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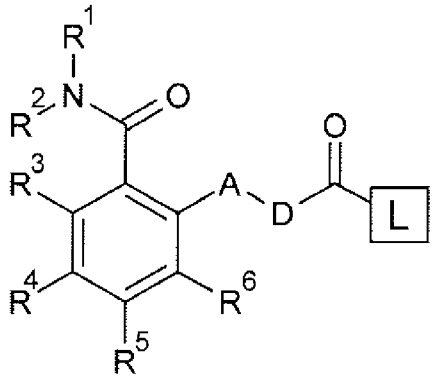
(54) Title: LIGANDS FOR ANTIBODY PURIFICATION BY AFFINITY CHROMATOGRAPHY



(I)

(57) Abstract: The present invention relates to the use, for affinity purification of a protein, of a compound according to the general formula (I), wherein A is selected from the group: -O-;-S-; >CR^7R^8; and NR^9; D is a linear or branched Ci to C6 alkyl group, which may comprise a cycloalkyl unit, wherein 1 or more C-atoms which are not connected to each other may be replaced by O or S, and wherein one or more C-atoms in the alkyl group may be substituted by one or more atoms independently from each other selected from the group F, C1, Br, I; L is the linking point via which the compound is attached to a support matrix, L optionally comprising a spacer group -Z-; R^1, R^2 are identical or different and are independently of each other selected from the group: H; -OH; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit; wherein in the alkyl 1 C-atom may be replaced by -O-, or a terminal C-atom may be replaced by -OH; wherein N may be part of a heterocycle having 3 to 7 members; and wherein one or more C- atoms in the alkyl may be substituted by one or more atoms independently from each other selected from the group F, C1, Br, I; R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein in the alkyl 1 C-atom may be replaced by -0-, or a terminal C-atom may be replaced by -OH; and wherein one or more C-atoms in each R^3, R^4, R^5 and R^6 may be substituted by one or more atoms independently

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from each other selected from the group F, C1, Br, I; and - $NR^{10}R^{11}$; R^7, R^8 are identical or different and are selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, wherein the alkyl group may optionally be substituted; R^9 is selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein the alkyl group may optionally be substituted; R^{10}, R^{11} can be identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein the alkyl group may optionally be substituted. Preferably, the entity to be separated of purified is an antibody, in particular an antibody of the Bevacizumab type or the Ranibizumab type.

Ligands for Antibody Purification by Affinity Chromatography

The present invention relates to the field of protein separation, preferably the purification of proteins with therapeutic properties, in particular antibodies and fragments thereof, by affinity separation techniques, in particular chromatography using small molecule ligands.

Immunoglobulins are a class of soluble proteins found in body fluids of humans and other vertebrates. They are also termed "antibodies" and play a key role in the processes of recognition, binding and adhesion of cells. Antibodies are oligomeric glycoproteins which have a paramount role in the immune system by the recognition and elimination of antigens, in general bacteriae and viruses.

The polymeric chain of antibodies is constructed such that they comprise so-called heavy and light chains. The basic immunoglobulin unit consists of two identical heavy and two identical light chains connected by disulfide bridges. There are five types of heavy chains (α, γ, δ, ε, μ), which determine the immunoglobulin classes (IgA, IgG, IgD, IgE, IgM). The light chain group comprises two subtypes, λ and κ.

IgGs are soluble antibodies, that can be found in blood and other body fluids. They are built by B-cell derived plasma cells as response to and to neutralize bacterial or other pathogens. An IgG is an Y-shaped glycoprotein with an approximate molecular weight of 150 kDa, consisting of two heavy and two light chains. Each chain is distinguished in a constant and in a variable region. The two carboxy terminal domains of the heavy chains are forming the Fc fragment ("constant fragment"), the amino terminal domains of the heavy and light chains are recognizing the antigen and are named Fab fragment ("antigen-binding fragment").

Therapeutic antibodies are used to treat various diseases, prominent examples include rheumatoid arthritis, psoriasis, multiple sclerosis and many forms of cancer. Therapeutic antibodies can be monoclonal or polyclonal antibodies. Monoclonal antibodies are derived from a single antibody producing cell line, showing identical specificity towards a single antigen. Possible treatments for cancer involve antibodies that are neutralizing tumour cell specific antigens. Bevacizumab (Avastin, Genentech) is a monoclonal antibody which neutralizes the vascular endothelial growth factor (VEGF), thereby preventing the growth of new blood vessels into the tumour tissue.

Protein bioseparation which refers to the recovery and purification of protein products from various biological feed streams is an important unit operation in the food, pharmaceutical and biotechnological industry. More and more therapeutic monoclonal antibodies (Mabs) and antibody fragments are entering the market or are currently in clinical development. Such proteins require an exceptionally high purity which is achieved by elaborate multi-step purification protocols. Downstream processing and purification constitute about 50 to 80 % of the manufacturing cost, hence considerable efforts are under way to develop new or improve existing purification strategies (**1**).

Affinity chromatography is one of the most effective chromatographic methods for protein purification. It is based on highly specific protein-ligand interactions. The ligand is immobilized covalently on the stationary phase which is used to capture the target protein from the feed stock solution. Affinity ligands can bind their target with high specificity and selectivity, enabling up to thousand fold higher enrichment at high yields even from complex mixtures.

