a concentration of 10 millimoles per litre and applied to a column consisting of 1 part of affinity matrix whose preparation is described in Example 2. The affinity column was then washed with phosphate buffer until UV monitoring of the wash liquors showed that all non-bound protein had been removed. The affinity matrix was
20 then washed with a citrate buffer having a pH of 3.8 and a concentration of 0.2 millimoles per litre until UV monitoring of the wash liquor showed that removal of bound protein had ceased. The protein contained in this wash liquor was shown to be immunoglobulin G.

    Table 1 gives further Examples of novel affinity ligands of the invention
25 which may be prepared by the method of Example 1 but replacing the 36.8 parts of cyanuric chloride used in Example 1 by the corresponding amount of the halogenoheterocyclic compound listed in Column II of the Table; replacing the 19.8 parts of aniline used in Example 1 by the corresponding amount of the amine listed in Column III of the Table and replacing the 2.74 parts of
30 tyramine used in Example 1 by the corresponding amount of the amine listed in Column IV of the Table. The number of the Example is given in Column I of the Table.

Table 1

I	II	III	IV
4	5-cyano-2,4,6-trichloropyrimidine	aniline	tyramine
5	5-chloro-2,4,6-trifluoropyrimidine	aniline	tyramine
6	cyanuric chloride	tyramine	tyramine
7	cyanuric chloride	aniline	4-hydroxy-benzylamine
8	cyanuric chloride	aniline	benzylamine
9	cyanuric chloride	N-methyl-aniline	4-hydroxy-benzylamine
10	cyanuric chloride	p-anisidine	p-aminophenol
11	cyanuric chloride	aniline	4-acetylamino-benzylamine
12	cyanuric chloride	p-toluidine	tyramine
13	cyanuric chloride	p-chloro-aniline	tyramine
14	cyanuric chloride	p-aminophenol	tyramine
15	cyanuric chloride	cyclohexyl-amine	tyramine
16	cyanuric chloride	β-phenyl-ethylamine	tyramine
17	cyanuric chloride	4-hydroxy-benzylamine	tyramine
18	cyanuric chloride	4-hydroxy-benzylamine	4-hydroxy-benzylamine
19	cyanuric chloride	benzylamine	tyramine
20	cyanuric chloride	tyramine	3-methylsulphonyl-aniline
21	cyanuric chloride	N-tert-butyl-benzylamine	tyramine
22	cyanuric chloride	N-isopropyl-benzylamine	tyramine
23	cyanuric chloride	p-amino-acetanilide	tyramine
24	cyanuric chloride	di-isopropyl-amine	tyramine
25	cyanuric chloride	aniline	N-benzyltyramine
26	cyanuric chloride	tyramine	N-benzyltyramine
27	cyanuric chloride	p-amino-benzamide	tyramine
28	cyanuric chloride	p-aminobenzene sulphonamide	tyramine
29	cyanuric chloride	p-amino-benzoic acid	tyramine
30	cyanuric chloride	p-amino-benzoic acid	1-amino-4-naphthol
31	cyanuric chloride	p-aminophenol	1-amino-5-naphthol
32	cyanuric chloride	p-amino-benzoic acid	1-amino-5-naphthol
33	cyanuric chloride	p-aminophenol	p-aminophenol
34	cyanuric chloride	m-amino-benzoic acid	aniline

35	cyanuric chloride	m-amino-benzoic acid	tyramine
36	cyanuric chloride	1-amino-5-naphthol	1-amino-5-naphthol
37	cyanuric chloride	m-aminophenol	1-amino-5-naphthol
38	cyanuric chloride	1-amino-4-naphthol	1-amino-5-naphthol

These ligands described in Examples 4-38 may be attached to an agarose matrix by the procedure detailed in Example 2 and used in the purification of immunoglobulin G by the procedure given in Example 3.

Example 39

6 Parts of ethylene diamine were added to a solution of 2.5 parts of 2-anilino-4-[β-(4′-hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine in 20 parts of toluene and the mixture heated at 100°C for 16 hours. The toluene was then evaporated off from the mixture under reduced pressure and the residue washed 5 times with 100 parts of water and then dried at 70°C.

Table 2 gives further Examples of novel affimity ligands of the invention which may be prepared by the method of Example 39 but replacing the 2.5 parts of 2-anilino-4-[β-(4′hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine used in Example 39 by the corresponding amount of the chlorotriazine listed in Column II of Table 2 and replacing the ethylene diamine used in Example 39 by the corresponding amount of the diamine or amino hydroxy compound listed in Column III of Table 2.

Table 2

I	II	III
40	Chlorotriazine described in Example 1	propylene diamine
41	Chlorotriazine described in Example 1	1,6-diaminohexane
42	Chlorotriazine described in Example 1	1,8-diaminooctane
43	Chlorotriazine described in Example 1	1,9-diaminononane
44	Chlorotriazine described in Example 1	1,10-diaminodecane
45	Chlorotriazine described in Example 1	1,12-diaminododecane
46	Chlorotriazine described in Example 14	1,6-diaminohexane
47	Chlorotriazine described in Example 30	1,6-diaminohexane
48	Chlorotriazine described in Example 31	1,6-diaminohexane
49	Chlorotriazine described in Example 33	1,6-diaminohexane
50	Chlorotriazine described in Example 35	1,6-diaminohexane
51	Chlorotriazine described in Example 38	1,6-diaminohexane
52	Chlorotriazine described in Example 1	N,N'-dimethylethylene diamine
53	Chlorotriazine described in Example 1	N-β-hydroxyethylethylene diamine
54	Chlorotriazine described in Example 1	piperazine
55	Chlorotriazine described in Example 1	ethanolamine
56	Chlorotriazine described in Example 1	diethanolamine
57	Chlorotriazine described in Example 1	isopropanolamine

40

Example 58

This Example illustrates the immobilisation of the compound produced in
Example 39 onto a solid phase support.

Sixty parts of agarose bearing epoxide groups was washed with 300
parts of water followed by 300 parts of a solution consisting of 5.95 parts of
potassium hydrogen carbonate dissolved in 180 parts of methanol and 120
parts of water. One part of the compound prepared according to Example 39
was dissolved in 60 parts of methanol and added to a solution of 40 parts of
aqueous potassium hydrogen carbonate solution. One hundred parts of this
aqueous methanol solution was then added to the prepared suspension of 60
parts of agarose bearing epoxy groups and the resulting suspension gently
agitated at ambient room temperature for 16 hours. The agarose based matrix
obtained was washed first with a mixture of 360 parts of methanol and 240
parts of water, and then with 600 parts of water.

Example 59

If Example 3 is repeated but replacing the column consisting of 1 part of
affinity matrix whose preparation is described in Example 2 by a column
consisting of 1 part of affinity matrix whose preparation is described in
Example 58. The protein contained in the pH 3.8 wash liquor was again shown
to be immunoglobulin G.

Example 60

This Example illustrates the immobilisation of the product obtained in
Example 39 onto a solid phase support.

Eighty parts of agarose was washed with 1050 parts of water followed
by 1050 parts of 30% aqueous acetone, then 1050 parts of 70% aqueous
acetone and finally 1425 parts of acetone. The eighty parts of agarose were

then suspended in 100 parts of acetone and the suspension warmed to 37°C. Fifteen parts of p-toluenesulphonyl chloride dissolved in 15 parts of pyridine and 25 parts of acetone was added to the agarose suspension and the mixture kept at 37°C for 8 hours. The activated agarose was then washed with 225

5   parts of acetone followed by 225 parts of 70% aqueous acetone, then 225 parts of 30% aqueous acetone and, finally, 225 parts of water.

Fortyfive parts of this activated matrix was washed with 225 parts of a solution consisting of 2.3 parts of potassium hydrogen carbonate dissolved in a mixture of 178 parts of methanol and 47 parts of water. A solution of 0.75

10   parts of the compound prepared according to Example 39 in a mixture of 45 parts of methanol and 30 parts of water containing 1.5 parts of potassium hydrogen carbonate was added to the activated agarose suspension and the mixture then left to stand for 16 hours at room temperature. The resulting affinity matrix was washed with 270 parts of methanol and 180 parts of water

15   containing 9 parts of potassium hydrogen carbonate, followed by 450 parts of water.

Prior to use, 115 parts of the resulting affinity matrix was suspended in a solution of 0.36 parts of sodium hydroxide in 45 parts of water.

20   Example 61

If Example 3 is repeated but replacing the column consisting of 1 part of the affinity matrix whose preparation is described in Example 2 by a column consisting of 1 part of the affinity matrix whose preparation is described in

25   Example 60, the protein contained in the pH 3.8 wash liquor was again shown to be immunoglobulin G.

                                     Example 62

30   Ten parts of the affinity matrix prepared according to Example 60 was allowed to stand in a phosphate buffer having a pH of 8.0 for 16 hours. Example 3 was repeated but replacing the matrix used therein with an

equivalent amount of this new affinity matrix and using cell free supernatant
from murine immunoglobulin cell culture medium in place of the human plasma
used in Example 3. The protein contained in the pH 3.8 wash liquor was again
shown to be murine immunoglobulin M.

5   Table 3 gives further Examples of the attachment of the novel affinity
ligands of the invention which may be prepared by the method of Example 58
but replacing the novel affinity ligand used in Example 58 by the corresponding
amount of the novel affinity ligand specified in Column II of Table 3 and by
replacing the 39 parts of epichlorohydrin activated agarose used in Example 58

10   by the corresponding amount of the carbohydrate listed in Column III of Table
3 activated by the reagent listed in Column IV of Table 3.