Typically, affinity chromatography on protein A is the first step in most Mab purification schemes. Protein A is a cell wall associated protein exposed on the surface of the bacterium *Staphylococcus aureus*. It binds with nanomolar affinity to the constant part (Fc domain) of immunoglobulins from various species, in particular to human subtypes IgG1, IgG2 and IgG4 (**2**). However, the use of Protein A is limited by leaching into the product and poor stability under harsh conditions applied for sanitization and cleaning in place procedures. The chemical stability of Protein A can be improved by using genetically engineered Protein A variants for Mab purification. Yet, the high costs of Protein A resins have resulted in the search for suitable alternatives. Furthermore, Protein A only recognizes the constant region Fc of IgG type proteins.

Antibody fragments (parts of whole antibody molecules) offer several advantages over whole antibodies, in particular in the therapeutic field. They are easier and more cost effective to manufacture, and have fewer side-effects in patients, due to the absence of the Fc (heavy chain) region, by reducing the risk of cytokine release and its associated toxicity. They can also be modified to include therapeutic payloads. There are several types of antibody fragments that are either lgG domains prepared by specific endopeptidase enzyme digestion or that have been genetically engineered in cell lines. These fragments are known to the person skilled in the art and include monovalent fragments such as Fab', Fab and scFv; bivalent fragments such as $F(ab')_2$ diabodies and minibodies; and multivalent fragments such as triabodies and tetrabodies.

The purification of recombinant antibody fragments like Fab, scFv, diabodies, minibodies and other variants parallels in many aspects the purification of Mabs. The downstream processing of antibody fragments can be divided into the initial isolation from host cells, followed by a capture step to concentrate the target protein and remove major host cell impurities and one or more polishing steps to obtain the pure protein product. Whereas Protein A based resins are used as standard material for the initial capture of Mabs, affinity ligands with broad applicability for capturing antibody fragments are not available.

Two bioaffinity ligands of bacterial origin could be used for the purification of selected Fab derived molecules. Protein L is a bacterial protein from *Peptococcus magnus* with affinity to the Fab portion of antibodies, specifically κ1, λ1 and λ2-light chains. Protein G which is isolated from group C and G streptococci interacts with the constant heavy domain of Fabs. This interaction is relatively weak and results in low dynamic binding capacities. In addition, harsh elution conditions are required which are not compatible with each target protein. Due to its binding specificity, protein G cannot be used to purify antibody fragments lacking the constant heavy and light chain domains e.g. scFvs.

Another approach for the purification of antibody fragments is the use of peptidic affinity tags that are engineered into the protein and are removed later by chemical or proteolytic cleavage. Examples are the hexahistidine tag for purification by metal-ion affinity chromatography (IMAC) or the Strep-tag, a biotin mimetic peptide that binds to immobilized streptavidin. However, a major disadvantage of affinity tags consists in the need to remove the tags, adding extra purification steps and possible contaminants to the final product.

Affinity purification using an immobilized antigen could be applied for small fragments such as scFv and analogues. However, protein derived antigens show generally low stabilities and could be very expensive, making this approach unsuitable for large-scale manufacturing processes.

New synthetic affinity ligands that are cheaper than protein-based ligands and are more robust under stringent conditions would provide a suitable alternative for antibody fragment purification.

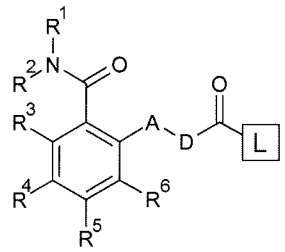
Synthetic small molecule affinity ligands are of particular interest for the purification of therapeutic proteins due to their generally higher chemical stability and their lower production costs. Depending on the target protein, such affinity ligands should either offer the same broad applicability as Protein A, recognizing the constant Fc region of IgG type immunoglobulins, or target the variable domain (Fab) of an antibody by mimicking the

epitope or binding to other, either variable or constant domains of the Fab fragments. The latter class of affinity ligands is perfectly suitable for the purification of individual Mabs and, in particular, they lend themselves to purify antibody fragments e.g. minibodies, diabodies or other multivalent fragments.

The problem underlying the present invention is the provision of a small compound affinity ligand binding to the Fab region of antibodies, in particular monoclonal antibodies. In one particular embodiment, the ligand shall bind to antibody fragment like Fab', Fab, scFv and $F(ab')_2$ diabodies.

The problem is solved by the embodiments of the present invention as laid out hereinafter.

In one aspect, the present invention is drawn to the use for affinity purification of a protein, of a compound according to the general formula (I),



(I)

wherein

A is selected from the group: –O-; -S-; $>CR^7R^8$; and $–N(R^9)$-;
D is a linear or branched C_1 to C_6 alkyl group, which may comprise a cycloalkyl unit, wherein 1 or more C-atoms which are not connected to each other may be replaced by O or S, and wherein one or more C-atoms in the alkyl group may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I;
L is the linking point via which the compound is attached to a support matrix, L optionally comprising a spacer group –Z-;
R^1, R^2 are identical or different and are independently of each other selected from the group: H; -OH; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit; wherein in the alkyl 1 C-atom may be replaced by –O-, or a terminal C-atom may be replaced by –OH; wherein N may be part of a heterocycle having 3 to 7 members; and wherein one or more C-

atoms in the alkyl may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I;
R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; -F; -Cl; -Br; -I; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein in the alkyl 1 C-atom may be replaced by –O-, or a terminal C-atom may be replaced by –OH; and wherein one or more C-atoms in each R^3, R^4, R^5 and R^6 may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I; and $-NR^{10}R^{11}$;
R^7, R^8 are identical or different and are selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, wherein the alkyl group may optionally be substituted;
R^9 is selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein the alkyl group may optionally be substituted;
R^{10}, R^{11} can be identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein the alkyl group may optionally be substituted.

In a sub-embodiment of the above-defined ligands, the N-heterocycle is a 3- to 5-membered heterocycle.

In a preferred embodiment, in the compound of the general formula (I), the symbols R^1 to R^6 and A, D have the following meaning:
A is selected from: –O-;-S-; $-CH_2-$; -NH, $-N(CH_3)$;
D is a linear or branched C_1 to C_6 alkyl group, wherein one or more C atoms not connected to each other may be replaced by –O-, and wherein one or more C-atoms may be substituted by one or more F atoms;
R^1, R^2 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit; wherein N may be part of a heterocycle having 3 to 5 members; and wherein one or more C-atoms in the alkyl may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I;
R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; -F; -Cl; -Br; -I; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein in the alkyl 1 C-atom may be replaced by –O-, and wherein one or more C-atoms in each R^3, R^4, R^5 and R^6 may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I.

In an even more preferred embodiment, in the compound of the general formula (I), the symbols R^1 to R^6 and A, D have the following meaning:
A is –O-;
D is a linear or branched C_1 to C_6 alkyl group;
R^1, R^2 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl;
R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, wherein 1 C-atom may be replaced by –O-.

In the above-defined embodiment, R^1, R^2 are preferably H; and R^3, R^4, R^5 and $R^6$are preferably H.

The terms used in the above definition of the compounds according to formula (I) are known to the person skilled in the art.

The term „linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit“ preferably denotes methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, sec-butyl, tert-butyl, cyclopropyl, methylcyclopropyl and cyclobutyl.

The term „linear and branched C_1 to C_6 alkyl, which may comprise a cycloalkyl unit“ preferably denotes methyl, ethyl, n-propyl, i-propyl, n-butyl, i-butyl, sec-butyl, tert-butyl, pentyl and its isomers, hexyl and its isomers , cyclopropyl and its alkyl-substituted derivatives e.g. methylcyclopropyl, cyclobutyl and its alkyl-substituted derivatives e.g. methylcyclobutyl, dimethyl cyclobutyl, ethyl cyclobutyl, cyclopentyl and methylcyclopentyl.

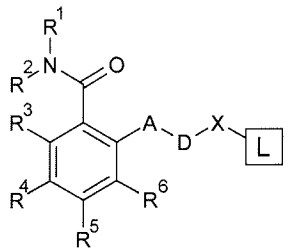
The term „heterocycle having 3 to 7 members“ include but are not restricted to pyrrole, pyrroline, imidazole, imidazoline, pyrazole, pyrazoline, pyrrolidine, imidazolidine, pyrazolidine, pyridine, pyridazine, pyrazine, pyrimidine, piperazine, piperidine, morpholine, tetrazole, tetrazoline, triazole, triazolidine, triazoline, tetrazolidine, azepine or homopiperazine, aziridine, azetidine, [1,2]- and [1,3]-oxazetidines, [1,2]- and [1,3]-thiazetidines, oxazoline, oxazolidine, isoxazoline, isoxazolidine, thiazoline, thiazolidine, isothiazoline, isothiazolidine, oxadiazoles, oxadiazolines, oxadiazolidines, thiadiazoles, thiadiazolines, thiadiazolidines.

In particular, the term: „alkyl group which may comprise a cycloalkyl unit“ refers to the case a) that the cycloalkyl group forms a part of a terminal alkyl group, wherein the cycloalkyl group may be connected to the neighboring atom, to which the terminal alkyl group is attached, by a direct bond, or that the cyloalkyl group is not connected to the neighboring

atom and forms a part of the alkyl group, which may be the terminal part or an intermediate part. The term: „alkyl group which may comprise a cycloalkyl unit" also refers to the case b) that the cycloalkyl group forms a part of a bridging alkyl group (i.e. an alkylene group), wherein the cycloalkyl group may be connected to one neighboring atom or to both neighboring atoms by one or two direct bond, or that the cyloalkyl group is not connected to any neighboring atom and forms a part of the alkyl group, which may be a terminal part or an intermediate part.

Some of the compounds of the invention may exist in different stereoisomeric forms. All of these forms are subjects of the invention.

The person skilled in the art is aware that the carboyl fragment [–C(O)-] present at the spacer terminus of the compounds according to the present invention is an electron withdrawing group and a potential hydrogen bond acceptor which is supposed to be of importance for the target protein affinity. The person skilled in the art is aware that attachment of any fragment which has hydrogen bond accepting properties (i.e. electronic and steric properties) similar or identical to that of the fragment [–C(O)-] , to the main part of the molecule will result in affinity ligands having similar or identical properties to those as defined in the present invention. An electron withdrawing group having similar or identical electronic and/or steric properties to those of fragment [–C(O)-) may, in the context of the present invention, also be designated by [-X-]. Accordingly, affinity ligands of the present invention may also be described by the formula (II)



(II)

wherein all symbols have the same meaning as defined for formula (I), and wherein X is an electron withdrawing group having electron delocalization properties (i.e. electronic and steric properties) similar or identical to that of the fragment –C(O)-

The compounds of the general formulae can be attached to the support matrix via L. L is the "linking point", also referred to as "point of attachment". The person skilled in the art is aware of appropriate linking points/points of attachment.