Table 3

I	II	III	IV
61	Ligand of Example 39	agarose	1,4-butanediol diglycidyl ether
62	Ligand of Example 39	agarose	cyanogen bromide
63	Ligand of Example 39	agarose	epichlorohydrin
64	Ligand of Example 39	agarose	sodium metaperiodate
65	Ligand of Example 39	agarose	2,2,2-trifluoro- -ethanesulphonyl- chloride
66	Ligand of Example 39	agarose	1,1'-carbonyldi- -imidazole
67	Ligand of Example 39	agarose	2-fluoro-1-methyl- -pyridinium toluene-4- sulphonate
68	Ligand of Example 39	agarose	divinyl sulphone
69	Ligand of Example 39	agarose	cyanuric chloride

15

43

Example 70

If the preparation of the ligand detailed in Example 1 is repeated but reacting the 2.74 parts of tyramine with 4.84 parts of 2-phenoxy-4,6-dichloro-
5   1,3,5-triazine instead of 4.82 parts of 2-anilino-4,6-dichloro-1,3,5-triazine, there is obtained 2-phenoxy-4-[β-(4′-hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine, which may be used in place of 2-anilino-4-[β-(4′-hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine in the preparation of an affinity matrix by the method of Example 2. Again, immunoglobulin G may be conveniently
10   isolated from human plasma by the technique described in Example 3.

Example 71

Use of 5.16 parts of 2-phenylthio-4,6-dichloro-1,3,5-triazine in place of
15   the 4.84 parts of 2-phenoxy-4,6-dichloro-1,3,5-triazine used in Example 70 gives 2-phenylthio-4-[β-(4′-hydroxyphenyl)-ethylamino]-6-chloro-1,3,5-triazine, which may be similarly used to purify immunoglobulin G.

Example 72

20

This Example illustrates an alternative approach to producing affinity ligand-matrix conjugates as exemplified in Example 2 and Example 58.

Ten parts of agarose bearing aminoethylamino groups were stirred at 0-5°C with 5 parts of acetone and 5 parts of an aqueous phosphate buffer
25   having a pH of 7 and a concentration of 0.5 moles per litre. To this suspension was added 2.5 parts of a solution comprising 1 part of cyanuric chloride in 10 parts of acetone and the mixture then agitated for an hour at a temperature of 0-5°C. The resulting dichlorotriazine activated agarose was washed sequentially with 50 parts of 50% aqueous acetone, 50 parts of water, 50
30   parts of 50% aqueous acetone and 100 parts of water. Twenty parts of the washed dichlorotriazine activated agarose was added to 20 parts of an aqueous solution comprising 1 part of tyramine in 200 parts of water and the

44

resulting suspension agitated for 16 hours at ambient room temperature. At the end of this period, the resulting derivatised matrix was washed with 200 parts of water and added to 20 parts of an aqueous solution comprising 0.55 parts of aniline dissolved in 200 parts of water. The resulting suspension was
5   agitated for 16 hours at 90°C after which time the derivatised matrix was washed with 200 parts of water.

Example 73

10   If Example 3 is repeated but replacing the column consisting of 1 part of the affinity matrix whose preparation is described in Example 2 by a column consisting of 1 part of the affinity matrix whose preparation is described in Example 72, the protein contained in the pH 3.8 wash liquor was again shown to be immunoglobulin G.

15

Example 74

        This example illustrates the synthesis of a library of affinity ligand-matrix conjugates of this invention which may be subsequently screened to determine
20   their protein binding properties.

        One part of agarose bearing amino groups was mixed with 1 part 1M potassium phosphate buffer, pH 7.0 and settled under gravity. The buffered amine agarose was transferred to a reaction vessel and mixed at 0-5°C with 0.5 parts 0.5M potassium phosphate buffer, pH 7.0 and 0.5 parts acetone.
25   One quarter of a part of a solution comprising 1 part cyanuric chloride in 10 parts acetone was added and the mixture stirred at 0-5°C for 1 hour, after which the mixture was filtered and washed sequentially with 10 parts 50% acetone, 6 parts water, 6 parts 50% acetone and 10 parts water to provide 2,4-dichloro-s-triazin-6-yl activated agarose.

30   One part of the 2,4-dichloro-s-triazin-6-yl activated agarose was added to 5 mole equivalents of the amine compound listed in Column II of Table 4 dissolved in 2 to 3 parts of a solution comprising 1 part acetone and 1 part

45

water and adjusted to neutral pH by addidtion of sodium hydroxide. The suspension was mixed for 24 hours at 30°C. The resulting monochloro-s-triazin-6-yl activated agaroses were washed sequentially with 10 parts of dimethylformamide, 10 parts of a solution comprising 3 parts propan-2-ol and

5   7 parts 0.2M sodium hydroxide, 10 parts of water and settled under gravity.

One part of the monochloro-s-triazin-6-yl-activated agarose was added to 5 mole equivalents of the amine compound listed in Column III of Table 4 dissolved in 2 to 3 parts of a solution comprising 1 part dimethylformamide and 1 part water and adjusted to neutral pH by addition of sodium hydroxide.

10   The suspension was mixed for 72 hours at 85 to 95°C. The resulting affinity ligand-matrix conjugates were washed sequentially with 10 parts of dimethylformamide, 10 parts of a solution comprising 3 parts propan-2-ol and 7 parts 0.2M sodium hydroxide, 10 parts of water and settled under gravity. A library of affinity ligand-matrix conjugates of this Invention was synthesised,

15   Examples of which are identified in Column I of Table 4.

46

Table 4

I	II	III
75	1,3-diaminopropane	tyramine
76	1,3-diaminopropane	N-benzylphenethylamine
77	1,4-diaminobutane	3-aminophenol
78	1,4-diaminobutane	tyramine
79	1,4-diaminobutane	1-amino-5-naphthol
80	1,4-diaminobutane	N-isopropylbenzylamine
81	1,4-diaminobutane	N-benzylphenethylamine
82	aniline	diaminopropane
83	aniline	aniline
84	aniline	3-aminophenol
85	aniline	3-aminobenzoic acid
86	aniline	tyramine
87	aniline	1-amino-4-naphthol
88	aniline	1-amino-2-naphthol
89	aniline	3-amino-2-naphthol
90	aniline	1-amino-6-naphthol ·
91	aniline	N-isopropylbenzylamine-4-phenylbutylamine
92	aniline	N-benzylphenethylamine
93	3-aminophenol	3-aminophenol
94	3-aminophenol	1-amino-5-naphthol
95	1,6-diaminohexane	tyramine
96	1,6-diaminohexane	4-phenylbutylamine
97	3-aminobenzoic acid	1-amino-5-naphthol
98	tyramine	tyramine
99	tyramine	1-amino-5-naphthol
100	tyramine	1-amino-7-naphthol
101	tyramine	1-amino-6-naphthol
102	1-amino-5-naphthol	aniline
103	1-amino-5-naphthol	3-aminophenol
104	1-amino-5-naphthol	4-aminobenzoic acid

105	1-amino-5-naphthol	3,5-diaminobenzoic acid
106	1-amino-5-naphthol	benzylamine
107	1-amino-5-naphthol	tyramine
108	1-amino-5-naphthol	1-amino-5-naphthol
109	1-amino-7-naphthol	3-aminophenol
110	1-amino-7-naphthol	1-amino-7-naphthol
111	1-amino-4-naphthol	3-aminophenol
112	1-amino-4-naphthol	1-amino-4-naphthol
113	1-amino-2-naphthol	3-aminophenol
114	1-amino-2-naphthol	1-amino-2-naphthol
115	3-amino-2-naphthol	3-aminophenol
116	3-amino-2-naphthol	3-amino-2-naphthol
117	1-amino-6-naphthol	3-aminophenol
118	1-amino-6-naphthol	1-amino-6-naphthol
119	2-amino-1-naphthol	3-aminophenol
120	2-amino-1-naphthol	2-amino-1-naphthol
121	6-amino-1-naphthol	3-aminophenol
122	6-amino-1-naphthol	6-amino-1-naphthol
123	1-amino-6-naphthalenesulphonic acid	3,5-diaminobenzoic acid
124	1-amino-6-naphthalenesulphonic acid	benzylamine
125	1-amino-6-naphthalenesulphonic acid	tyramine
126	3-amino-2-napthoic acid	3-aminophenol
127	3-amino-2-napthoic acid	3-amino-2-napthoic acid

Example 128

This example demonstrates the screening process by which the protein

5 binding properties of affinity ligand-matrix conjugates described in example 74

may be identified.

Chromatography columns of 1 ml total volume were packed with affinity

ligand-matrix conjugates of Examples 75-127. The columns were equilibrated

48

by flushing with 10 ml of 50 mM sodium phosphate buffer, pH 8.0. One ml of
a solution comprising 1.5 mg of human IgG per ml of 50 mM sodium
phosphate buffer, pH 8.0 was applied to each chromatography column which
were subsequently washed with 10 ml of 50 mM sodium phosphate buffer, pH

5   8.0 to remove non-bound IgG. Bound IgG was eluted by flushing each column
with 5 ml of 50 mM sodium citrate buffer, pH 3.0. The IgG content of the
wash and elution fractions was determined by measurement of absorbance at
280 nm against a buffer blank. Analysis of the results revealed affinity ligand-
matrix conjugates of Examples 75 to 127 all exhibited human IgG binding. Of

10 these, almost quantitative elution of bound IgG was achieved for affinity
ligand-matrix conjugates of Examples 92, 99, 101, 102, 103, 111, 113 and
119 which, as a consequence, are considered to be of exceptional value in the
separation, isolation or purification of human IgG.