In one embodiment, L is a bond, preferably a single bond, which is directly attached to the matrix, in general via an appropriate functional group on the matrix. In this context, the term "matrix" refers to polymers known to the person skilled in the art and available on the market which lend themselves for the purposes of the present invention. A definition of "matrix" is provided below. In general, the matrix comprises functional groups for the attachment of molecules, in general those of the present invention. In the context of the present invention, the functional groups on the matrix are regarded as a part of the matrix; this also applies in cases where the compounds of the present invention are connected to the matrix via a bond (i. e. L is a bond and does not comprise a "spacer group, see below), and wherein the bond is directly formed between the respective compound according to the invention and the functional group on the matrix.

"Functional group" in the present context refers, on the one hand, to appropriate groups ("precursor groups") on the matrix and, on the other hand, to appropriate groups ("precursor groups") present in the compound according to the invention (e.g. present in alpha position to the carbonyl group, or present on the spacer Z). "Precursor groups" in the present context are groups which are capable of a chemical reaction with "complementary groups", under formation of a chemical bond. As is clear from the foregoing, during the reaction the functional groups/precursor groups are transformed into a chemical bond under a transformation of the original functionality into a "final functionality". From the foregoing, it is clear that a functional group/precursor group also is or can be a "complementary group".

Examples of functional groups on the matrix include, but are not limited to –OH, –SH, $-NH_2$, -COOH, $-SO_3H$, -CHO, $-NHNH_2$, $-P(=O)(OH)_2$, -O-PH(=O)(OH), $-P(OH)_2$, $-O-P(=O)(OH)_2$ ester, ether, thioether, carboxylic acid, epoxide, amine, amide, amino acids, carboxylic amides, maleimide, aldehyde, ketone, sulfonic acid, sulfoxide, sulfone, urea, isourea, imidocarbonate, sulphonamide, sulfonic ester, phosphate, phosphonate, phosphoric amide, phosphonic amide, hydrazine, oxime, azide, Cl, Br I, alkene and alkyne groups.

Examples of functional groups attached to the carbonyl function on the compounds of the invention include, but are not limited to hydroxy, thiol, Cl, Br, ester, ether, thioether, carboxylic acid, epoxide, amine, amide, amino acids, carboxylic amides, maleimide, aldehyde, ketone, sulfonic acid, sulfoxide, sulfone, urea, isourea, imidocarbonate,

sulphonamide, sulfonic ester, phosphate, phosphonate, phosphoric amide, phosphonic amide, hydrazine, oxime, azide and alkyne groups.

In this embodiment of the invention, the bond which is formed as such constitutes the linking point L by which the compounds of the invention are attached to the matrix.

In a further embodiment of the invention, L includes a spacer –Z-. In this embodiment, L is a chemical unit resulting from the reaction of an appropriate functional group on the matrix with an appropriate functional group (or a "complementary group") which is present on the spacer group Z of the respective compound according to the invention. Accordingly, the chemical reaction between the functional group on the matrix and the functional group (or "complementary group") on the spacer group Z incorporates Z into the linking point L of the compound according to the invention with the matrix.

"Final functionality" in the present context designates a unit formed in the reaction between the precursor groups (functional group) on the matrix and on the compound of the invention and/or on the spacer group Z. The "final functionality" incorporates the bond between the matrix and the compounds of the present invention (which may include the spacer Z).

Suitable functional groups on the spacer group Z include –OH, –SH, $-NH_2$, -COOH, $-SO_3H$, -CHO, $-NHNH_2$, $-P(=O)(OH)_2$, $-O-PH(=O)(OH)$, $-P(OH)_2$, $-O-P(=O)(OH)_2$, natural or non natural amino acids, carboxylic amides, ethers, hydroxy, thiol, ester, thioether, carboxylic acid, epoxide, amine, amide, hydroxyl, maleimide, aldehyde, ketone, sulfonic acid, sulfoxide, sulfone, urea, isourea, imidocarbonate, sulphonamide, sulfonic ester, phosphate, phosphonate, phosphoric amide, phosphonic amide, hydrazine, oxime, azide and alkyne groups.

The spacer group Z preferably is a hydrocarbon group which may contain, in addition to C and H atoms, further atoms. Appropriate further atoms are known to the person skilled in the art. Preferred atoms include O, S, N, P, Si. The hydrocarbon group may be linear or branched or cyclic. Z is linked to the carbonyl group of the compounds according to the invention, in general Z is found in alpha-position to the carbonyl group. Furthermore, Z contains functional groups (precursor groups) by which the compounds of the invention can be covalently linked with the chromatography matrix in a chemical reaction under formation of a final functionality, and wherein Z is present in the linking point L. Furthermore, Z contains the final functionality

Z may also be branched, allowing the linkage of more than one ligand to one chromatography matrix attachment point (linking point) L. Typically the functional groups are attached to a

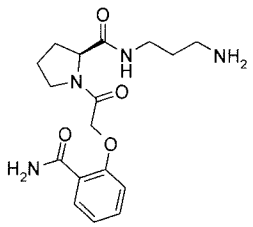
hydrocarbon group which may contain one or more heteroatoms such as N, O, P, S, Si. Preferably the atom which is linked to the carbonyl group of the compounds of the invention is nitrogen, oxygen, carbon or sulfur. Preferred units of the spacer group Z include but are not restricted to amines, preferably organic amines, diamines, like ethylenediamine, piperazine, homopiperazine, proline, alanine, -NH-$((CH_2)_2O)_n$-CH_2-NH-; -NH-$(CH_2)_n$-NH-, -NH-$(CH_2)_n$- wherein n is an integer from1-12, preferably 1-6. The named units and functional groups are thus part of the spacer group Z and of the linking point L., Particularly preferred examples of spacer groups Z are -NH-$(CH_2)_n$NH-, piperazine, homopiperazine, -NH-$((CH_2)_2O)_n$-CH_2-NH-; -NH-$(CH_2)_n$-NH-proline, -NH-$(CH_2)_n$-NH-alanine, wherein n is integer from -1-12, preferably 1-6.