15   **Example 129**

Selective binding and elution of Recombinant Coagulation Factor VIIa (rFVIIa)
applied to the affinity matrix according to Example 181 from cell culture media.

20 *Procedure:*

0.85 ml settled volume of the affinity matrix prepared as in example 181 was
packed into a 5 by 50 mm column (Pharmacia HR 5/5) and equilibrated with
20 mL of buffer A: 20 mM Tris, 50 mM NaCl, 5 mM CaCl$_2$, pH 8.0.
5 ml of conditioned BHK Cell culture supernatant enriched with 1.4 mg rFVIIa

25 was applied to the packed column.
After washing off non-binding proteins with 10 mL of buffer A, rFVIIa was
eluted by applying 5 mL of buffer B: 20 mM Tri-Sodium-Citrate, 50 mM Tris
pH 8.0.

30 The flow rate during the chromatographic procedure was 0.3 mL/ minute.

The column effluent was passed through an in line UV-monitor and collected in

49

1 mL fraction, and each fraction analyzed for the content of rFVIIa and total protein by analytical reversed phase High Performance Liquid Chromatography(RP-HPLC)

5   *Results:*

The UV- monitor out put showed that most of the UV (280nm) absorbing material came out during applying the supernatant and the following wash with buffer A. During the following elution with buffer B a distinct peak was monitored, that matched the expected size for the applied amount of rFVIIa.

10

The RP-HPLC analysis of the collected fractions showed that 90 % of the applied amount of rFVIIa came out in the fractions during elution with buffer B. The purity of rFVIIa in these fractions were above 95%.

15   The results show that selective binding of rFVIIa from enriched culture media to the used ligand is achieved.

Example 130

20   **Purification of insulin B-chain$^{1-29}$-A-A-K-insulin A-chain$^{1-21}$ on an affinity ligand-matrix conjugate according to Example 171:**

255 mg of insulin B-chain$^{1-29}$-Ala-Ala-Lys-insulin A-chain$^{1-21}$, (batch A202558) was suspended in 51 ml of H_2O. 10 drops of 1 M acetic acid were added to

25   solubilize the precursor. 0.2 M potassium citrate pH 5.5 was added to a total volume of 510 ml resulting in a solution of 0.12 mg/ml (by RP-HPLC analysis). pH was measured to 5.53, the ionic strength to 30.0 mS/cm and the redox potential to 273 mV.

400 ml of the above solution was applied a Pharmacia K16 (1.6 x 6 cm)

30   column packed with 12 ml of the above matrix conjugate, equilibrated in 0.2 M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The column was washed in 50 ml of 0.2 M potassium citrate pH 5.5 and then eluted with 0.1 M acetic acid at 1.8 ml/min. 12 fractions of 5.0 ml were

collected.

The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50 ml of 0.1 M citric acid, 60% v/v ethanol.

Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to
5   analysis.

Sample	Identification	MI3 conc.	Volume	Amount	%
Application	E091b	0.12 mg/ml	400 ml	48.8 mg	100
Run through	E091c	0.00 mg/ml		0.0 mg	0
Wash	E093a	0.00 mg/ml		0.0 mg	0
Fraction 3	E093b	0.01 mg/ml	5.0 ml	0.1 mg	
Fraction 4	E093c	5.00 mg/ml	5.0 ml	25.0 mg	
Fraction 5	E093d	2.62 mg/ml	5.0 ml	13.1 mg	
Fraction 6	E093e	0.52 mg/ml	5.0 ml	2.6 mg	
Fractions				40.8 mg	83

Thus a total recovery of 83% was obtained.

10   The purity of the product was determined to 94% by RP-HPCL analysis. The remaining impurities were insulin related.

Example 131

15

Purification of des-Thr^{B30}-insulin on an affinity ligand-matrix conjugate according to Example 171:

150 mg of des-Thr^{B30}-insulin (INS-J-009) precipitate was suspended in 50 ml
20   H_2O. 5 drops of 2 M acetic acid were added to dissolve the suspension. 0.2 M of potassium citrate pH 5.5 was added to a volume of 500 ml resulting in a concentration of 0.076 mg/ml (by RP-HPLC analysis). pH was measured to 5.52, the ionic strength to 30.0 mS/cm and the redox potential to 264 mV. 400 ml of the above solution was applied a Pharmacia K16 (1.6 x 6 cm)
25   column packed with 12 ml of the above matrix conjugate, equilibrated in 0.2

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M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The column was washed in 50 ml of 0.2 M potassium citrate pH 5.5 and then eluted with 0.1 M acetic acid at 1.8 ml/min. 10 fractions of 5.0 ml were collected.

5   The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50 ml of 0.1 M citric acid, 60% v/v ethanol.

Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to analysis.

Sample	Identification	MI3 conc.	Volume	Amount	%
Application	E105a	0.076mg/ml	400 ml	30.0mg	100
Run through	E105b	0.00 mg/ml		0.0 mg	0
Wash	E105d	0.00 mg/ml		0.0 mg	0
Fraction 3	E105e	0.00 mg/ml	5.0 ml	0.0 mg	0
Fraction 4	E105f	0.41 mg/ml	5.0 ml	2.1mg	
Fraction 5	E105n	1.93 mg/ml	5.0 ml	9.7 mg	
Fraction 6	E105o	1.14 mg/ml	5.0 ml	5.7 mg	
Fraction 7	E105p	0.48 mg/ml	5.0 ml	2.4 mg	
Fraction 8	E105j	0.31 mg/ml	5.0 ml	1.6 mg	
Fractions				21.5 mg	71%

10

Thus a total recovery of 71% was obtained.

The purity of the product was determined to 91% by RP-HPCL analysis. the remaining impurities were insulin related.

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Example 132

Purification of insulin B-chain$^{1-29}$-A-A-K-insulin A-chain$^{1-21}$ on an affinity ligand-matrix conjugate according to Example 145:

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2 l of centrifuged broth (batch 628) was adjusted to pH 5.5 with 5 M NaOH and filtered through a Leitz Tiefenfilter (Seitz EK) filter followed by filtration through a Leitz Tiefenfilter (Seitz EKS) filter. The concentration was measured to 0.006 mg/ml by RP-HPLC. The ionic strength was measured to 12.2 mS/cm

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and the redox potential to 316 mV.

1000 ml of the above solution was applied a Pharmacia K16 (1.6 x 6 cm) column packed with 12 ml of the above matrix conjugate, equilibrated in 0.2 M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The column was washed in 50 ml of 0.2 M potassium citrate pH 5.5 and then eluted with 0.1 M acetic acid at 1.8 ml/min. 11 fractions of 5.0 ml were collected.

The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50 ml of 0.1 M citric acid, 60% v/v ethanol.

Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to analysis.

Sample	Identification	MI3 conc.	Volume	Amount	%
Application	E111e	0.006mg/ml	1000 ml	6.0mg	100
Run through	E115a	0.00 mg/ml		0.0 mg	0
Wash	E115b	0.00 mg/ml		0.0 mg	0
Fraction 3	E115c	0.00 mg/ml	5.0 ml	0.0 mg	0
Fraction 4	E115d	0.58 mg/ml	5.0 ml	2.9mg	
Fraction 5	E115e	0.11 mg/ml	5.0 ml	0.6 mg	
Fraction 6	E115f	0.04 mg/ml	5.0 ml	0.2 mg	
Fraction 7	E115g	0.02 mg/ml	5.0 ml	0.1 mg	
Fractions				3.8 mg	63%

Thus a total recovery of 63% was obtained.

The purity of the product was determined to 88% by RP-HPLC analysis. The remaining impurities were insulin related.

Example 133

Purification of insulin B-chain$^{1-29}$-A-A-K-insulin A-chain$^{1-21}$ on an affinity ligand-matrix conjugate according to Example 171:

2 l of centrifuged broth (batch 628) was adjusted to pH 5.5 with 5 M NaOH
and filtered through a Leitz Tiefenfilter (Seitz EK) filter followed by filtration
through a Leitz Tiefenfilter (Seitz EKS) filter. The concentration was measured

5   to 0.006 mg/ml by RP-HPLC. The ionic strength was measured to 12.2 mS/cm
and the redox potential to 316 mV.
1000 ml of the above solution was applied a Pharmacia K16 (1.6 x 6 cm)
column packed with 12 ml of the above matrix conjugate, equilibrated in 0.2
M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The

10 column was washed in 50 ml of 0.2 M potassium citrate pH 5.5 and then
eluted with 0.1 M acetic acid at 1.8 ml/min. 11 fractions of 5.0 ml were
collected.
The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50
ml of 0.1 M citric acid, 60% v/v ethanol.

15 Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to
analysis.

Sample	Identification	MI3 conc.	Volume	Amount	%
Application	E111e	0.006mg/ml	830 ml	5.0mg	100
Run through	E113a	0.00 mg/ml		0.0 mg	0
Wash	E113b	0.00 mg/ml		0.0 mg	0
Fraction 3	E113c	0.00 mg/ml	5.0 ml	0.0 mg	0
Fraction 4	E113d	0.58 mg/ml	5.0 ml	2.9mg	
Fraction 5	E113e	0.10 mg/ml	5.0 ml	0.5 mg	
Fraction 6	E113f	0.04 mg/ml	5.0 ml	0.2 mg	
Fraction 7	E113g	0.02 mg/ml	5.0 ml	0.1 mg	
Fractions				3.7 mg	74%

Thus a total recovery of 74% was obtained.