In one embodiment of the present invention, a substituted or non substituted heterocycle having 3 to 7 members and containing nitrogen, oxygen, carbon or sulfur may be present in alpha position to the carbonyl group of the compound of the invention. The heterocycle may form the hydrocarbon group of which Z is composed alone, or the heterocycle may be attached to a further hydrocarbon chain present in Z which connects the compound of the invention with the matrix. "Heterocycle" includes but is not restricted to cyclopentane, cyclohexane or cycloheptane ring that may contain up to the maximum number of double bonds (aromatic or non-aromatic ring which is fully or partially unsaturated) wherein at least one carbon atom up to 4 carbon atoms are replaced by a heteroatom selected from the group consisting of nitrogen, sulfur, oxygen. Examples for a heterocycle include but are not restricted to furan, thiophene, pyrrole, pyrroline, imidazole, imidazoline, pyrazole, pyrazoline, oxazole, oxazoline, isoxazole, isoxazoline, thiazole, thiazoline, isothiazole, isothiazoline, thiadiazole, thiadiazoline, tetrahydrofuran, tetrahydrothiophene, pyrrolidine, imidazolidine, pyrazolidine, oxazolidine, isoxazolidine, thiazolidine, isothiazolidine, thiadiazolidine, sulfolane, pyran, dihydropyran, tetrahydropyran, imidazolidine, pyridine, pyridazine, pyrazine, pyrimidine, piperazine, piperidine, morpholine, tetrazole, triazole, triazolidine, tetrazolidine, azepine or homopiperazine. "Heterocycle" means also azetidine, azocine or diazocine.

The final functionality incorporated in L and/or Z includes the following groups: ether, thioether, C-C single bond, alkenyl, alkynyl, ester, carboxylic amide, sulfonic ester, sulfonic amide, phosphonic ester, phosphonic amide, phosphoric ester, phosphoric amide, phosphoric thioester, thiophosphoric ester, secondary amine, secondary alcohol, tertiary alcohol, 2-hydroxyamine, urea, isourea, imidocarbonate, alkylthiosuccinic imide, dialkylsuccinic imide, triazole, ketone, sulfoxide, sulfone, oxime ether, hydrazone, silyl ether.

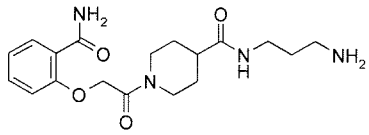
The present invention is also drawn to compounds of the formula (I) as defined beforehand, wherein the symbols have the meanings defined beforehand for the general, preferred and even more preferred embodiments, and to their synthesis.

Preferred compounds according to the present invention are:



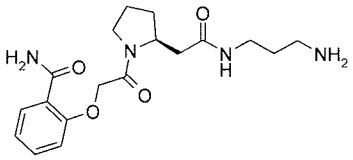
(III)

(*S*)-*N*-[(2-Carbamoylphenyloxy)acetyl]pyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide III (Ligand A)



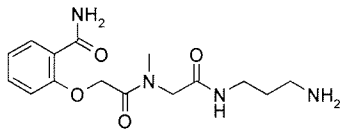
(IV)

N-[(2-Carbamoylphenyloxy)acetyl]piperidine-4-carboxylic acid 3-aminoprop-1-yl amide IV (Ligand B)



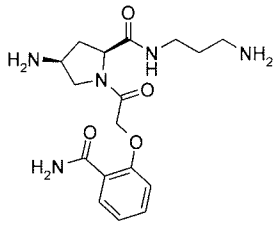
(V)

(*S*)-*N*-[(2-Carbamoylphenyloxy)acetyl]pyrrolidine-2-ylacetic acid 3-aminoprop-1-yl amide V (Ligand D)



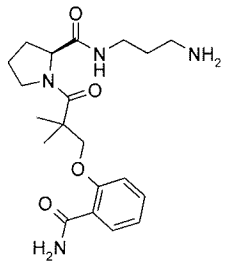
(VI)

N-[(2-Carbamoylphenyloxy)acetyl]-*N*-methylglycine 3-aminoprop-1-yl amide VI (Ligand E)



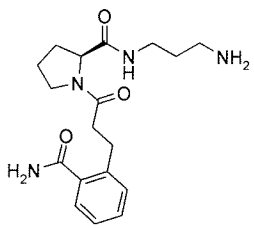
(VII)

(*S*,*S*)-1-*N*-[(2-Carbamoylphenyloxy)acetyl]-4-aminopyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide VII (Ligand G)



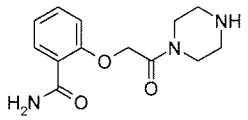
(VIII)

(*S*)-*N*-[3-(2-Carbamoylphenyloxy)-2,2-dimethylpropionyl]pyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide VIII (Ligand C)