20

The purity of the product was determined to 86% by RP-HPLC analysis. The
remaining impurities were insulin related.

Example 134

Purification of EEAEPK-insulin B-chain(1-29)-AAK-insulin A-chain(1-21) on an affinity ligand-matrix conjugate according to Example 171:

Centrifuged yeast broth (batch Y44) was filtered through a Leitz Tiefenfilter (Seitz EK) filter resulting in a concentration of 0.35 mg/ml (by RP-HPLC analysis). pH was measured to 5.27, the ionic strength to 7.38 mS/cm and the redox potential to 221 mV.

120 ml of the above solution was applied a Pharmacia K16 (1.6 x 6 cm) column packed with 12 ml of the above matrix conjugate, equilibrated in 0.2 M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The column was washed in 50 ml of 0.2 M potassium citrate pH 5.5 and then eluted with 0.1 M acetic acid at 1.8 ml/min. 11 fractions of 5.0 ml were collected.

The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50 ml of 0.1 M citric acid, 60% v/v ethanol.

Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to analysis.

Sample	Identification	MI3 conc.	Volume	Amount	%
Application	E127b	0.35 mg/ml	120 ml	42.0mg	100
Run through	E127c	0.00 mg/ml		0.0 mg	0
Wash	E127d	0.00 mg/ml		0.0 mg	0
Fraction 3	E127e	2.26 mg/ml	5.0 ml	11.3 mg	
Fraction 4	E127f	2.07 mg/ml	5.0 ml	10.4mg	
Fraction 5	E127g	1.12 mg/ml	5.0 ml	5.6 mg	
Fraction 6	E127h	1.27 mg/ml	5.0 ml	6.4 mg	
Fractions				33.6mg	79%

Thus a total recovery of 79% was obtained.

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The purity of the product was determined to 93% by RP-HPLC analysis. The remaining impurities were insulin related.

5   **Example 135**

Purification of [Asp^{B28}]-insulin-B-chain$^{1-29}$-A-A-K-insulin-A-chain$^{1-21}$ on an affinity ligand-matrix conjugate according to Example 171:

10 Centrifuged broth (batch GSG9414) was adjusted to pH 5.5 with 5 M NaOH and filtered through a Leitz Tiefenfilter (Seitz EK) filter followed by filtration through a Leitz Tiefenfilter (Seitz EKS) filter. The concentration was measured to 0.02 mg/ml by RP-HPLC. The ionic strength was measured to 17.0 mS/cm and the redox potential to 308 mV.

15 800 ml of the above solution was applied a Pharmacia K16 (1.6 x 6 cm) column packed with 12 ml of the above matrix conjugate, equilibrated in 0.2 M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The column was washed in 50 ml of 0.2 M potassium citrate pH 5.5 and then eluted with 0.1 M acetic acid at 1.8 ml/min. 12 fractions of 5.0 ml were

20 collected.

The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50 ml of 0.1 M citric acid, 60% v/v ethanol.

Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to analysis.

Sample	Identification	MI3 conc.	Volume	Amount	%
Application	E107b	0.02mg/ml	800 ml	16.0mg	100
Run through	E107c	0.00 mg/ml		0.0 mg	0
Wash	E107d	0.00 mg/ml		0.0 mg	0
Fraction 2	E109a	0.00 mg/ml	5.0 ml	0.0 mg	0
Fraction 3	E109b	0.12 mg/ml	5.0 ml	0.6 mg	
Fraction 4	E109c	0.76 mg/ml	5.0 ml	3.8 mg	
Fraction 5	E109d	1.04 mg/ml	5.0 ml	5.2 mg	
Fraction 6	E109e	0.31 mg/ml	5.0 ml	1.6 mg	
Fraction 7	E109f	0.10 mg/ml	5.0 ml	0.5 mg	
Fraction 8	E109g	0.06 mg/ml	5.0 ml	0.3 mg	
Fractions				12 mg	75%

Thus a total recovery of 75% was obtained.

5   The purity of the product was determined to 84% by RP-HPLC analysis. The
remaining impurities were insulin related.

Example 136

10   This example further demonstrates the screening process by which the protein
binding properties of affinity ligand-matrix conjugates of this invention may be
identified.

A library of affinity ligand matrix conjugates of this invention were
synthesised according to example 74 except that the 5 mole equivalents of the
15   amine compound listed in Column II of Table 4 were replaced by the
corresponding amount of the amine compound listed in Column II of Table 5
and the 5 mole equivalents of the amine compound listed in Column III of Table
4 were replaced by the corresponding amount of the amine compound listed in
Column III of Table 5. A library of affinity ligand-matrix conjugates of this
20   invention was synthesised, Examples of which are identified in Column I of
Table 5.

Chromatography columns of 1 ml total volume were packed with affinity ligand-matrix conjugates of Examples 137-180. The columns were equilibrated by flushing with 10 ml of 0.2 M sodium acetate, 0.1 M sodium chloride buffer, pH 5.0. Twelve ml of clarified fermenter broth containing 50 mg/ml human

5   insulin precursor was applied to each chromatography column which were subsequently washed with 12 ml of 0.2 M sodium acetate, 0.1 M sodium chloride buffer, pH 5.0 to remove non-bound material. Bound human insulin precursor was eluted by flushing each column with 3 ml of 2M acetic acid. The human insulin precursor content of the flow-through, wash and elution

10 fractions was determined by high performance liquid chromatography (HPLC) using a C18 reverse-phase silica column (4 x 250 mm) and a solvent system comprising buffer A (0.2 M sodium sulphate, 40 mM phosphoric acid and 10% (v/v) acetonitrile, pH 2) and buffer B (50% (v/v acetonitrile) delivered at a flow rate of 1 ml per minute in the proportions 55% buffer A to 45% buffer B. The

15 elution time of human insulin precursor was determined by comparison to a reference standard.

Analysis of the results revealed affinity ligand-matrix conjugates of Examples 139, 140, 145, 148, 153, 159, 162, 163, 164, 166, 167, 170, 171, 173 all bound human insulin precursor which was eluted under the

20 conditions described in this Example. As a consequence, affinity ligand-matrix conjugates of Examples 139, 140, 145, 148, 153, 159, 162, 163, 164, 166, 167, 170, 171, 173 are considered to be of exexptional value in the separation, isolation or purification of human insulin precursor.

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Table 5

I	II	III
137	1-amino-6-naphthalenesulphonic acid	benzylamine
138	1-amino-6-naphthalenesulphonic acid	3,5-diaminobenzoic acid
139	1-amino-5-naphthol	benzylamine
140	1-amino-5-naphthol	3,5-diaminobenzoic acid
141	benzylamine	3-aminobenzoic acid
142	benzylamine	4-aminobenzoic acid
143	tyramine	3-aminobenzoic acid
144	tyramine	4-aminobenzoic acid
145	1-amino-5-naphthol	1-amino-5-naphthol
146	1-amino-5-naphthol	3-aminobenzoic acid
147	1-amino-5-naphthol	4-aminobenzoic acid
148	1-amino-5-naphthol	tyramine
149	1-amino-6-naphthalenesulphonic acid	tyramine
150	1-amino-5-naphthol	3-amino-1,2-propanediol
151	1-amino-5-naphthol	3-aminopropan-1-ol
152	1-amino-5-naphthol	5-aminopentan-1-ol
153	1-amino-5-naphthol	3-aminophenol
154	1-amino-5-naphthol	6-aminocaproic acid
155	1-aminonaphthalene	benzylamine
156	1-aminonaphthalene	3,5-diaminobenzoic acid
157	1-aminonaphthalene	3-aminobenzoic acid
158	1-aminonaphthalene	4-aminobenzoic acid
159	3-amino-2-naphthoic acid	3-aminophenol
160	4-amino-1-naphthol	3-aminophenol
161	1-amino-2-naphthol	3-aminophenol
162	3-amino-2-naphthol	3-aminophenol
163	1-amino-6-naphthol	3-aminophenol
164	1-amino-7-naphthol	3-aminophenol
165	2-amino-1-naphthol	3-aminophenol

166	6-amino-1-naphthol	3-aminophenol
167	3-amino-2-naphthoic acid	3-amino-2-naphthoic acid
168	4-amino-1-naphthol	4-amino-1-naphthol
169	1-amino-2-naphthol	1-amino-2-naphthol
170	3-amino-2-naphthol	3-amino-2-naphthol
171	1-amino-7-naphthol	1-amino-7-naphthol
172	2-amino-1-naphthol	2-amino-1-naphthol
173	6-amino-1-naphthol	6-amino-1-naphthol
174	3-amino-2-naphtoic acid	1-amino-5-naphthol
175	3-amino-2-naphthoic acid	4-amino-1-naphthol
176	3-amino-2-naphthoic acid	2-amino-1-naphthol
177	1-amino-7-naphthol	1-amino-5-naphthol
178	1-amino-7-naphthol	3-amino-2-naphthoic acid
179	1-amino-7-naphthol	4-amino-1-naphthol
180	1-amino-7-naphthol	2-amino-1-naphthol

Example 181

This example demonstrates the process by which affinity ligand-matrix

5   conjugates of this invention may be synthesised which are of value for the

purification of Factor VII.

An affinity ligand-matrix conjugate of this invention was synthesised

according to Example 74 except that the 5 mole equivalents of the amine

compound listed in Column II of Table 4 were replaced by the corresponding

10   amount of 2-aminobenzimidazole and the 5 mole equivalents of the amine

compound listed in Column III of Table 4 were replaced by 3-amino-2-

naphthoic acid. This affinity ligand-matrix conjugate may be used for the

purification of Factor VIIa according to Example 129 of this invention.

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Example 182

Purification of EEAEPK-insulin B-chain$^{1-29}$-A-A-K-insulin A-chain$^{1-21}$ on an affinity ligand-matrix conjugate according to Example 171:

50 ml of ionexchange purified insulin precursor (EEAEPK-insulin B-chain$^{1-29}$-A-A-K-insulin A-chain$^{1-21}$ at 2.2 mg/ml) was applied a Pharmacia K16 (1.6 x 6 cm) column packed with 12 ml of the conjugate matrix, equilibrated in 0.2 M potassium citrate pH 5.5, at 1.8 ml/min at ambient temperature. The column was washed in 50 ml of 0.1 M potassium citrate, 0.2 M potassium sulfate pH 5.5 and then eluted with 0.1 M acetic acid at 1.8 ml/min. 5.0 ml fractions were collected.

The column was cleaned with 50 ml of 0.5 M NaOH and regenerated with 50 ml of 0.1 M citric acid, 60% v/v ethanol.

Samples for RP-HPLC analysis were diluted with 2 M acetic acid prior to analysis.

Sample	Identification	Conc. mg/ml	Volume ml	Amount mg	%
Application	R-029e	2.27	50	114	100
Run through	R-033a	0.03	50	1.4	1.2
Wash	R-033b	0.09	50	4.3	3.8
Pool 1	R-033c	5.61	15	84.2	74
Pool 2	R-033d	0.55	10	5.5	4.8

A dynamic binding capacity of 9.5 mg/ml matrix was demonstrated with a yield of 88% of the precursor.

Claims

1.   Affinity ligand-matrix conjugates comprising a ligand with the general formula (a):

5

(a) $R_1 - (CH_2)_{\overline{p}} - Y$ X $Z - (CH_2)_{\overline{n}} - Q - R_6$

with the ring bearing R_4, R_5 substituents and the N—X ring substituted with A

A
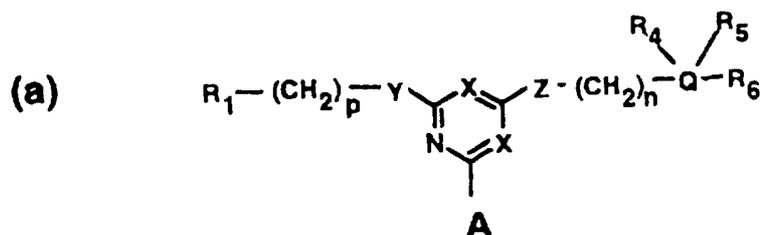
wherein

R₁ represents a hydrogen atom, an alkyl group containing from 1 to 6

10   carbon atoms, a hydroxyalkyl group containing from 1 to 6 carbon atoms, a cyclohexyl group, an amino group, a phenyl group, naphthyl group, 1-phenylpyrazole, indazole, benzthiazole group, benzoxazole group or a benzimidazole group, each of which benzene, naphthalene, 1-phenylpyrazole, indazole, benzthiazole, benzoxazole or benzimidazole ring is optionally

15   substituted with one or more substituents independently selected from the group consisting of alkyl groups containing from 1 to 6 carbon atoms, alkoxy groups containing from 1 to 6 carbon atoms, acyloxy or acylamino groups containing from 1 to 6 carbon atoms, amino groups, hydroxyl groups, carboxylic acid groups, sulphonic acid groups, carbamoyl groups, sulphamoyl

20   groups, alkylsulphonyl groups containing from 1 to 6 carbon atoms and halogen atoms;

Y represents an oxygen atom, a sulphur atom or a group N-R₂;

Z represents an oxygen atom, a sulphur atom or a group N-R₃;

R₂ and R₃ each independently represent a hydrogen atom, an alkyl group

25   containing from 1 to 6 carbon atoms; a hydroxyalkyl group containing from 1 to 6 carbon atoms, a benzyl group or a β-phenylethyl group;

R₄, R₅ and R₆ each independently represent a hydrogen atom, a hydroxyl group, an alkyl group containing from 1 to 6 carbon atoms, an alkoxy group containing from 1 to 6 carbon atoms, an amino group, an acyloxy or acylamino

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group containing from 1 to 6 carbon atoms, a carboxylic acid group, a
sulphonic acid group, a carbamoyl or sulphamoyl group, an alkylsulphonyl
group containing from 1 to 6 carbon atoms or a halogen atom;

one of the symbols X represents a nitrogen atom and the other symbol

5   X represents a nitrogen atom or a carbon atom carrying a chlorine atom or a
cyano group;

Q represents a benzene, naphthalene, 1-phenylpyrazole, indazole,
benzthiazole, benzoxazole or benzimidazole ring;

n is an integer between 0 and 6;

10   p is an integer between 0 and 20;

and

which ligand is attached to a support matrix in position (A), optionally through
a spacer arm interposed between the matrix and ligand.

15 2.   Affinity ligand-matrix conjugates according to claim 1 wherein the
optional spacer arm interposed between the ligand and the matrix is
represented by the general formula (b)

$$-T-[-L-V-]_m-\qquad\text{(b)}$$
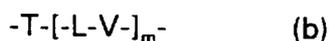
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wherein T represents an oxygen atom, a sulphur atom or a group $N-R_7$;
wherein R_7 represents a hydrogen atom or an alkyl group containing from 1 to
6 carbon atoms;

V represents an oxygen atom, a sulphur atom, a -COO- group, a CONH

25 group or an NHCO group or a $-PO_3H-$ group , an $NH-arylene-SO_2-CH_2-CH_2$ group
or an $N-R_8$ group; wherein R_8 represents a hydrogen atom or an alkyl group
containing from 1 to 6 carbon atoms;

L represents an optionally substituted hydrocarbon linkage containing
from 2 to 20 carbon atoms; and

30   m is 0 or 1.

3.   Affinity ligand-matrix conjugates which are represented by the General

Formula (I):

$$R_1 - (CH_2)_p - Y \diagdown X \diagup Z - (CH_2)_n - Q - R_6 \qquad R_4 \diagup R_5$$

(I)

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$$N \diagdown X$$

$$T - [\text{-L-V-}]_m - M$$
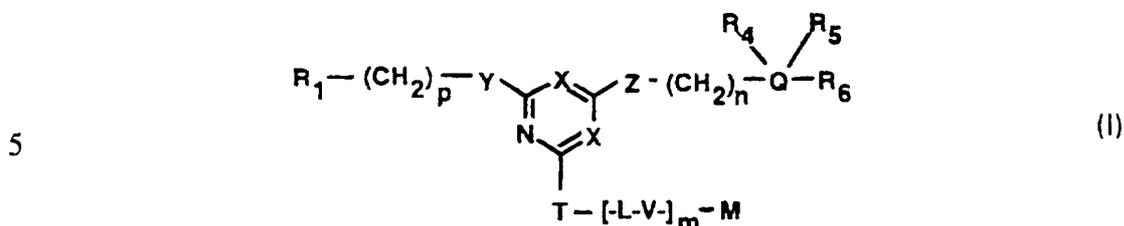
wherein

10   R_1, Y, Z, R_2, R_3, R_4, R_5, R_6, X, Q, n and p are as defined above

T represents an oxygen atom, a sulphur atom or a group $N-R_7$;

V represents an oxygen atom, a sulphur atom, a -COO- group, a CONH group or an NHCO group or a $-PO_3H-$ group , an NH-arylene-$SO_2-CH_2-CH_2$ group or an $N-R_8$ group;

15   R_7 and R_8 each independently represents a hydrogen atom or an alkyl group containing from 1 to 6 carbon atoms;

L represents an optionally substituted hydrocarbon linkage containing from 2 to 20 carbon atoms;

m is 0 or 1; and

20   M represents the residue of a support matrix.

4.   Affinity ligand matrix conjugates according to anyone of the preceding claims wherein M represents the residue of a support matrix which may be any compound or material, particulate or non particulate, soluble or insoluble,

25   porous or non-porous which may be used in conjunction with affinity ligands to form a novel affinity ligand-matrix conjugate according to anyone of the preceding claims and which provides a convenient means of separating the affinity ligands from solutes in a contacting solution.

30   5.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_1 represents a phenyl or naphthyl group each of which is optionally substituted on the benzene or naphthalene ring with one or more

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independently selected from hydroxyl groups and carboxylic acid groups.

6.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_2 represents a hydrogen atom.

7.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_3 represents a hydrogen atom.

8.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_4 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group, or an amino group.

9.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_5 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group, or an amino group.

10.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_6 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group, or an amino group.

11.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein R_7 represents a hydrogen atom.

12.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein T represents an oxygen atom or an NH group.

13.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein Y represents N-R_2 wherein R_2 is as defined above.

14.   Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein Z represents N-R_3 wherein R_3 is as defined above.

15. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein both X represents a nitrogen atom.

16. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein Q represents a benzene or naphthalene ring.

17. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein n represents 0 or 2.

18. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein p represents 0 or 2.

19. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein m represents 0 or 1.

20. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein L represents an ethyl, propyl, hydroxy propyl, butyl, pentyl, hexyl, octyl or decyl group and V and m are as defined above.