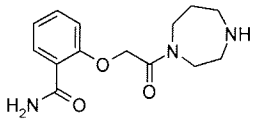
(IX)

(*S*)-*N*-[3-(2-Carbamoylphenyl)propionyl]pyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide IX (Ligand F)



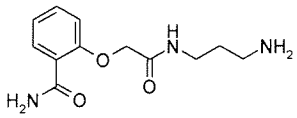
(X)

N-[(2-Carbamoylphenoxy)acetyl]piperazine (Ligand H)



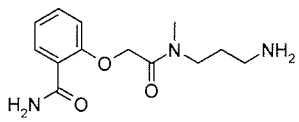
(XI)

N-[(2-Carbamoylphenoxy)acetyl]homopiperazine (Ligand I)



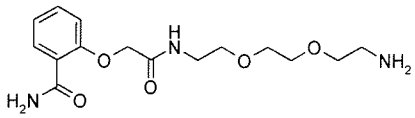
(XII)

2-Carbamoylphenoxyacetic acid (3-amino-1-propyl)amide (Ligand J)



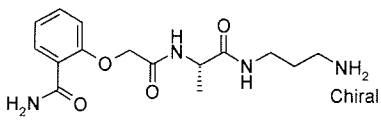
(XIII)

2-Carbamoylphenoxyacetic acid *N*-(3-amino-1-propyl)-*N*-methyl amide (Ligand K)



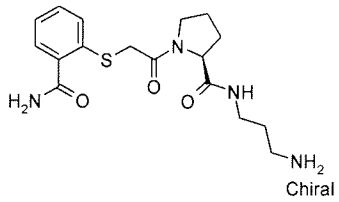
(XIV)

2-Carbamoylphenoxyacetic acid (8-amino-3,6-dioxa-1-octyl)amide (Ligand L)



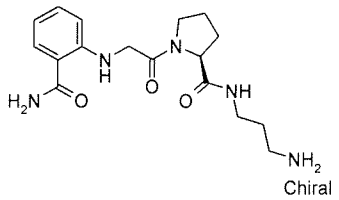
(XV)

(*S*)-*N*-[(2-Carbamoylphenoxy)acetyl]alanine 3-aminopropyl amide (Ligand M)



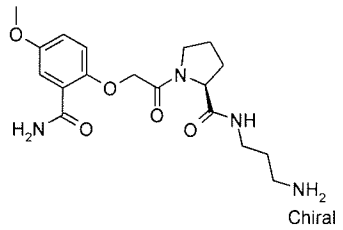
(XVI)

(*S*)-*N*-[(2-Carbamoylphenylthio)acetyl]proline 3-aminopropyl amide (Ligand N)



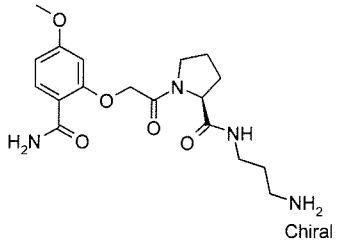
(XVII)

(*S*)-*N*-[(2-Carbamoylphenylamino)acetyl]proline 3-aminopropyl amide (Ligand O)



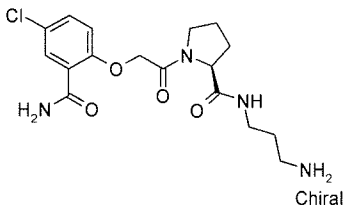
(XVIII)

(*S*)-*N*-[(2-Carbamoyl-4-methoxyphenoxy)acetyl]proline 3-aminopropyl amide (Ligand P)



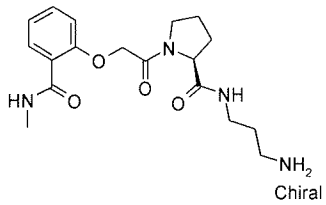
(XIX)

(*S*)-*N*-[(2-Carbamoyl-5-methoxyphenoxy)acetyl]proline 3-aminopropyl amide (Ligand Q)



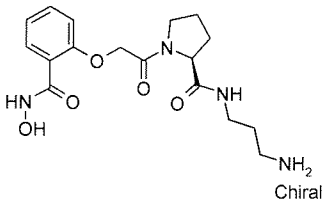
(XX)

(*S*)-*N*-[(2-Carbamoyl-4-chlorophenoxy)acetyl]proline 3-aminopropyl amide (Ligand R)



(XXI)

(*S*)-*N*-{[2-(*N*-Methylcarbamoyl)phenoxy]acetyl}proline (3-amino-1-propyl)amide (Ligand S)



(XXII)

(*S*)-*N*-{[2-(*N*-Hydroxycarbamoyl)phenoxy]acetyl}proline (3-amino-1-propyl)amide (Ligand T)

The synthesis of the compounds (I) can be carried out on insoluble supports, preferably pre-loaded with the desired linker bearing a reactive group, e. g. an amino group, to which the spacer group Z, e. g. an Fmoc protected amino acid, is attached by amide formation. After deprotection of the spacer group, the remaining parts of the molecule, including the substituted phenylene moiety and the A and D groups are attached by amide formation and, where necessary, additional synthetic steps, e. g. nucleophilic displacement reactions. Finally, the compounds including the linker moieties are released from the insoluble support by a suitable cleavage protocol known to the person skilled in the art and purified by chromatographic methods also known to the person skilled in the art.

The term "antibody" means an immunoglobulin, including both natural or wholly or partially synthetically produced and furthermore comprising all fragments and derivatives thereof which maintain specific binding ability. Typical fragments are Fc, Fab, heavy chain, and light chain. The term also comprises any polypeptide having a binding domain which is

homologous or largely homologous, such as at least 95% identical when comparing the amino acid sequence, to an immunoglobulin binding domain. These polypeptides may be derived from natural sources, or partly or wholly synthetically produced. An antibody may be monoclonal or polyclonal. The antibody may be a member of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and IgE. Derivatives of the IgG class, however, are preferred in the context of the present invention.

The term "antibody fragment" refers to any derivative of an antibody which is less than full-length. Preferably, the antibody fragment retains at least a significant portion of the specific binding ability of the full-length antibody. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, scFv, Fv, dsFv diabody, and Fd fragments, The antibody fragment may be produced by any means. For instance, the antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody or it may be recombinantly produced from a gene encoding the partial antibody sequence. Alternatively, the antibody fragment may be wholly or partially synthetically produced. The antibody fragment may optionally be a single chain antibody fragment. Alternatively, the fragment may comprise multiple chains which are linked together, for instance, by disulfide linkages. The fragment may also optionally be a multimolecular complex. A functional antibody fragment will typically comprise at least about 50 amino acids and more typically will comprise at least about 200 amino acids.

The Fab regions comprise the "arms" of the antibody, which are critical for antigen binding. The Fc region comprises the "tail" of the antibody and plays a role in immune response, as well as serving as a useful "handle" for manipulating the antibody during some immunochemical procedures. The number of F(ab) regions on the antibody, corresponds with its subclass, and determines the "valency" of the antibody (loosely stated, the number of "arms" with which the antibody may bind its antigen).

"Single-chain Fvs" (scFvs) are recombinant antibody fragments consisting of only the variable light chain (V_L) and variable heavy chain (V_H) covalently connected to one another by a polypeptide linker. Either V_L or V_H may be the amino-terminal domain. The polypeptide linker may be of variable length and composition so long as the two variable domains are bridged without serious steric interference. Typically, the linkers comprised primarily of stretches of glycine and serine residues with some glutamic acid or lysine residues interspersed for solubility. "Diabodies" are dimeric scFvs. The components of diabodies typically have shorter peptide linkers than most scFvs and they show a preference for associating as dimmers. An "Fv" fragment is an antibody fragment which consists of one V_H and one V_L domain held together by non-covalent interactions. The term "dsFv" is used

herein to refer to an Fv with an engineered intermolecular disulfide bond to stabilize the V_H-V_L pair. A "$F(ab')_2$" fragment is an antibody fragment essentially equivalent to that obtained from immunoglobulins (typically IgG) by digestion with an enzyme pepsin at pH 4.0-4.5. The fragment may be recombinantly produced. A "Fab" fragment is an antibody fragment essentially equivalent to that obtained by reduction of the disulfide bridge or bridges joining the two heavy chain piece in the $F(ab')_2$ fragment. The Fab' fragment may be recombinantly produced. A "Fab" fragment is an antibody fragment essentially equivalent to that obtained by digestion of immunoglobulins (typically IgG) with the enzyme papain. The Fab fragment may be recombinantly produced. A "Fc" region is a highly conserved region of a particular class of antibody.

The present invention is drawn to the affinity purification, preferably by affinity chromatography, of antibodies or fragments thereof from complex mixtures e.g. fermentation supernatants, making use of the affinity ligands of formula (I) and preferred embodiments thereof, as disclosed elsewhere in the specification.

Accordingly, in a preferred embodiment, the present invention comprises a method for purifying a protein, preferably an immunoglobulin, more preferably an IgG antibody, by affinity purification, preferably affinity chromatography. The affinity ligands according to the present invention bind to the Fab region of an antibody.

In one preferred embodiment of the present invention, the IgG antibody is in particular a monoclonal IgG antibody or a fragment thereof, more preferably a Fab fragment of an IgG, or a part of the Fab fragment of an IgG.

In a particular preferred embodiment, the antibody, accordingly, is an antibody of the Bevacizumab type or an antibody of the Ranibizumab type.

Bevacizumab (Avastin®, Genentech/Roche) is a humanized monoclonal antibody. It stops tumor growth by preventing the formation of new blood vessels by targeting and inhibiting the function of the protein vascular endothelial growth factor-A (VEGF-A) that stimulates new blood vessel formation (angiogenesis).

Blood vessels grow uncontrollably in cancer, retinal proliferation of diabetes in the eye, and other diseases. Bevacizumab blocks VEGF-A from creating new blood vessels. It is approved for combination use with standard chemotherapy for metastatic colon cancer and non-small cell lung cancer for use in metastatic breast cancer and for treatment of recurring Glioblastoma Multiforme. Clinical studies are underway in non-metastatic breast cancer, renal

cell carcinoma, glioblastoma multiforme, ovarian cancer, castrate-resistant prostate cancer, non-metastatic unresectable liver cancer and metastatic or unresectable locally advanced pancreatic cancer.

Ranibizumab (Lucentis®) a Fab fragment derived from the same parent molecule as bevacizumab, has been developed by Genentech for intraocular use and now has FDA approval.

In the context of the present invention, the term "antibody of the Bevacizumab type" denotes an antibody having a Fab domain in which the amino acid sequence is identical or essentially identical with that of Bevacizumab, in particular in which the peptide sequence of the part to which the ligands of the present invention bind are identical or essentially identical with that Bevacizumab. "Essentially identical" in the context of the present invention means a amino acid sequence identity of 70 %, preferably 80 %, more preferably 90 %, even more preferably 95 %, still more preferably 98 %, most preferably 99 % relative to Bevacizumab.