21. An affinity ligand-matrix conjugate according to anyone of the preceding claims wherein V represents an oxygen atom or an NH group and L and m are as defined above.

22. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein m represents 1 and L and V are as defined above.

23. Affinity ligand-matrix conjugates according to anyone of the preceding claims wherein the residue of a support matrix M represents optionally activated agarose, silica, cellulose, glass, toyopearl, hydroxyethylmethacrylate, polyacrylamide, styrenedivinylbenzene, Hyper D, perfluorocarbons.

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24. Affinity ligand-matrix conjugates according to claim 23 wherein M represents optionally tresyl activated, sulphonylchloride activated, tosyl activated, vinylsulphone activated or epoxy activated agarose.

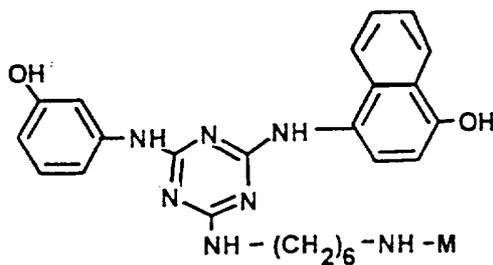5 25. Affinity ligand-matrix conjugates according to anyone of the preceding claims selected from:
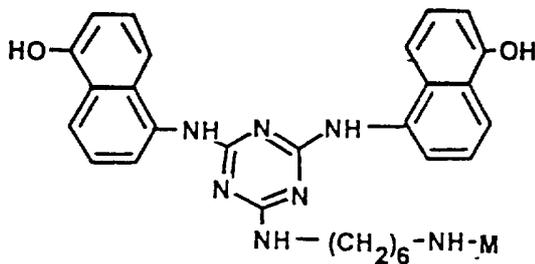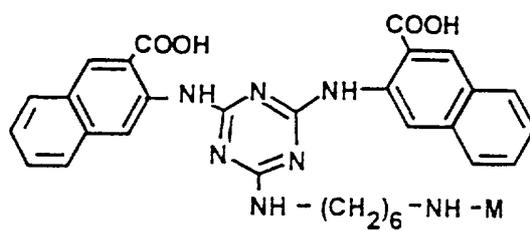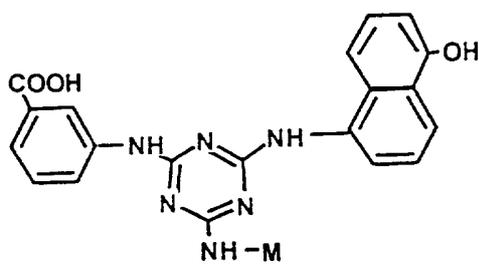
i)

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ii)

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iii)

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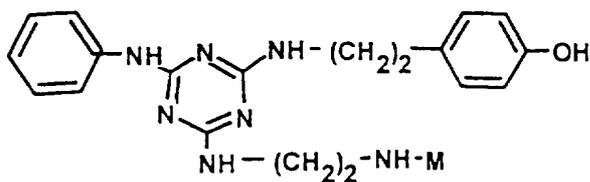
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iv)

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v)

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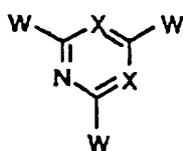
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25 vii)

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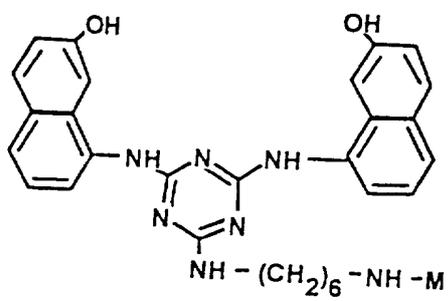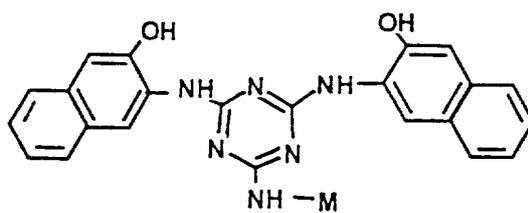
viii)

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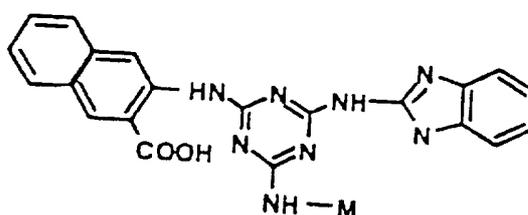
wherein M is as defined above.

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26.   A method for the manufacture of the novel affinity ligand-matrix conjugates as defined in anyone of the preceding claims which comprises reacting, in any order, a halogenoheterocylic compound of General Formula (II):

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(II)

20   wherein the symbols X have the meaning specified in anyone of the preceding claims and W represents a halogen atom with
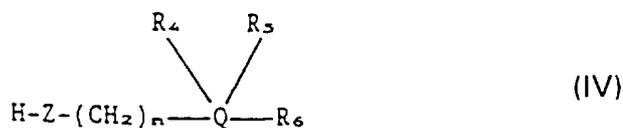
    (i) a compound of General Formula (III):

25   R_1-$(CH_2)_p$-Y-H   (III)

wherein the symbols R_1, p and Y have the meanings specified in anyone of the preceding claims,

30   (ii) a compound of General Formula (IV)

$$\text{H-Z-(CH}_2\text{)}_n\text{——Q——R}_6 \quad \overset{\displaystyle R_4 \quad R_5}{\diagdown \diagup} \qquad \text{(IV)}$$

5

wherein the symbols R_4, R_5, R_6, Q, Z and n have the meanings specified in anyone of the preceding claims and

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   (iii) an optionally derivatised support matrix of General Formula V

$$\text{H-T-[-L-V-]}_m\text{-M} \qquad \text{(V)}$$

15   wherein the symbols L, M, V, T, and m have the meanings specified in anyone of the preceding claims.
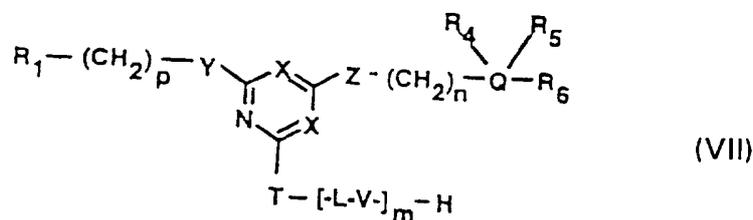
27.   A method for the manufacture of the novel affinity ligand-matrix conjugates as defined in anyone of the preceding claims which comprises

20   reacting, in any order, a halogenoheterocyclic compound of General Formula (II), as defined in claim 26, with

   (i)   a compound of General Formula (III), as defined in Claim 26
   (ii)   a compound of General Formula (IV), as defined in Claim 26 and
25   (iii)   with a linking unit of General Formula (VI)

$$\text{H-T-L-V-H} \qquad \text{(VI)}$$

wherein the symbols L, V and T have the meanings specified in anyone of the
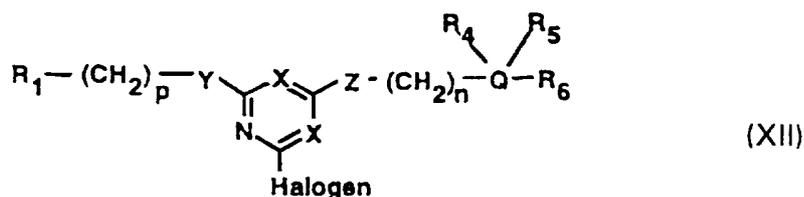30   preceding claims to give a compound of General Formula (VII):

$$R_1-(CH_2)_p-Y \diagdown \begin{matrix} X \\ \| \\ N \end{matrix} \diagup X - Z-(CH_2)_n-Q-R_6 \quad \begin{matrix} R_4 \diagdown \diagup R_5 \\ \end{matrix}$$

T — [-L-V-]$_m$ — H

(VII)

wherein R_1, R_4, R_5, R_6, T, Q, L, V, X, Y, Z, m, n and p have the meanings specified in anyone of the preceding claims followed by reaction of the compound of General Formula (VII) with a matrix support.
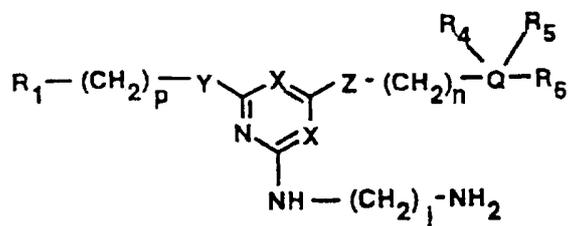
28.   Novel affinity ligands of General Formula (XII):

$$R_1-(CH_2)_p-Y \diagdown \begin{matrix} X \\ \| \\ N \end{matrix} \diagup X - Z-(CH_2)_n-Q-R_6 \quad \begin{matrix} R_4 \diagdown \diagup R_5 \\ \end{matrix}$$

Halogen

(XII)

wherein R_1, R_4, R_5, R_6, Q, X, Y, Z, n and p have the meanings specified in anyone of the preceding claims and Halogen represents a fluorine, chlorine, bromine or iodine atom.

29.   A method of attaching novel affinity ligands of General Formula (XII) as claimed in Claim 28 to a matrix of General Formula (V) as defined in Claim 26 by reacting the novel affinity ligands with the matrix at temperatures between -20°C and 121°C, optionally in the presence of an acid binding agent.

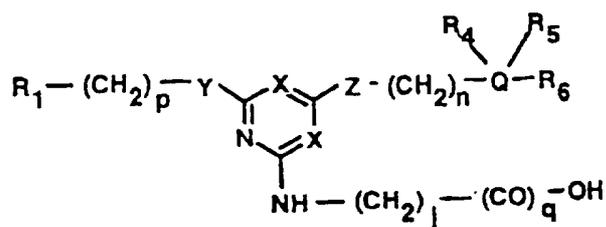30.   Novel affinity ligands of General Formula (XIII):

$$R_1-(CH_2)_{\overline{p}}-Y\diagdown X \diagup Z-(CH_2)_{\overline{n}}-Q-R_6$$

$$R_4 \diagdown \diagup R_5$$

(XIII)

$$NH-(CH_2)_j-NH_2$$

wherein R_1, R_4, R_5, R_6, Q, X, Y, Z, m, n and p have the meanings specified in anyone of the preceding claims and j is an integer between 2 and 20.

31.   A method of preparing novel affinity ligands of General Formula (XIII) as claimed in Claim 30 by reacting a compound of General Formula (XII) as claimed in Claim 28 with an alkylene diamine of General Formula $H_2N-(CH_2)_j-NH_2$ at temperatures between 0°C and 100°C in the presence of an acid binding agent.

32.   Novel affinity ligands of General Formula (XIV):

$$R_1-(CH_2)_{\overline{p}}-Y\diagdown X \diagup Z-(CH_2)_{\overline{n}}-Q-R_6$$

$$R_4 \diagdown \diagup R_5$$

(XIV)

$$NH-(CH_2)_j-(CO)_q-OH$$

wherein R_1, R_4, R_5, Q, X, Y, Z, m, n and p have the meanings specified in anyone of the preceding claims, q is 0 or 1 and j is an integer between 2 and 20.

33.   A method of preparing novel affinity ligands of General Formula (XIV) as claimed in Claim 32 by reacting a compound of General Formula (XII) as claimed in Claim 28 with an amino hydroxy compound of General Formula H_2N-
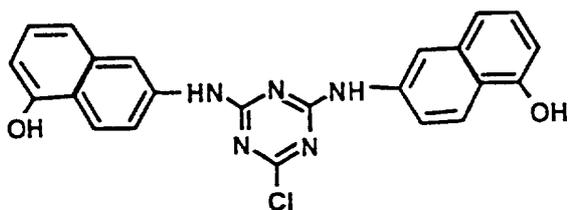
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$(CH_2)_i$-$(CO)_q$-OH at temperatures between 0°C and 100°C, optionally in the presence of an acid binding agent.

34.   Novel affinity ligands of General Formula (VIII):

$$R_1 - (CH_2)_p - Y \quad X \quad Z - (CH_2)_n - Q - R_6 \quad \begin{matrix} R_4 \diagdown \diagup R_5 \end{matrix}$$

(VIII)

with the triazine ring bearing N, X positions and substituent $T - [-L-V-]_m - R_{10} - \overset{R_9}{\underset{|}{C}} = CH_2$

wherein R_1, R_4, R_5, R_6, L, Q, T, V, X, Y, Z, m, n and p have the meanings specified in anyone of the preceding claims; R_9 represents a hydrogen atom or an alkyl group containing from 1 to 6 carbon atoms; R_{10} represents a carbonyl group, a methylene group, an -NH-CH$_2$- group or an -S-CH$_2$- group.

35.   Novel affinity ligands of General Formula (XV):

$$R_1 - (CH_2)_p - Y \quad N \quad Z - (CH_2)_n - Q - R_6 \quad \begin{matrix} R_4 \diagdown \diagup R_5 \end{matrix}$$

(XV)

with the triazine ring bearing N, N positions and Cl substituent

wherein R_1, R_4, R_5, R_6, Q, n and p have the meanings specified in anyone of the preceding claims.

36.   Novel affinity ligands of General Formula (XVI):

$$R_1 - (CH_2)_p - NH \overset{\displaystyle N}{\underset{\displaystyle N}{\bigcirc}} NH \cdot (CH_2)_n - Q - R_6$$

with R_4, R_5 branching off at the top right, and $NH - (CH_2)_j - NH_2$ below

(XVI)

wherein R_1, R_4, R_5, R_6, Q, n and p have the meanings specified in anyone of the preceding claims and j is an integer between 2 and 20.

37.   Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein R_1 represents a phenyl or naphthyl group each of which is optionally substituted on the benzene or naphthalene ring with one or more independently selected from hydroxyl groups and carboxylic acid groups.

38.   Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein R_4 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group or an amino group.

39.   Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein R_5 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group or an amino group.

40.   Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein R_6 represents a hydrogen atom, a hydroxyl group, a carboxylic acid group or an amino group.

41.   Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein Q represents a benzene or naphthalene ring.

42.   Affinity ligands according to anyone of claims 28, 30, 32, or 34 wherein X represents a nitrogen atom.

43. Affinity ligands according to anyone of claims 28, 30, 32, 34 or 35 wherein Y represents a -NH- group.

44. Affinity ligands according to anyone of claims 28, 30, 32, 34 or 35 wherein Z represents a -NH- group.

45. Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein n is 0 or 2.

46. Affinity ligands according to anyone of claims 28, 30, 32, 34, 35 or 36 wherein p is 0 or 2.

47. Affinity ligands according to anyone of claims 30, 32, or 36 wherein j is 2, 4 or 6.

48. Affinity ligands according to claim 34 wherein L is an ethyl, butyl, or hexyl group.

49. Affinity ligands according to claim 34 wherein T represents a -NH- group.

50. Affinity ligands according to claim 34 wherein V represents a -NH- group.

51. Affinity ligands according to claim 34 wherein m is 1.

52. Novel affinity ligands of General Formula (XI):

(XI)
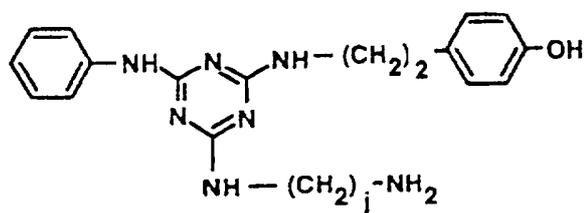
wherein j is an integer betweem 2 and 20.

53. Affinity ligands according to anyone of claims 28 to 51 selected among the following:

(1)

(2)

(3)

(4)

(5)

(6)
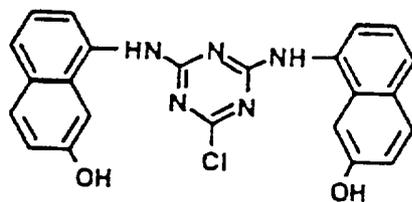

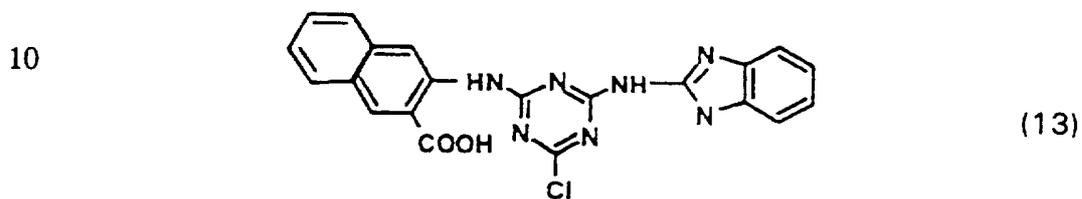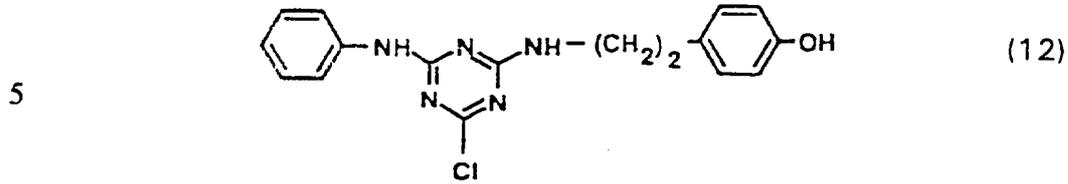(7)

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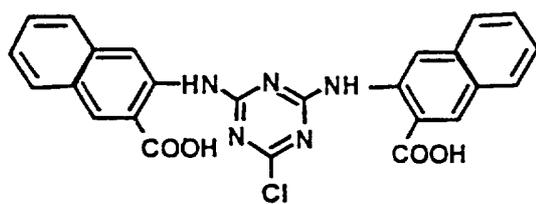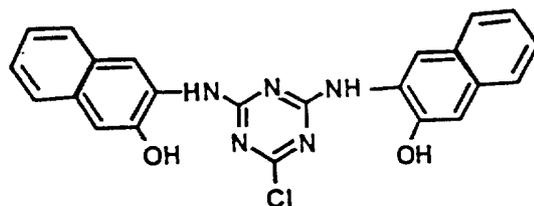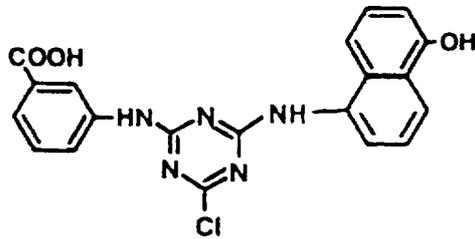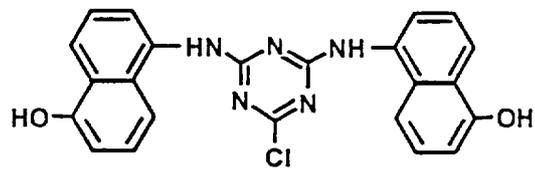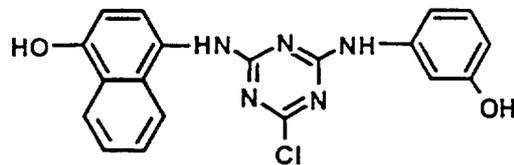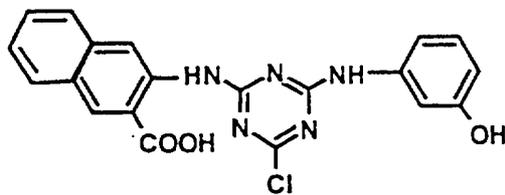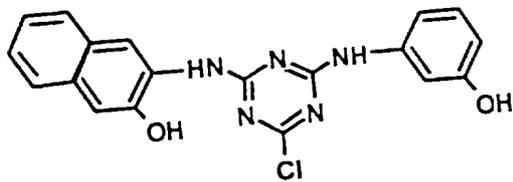
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(9)

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(10)

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(11)

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(12)

(13)
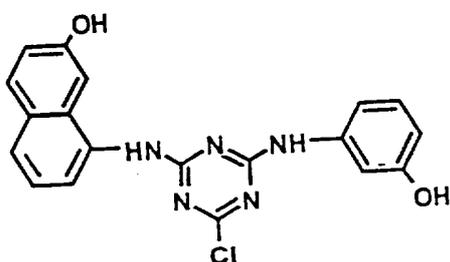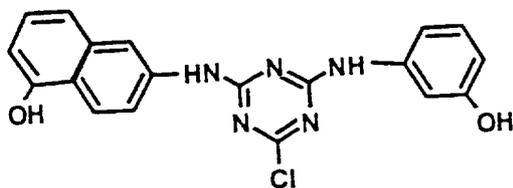
54.   The use of affinity ligands according to anyone of the preceding claims for the preparation of affinity ligand matrix conjugates

55.   A method of attaching the novel affinity ligands of General Formulae (VII) as defined in claim 27 and (XIII) as defined in Claim 30, (XVI) as defined in Claim 36 and (XI) as defined in Claim 52 to carbohydrate or organic polymer matrices by reacting the carbohydrate or organic polymer matrix with an activating agent followed by reaction of the activated matrix with the novel affinity ligand, optionally in the presence of an acid binding agent.

56.   A method of attaching the novel affinity ligands of General Formulae (XIV) as defined in Claim 32 to carbohydrate or organic polymer matrices by condensation with the matrix.

57.   A method of attaching the novel affinity ligands of General Formulae (VII) as defined in Claim 27 and (XIII) as defined in Claim 30, (XVI) as defined in Claim 36 and (XI) as defined in Claim 52 to metal oxide, glass or silica matrices, optionally coated with an organic polymer by reacting the optionally

coated metal oxide, glass or silica matrix with an activating agent followed by reaction of the activated matrix with the novel affinity ligand, optionally in the presence of an acid binding agent.

5   58.   A method of attaching the novel affinity ligands of General Formulae (XIV) as defined in Claim 32 to metal oxide, glass or silica matrices, optionally coated with an organic polymer by condensation with the matrix.

59.   A method of attaching novel affinity ligands of General Formula (XV) as
10 defined in Claim 35 and (XII) as defined in Claim 28 to a matrix of General Formula (V) as defined in Claim 26 by reacting the novel affinity ligands with the matrix at temperatures between -20°C and 121°C, optionally in the presence of an acid binding agent.

15 60.   Affinity ligand-matrix conjugates, prepared as claimed in Claims 26, 27, 29, 54, 55, 56, 57 and 58.

61.   The use of affinity ligand-matrix conjugates, according to anyone of the preceding claims to affinity ligand-matrix conjugates for the separation,
20 isolation, purification, characterisation, identification or quantification of proteins.

62.   . The use according to claim 61 wherein the proteinaceous material is I_gG, I_gM, I_gA, insulins, Factor VII,or Human Growth Hormone or analogues,
25 derivatives and fragments thereof and precursors.

63.   A process for the separation or purification of proteinaceous materials comprising carrying out affinity chromatography using as the biospecific ligand a ligand of general formula (a) as defined above.
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64.   The use according to claim 61 wherein the proteinaceous material is immunoglobulins or subclasses, fragments, precursors or derivatives thereof,

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whether derived from natural or recombinant sources.

65.   The use according to claim 61 wherein the proteinaceous material is immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA) or
5 subclasses, fragments, precursors or derivatives thereof, whether derived from natural or recombinant sources.

66.   The use according to claim 61 wherein the proteinaceous material is insulins or insulin analogues, derivatives and fragments thereof and precursors,
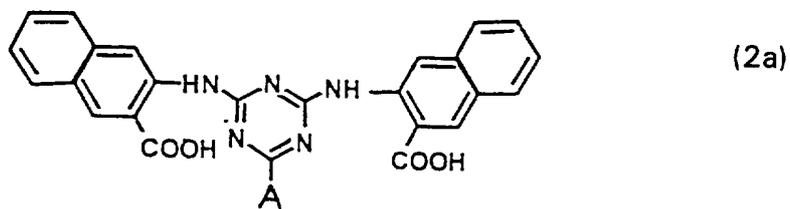10 whether derived from natural or recombinant sources.

67.   The use according to claim 66 wherein the affinity ligand-matrix conjugates comprises a ligand selected among the following:
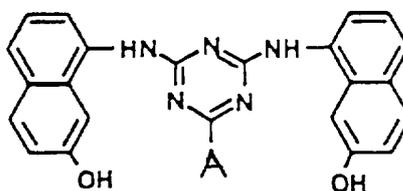
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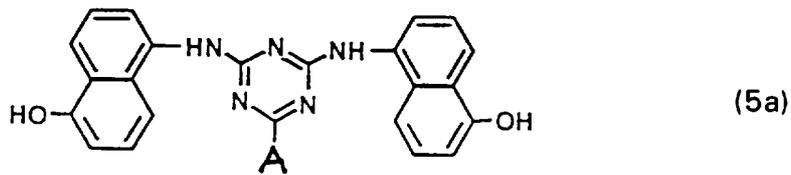
(5a)

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(2a)

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(11a)

which ligand is attached to a support matrix in position (A), optionally through a spacerarm represented by the general formula (b) as specified above.

68. The use according to claim 67 wherein the ligand is 11a.

69. The use according to claim 67 or 68 wherein the support matrix is optionally activated agarose, cellulose, silica or glass.

70. The separation, isolation, purification, characterisation, identification or quantification of immunoglobulins by any process whereby the said immunoglobulins are applied to affinity ligand-matrix conjugates, as defined in anyone of above affinity ligand-matrix conjugate claims at a pH in the range 5.0 to 12.0 and subsequently removed, eluted or desorbed by reducing the pH to 4.9 or lower.

71. The separation, isolation, purification, characterisation, identification or quantification of insulins or insulin analogues or derivatives thereof and precursors by any process whereby the said insulins, insulin derivatives, analogues, and precursors are applied to affinity ligand-matrix conjugates, as defined in anyone of above affinity ligand-matrix conjugate claims at a pH in the range 4,0 to 9,0 and subsequently removed, eluted or desorbed by reducing the pH to 3,99 or lower or to 9,01 or higher.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00399

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: B01D 15/08, C07D 251/54

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)

IPC6: B01D, 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)

CA, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Arch. Immunol. Ther. Exp., Volume 30, No 1, 1982, M. Konieczny et al, "Search for Carriers for Non-Covalent Binding of Interferon Among 1,3, 5-Triazine Derivatives of Dextran" page 1 - page 9	1-24,26-52, 54-66,68-71
X	J. Mol. Recogn., Volume 5, 1992, N.P. Burton and C.R. Lowe, "Design of Novel Affinity Adsorbents for the Purification of Trypsin-like Proteases" page 55 - page 68	1-24,26-52, 54-66,68-71

[X] Further documents are listed in the continuation of Box C.	[X] See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	erlier document but published on or after the international filing date	"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
"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)	"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
"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	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 1996	0 9 -01- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Teddy Ercegovic Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00399

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. Chrom. Vol. 508, 1990, S.J. Burton et al., "Design and Applications of Biomimetic Anthraquinone Dyes. III. Anthraquinone-immobilised C.I. Reactive Blue 2 Analogues and Their Interaction With Horse Liver Alcohol Dehydrogenase and Other Adenine Nucleotide -Binding Proteins", page 109 - page 125	1-71
A	A. Atkinson et al., "The Potential of Organic Dyes as Affinity Ligands in Protein Studies", In: Affinity Chromatography and Related Techniques, Proc. 4th Int. Symp. Edited by T.C.J. Gribnau et al., Elsevier Sci. Publ., Amsterdam, Anal. Chem. Symp. Series, 1982, Vol. 9. page 399 - page 410	1-71
A	EP 0302503 A2 (BOEHRINGER MANNHEIM GMBH), 8 February 1989 (08.02.89)	1-71

International application No.
PCT/DK 96/00399

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0302503	08/02/89	DE-A-	3726019	16/02/89
		DE-A-	3871277	25/06/92
		JP-A-	1067187	13/03/89
		JP-C-	1670183	12/06/92
		JP-B-	3036513	31/05/91