In the context of the present invention, the term "antibody of the Ranibizumab type" denotes an antibody having a Fab domain in which the amino acid sequence is identical or essentially identical with that of Ranibizumab, in particular in which the peptide sequence of the part to which the ligands of the present invention bind are identical or essentially identical with that Ranibizumab. "Essentially identical" in the context of the present invention means a amino acid sequence identity of 70 %, preferably 80 %, more preferably 90 %, even more preferably 95 %, still more preferably 98 %, most preferably 99 % relative to Ranibizumab.

Preferably, the ligand binds to an antibody having the same specificity, i.e. binding to the same epitope, as bevacizumab and ranibizumab.

More preferably, the ligand binds to an antibody comprising in its light change a sequence as defined by SEQ ID NO: 1 and in its heavy chain a sequence as defined by SEQ ID NO: 2. Also preferably, the ligand binds to a Fab-fragment comprising the aforementioned sequences or essentially consisting of them. SEQ ID NO: 1 represents the consensus sequence of the light chains of bevacizumab and ranibizumab and SEQ ID NO: 2 represents the consensus sequence of the Fab-fragments of the heavy chains of bevacizumab and ranibizumab.

Most preferably, the ligand binds to an antibody comprising in its light chain a sequence as defined by SEQ ID NO: 3 or SEQ ID NO: 4 and in its heavy chain a sequence as defined by SEQ ID NO: 5 or SEQ ID NO: 6. SEQ ID NO: 3 represents the light chain of bevacizumab and SEQ ID NO: 4 represents the light chain of ranibizumab. Preferably, the antibody consists

of a light chain comprising a sequence as defined by SEQ ID NO: 3 and a heavy chain comprising a sequence as defined by SEQ ID NO: 5 or it consists of a light chain comprising a sequence as defined by SEQ ID NO: 4 and a heavy chain comprising a sequence as defined by SEQ ID NO: 6. SEQ ID NO: 5 represents the Fab-fragment of the heavy chain of of bevacizumab and SEQ ID NO: 6 represents the Fab-fragment of the heavy chain of ranibizumab. Also preferably, the ligand binds to a Fab-fragment comprising the aforementioned sequences or essentially consisting of them. The sequences are identical to preferably 90 %, even more preferably 95 %, still more preferably 98 %, most preferably 99 % relative to the aforementioned sequences.

When practicing the invention, the compounds according to the general formula (I) are attached to a support matrix of an appropriate support material, resulting in a matrix for affinity purification, preferably affinity chromatography (also referred to as affinity matrix in the context of the present invention) for protein separation. The compounds of the general formula are attached to the support matrix via L, optionally including a spacer –Z-.

Accordingly, the present invention includes an affinity matrix for protein separation, comprising a support matrix comprising a support material and at least one compound as specified in the specification beforehand, wherein the compound is attached thereto via group L, and wherein L optionally contains a spacer group Z.

The support matrix may comprise any appropriate support material which is known to the person skilled in the art. The material may be soluble or insoluble, particulate or non-particulate, or of a monolithic structure, including fibers and membranes, porous or non-porous. It provides a convenient means of separating ligands of the invention from solutes in a contacting solution. Examples of support matrix include carbohydrate matrices such as agarose, cellulose, dextran, starch, alginate or carrageenan; synthetic polymer matrices such as polystyrene, styrene-divinylbenzene copolymers, polymethacrylates, (e.g. poly(hydroxyethylmethacrylate), polyvinyl alcohol, polyamides or perfluorocarbons; inorganic matrices such as glass, silica or metal oxides; and composite materials.

The affinity matrix is prepared by providing a support matrix of an appropriate support material and attaching a compound of formula (I) thereto. Methods for attaching the compound (I) to the support matrix are known to the person skilled in the art.

The term “affinity purification” (which is used interchangeably with the term “affinity separation”) refers to any separation technique involving molecular recognition of a protein by a compound of formula (I). The compound may be immobilized on a solid support or

coupled to a functional moiety facilitating separation of the ligand-antibody complex later on. Separation techniques may include, but are not limited to, affinity chromatography on packed columns, monolithic structures or membranes. The term further includes adsorption in batch-mode or affinity precipitation.

Independently of the flavour, purification techniques are composed by an initial recognition phase where ligand is contacted with antibody in crude. In a second phase, either impurities are separated from the ligand-antibody complex or (e.g. column chromatography) or ligand-antibody complex is separated from impurities (e.g. affinity precipitation). In a third step, the antibody is released from the ligand-antibody complex by alteration of chemical and/or physical conditions like change in pH, ionic strength and/or addition of modifiers like organic solvents, detergents or chaotropes.

The invention will now be illustrated by the following examples, which shall not be construed to limit the invention.

Documents cited in the specification:

1. A.Cecilia, A.Roque, C.R. Lowe, M.A. Taipa: Antibodies and Genetically Engineered Related Molecules: Production and Purification, Biotechnol. Prog. **2004**, 20, 639-654

2. K.L.Carson: Flexibility-the guiding principle for antibody manufacturing, Nature Biotechnology, **2005**, 23, 1054-1058; S.Hober, K.Nord, M. Linhult: Protein A chromatography for antibody purification, J. Chromatogr. B, **2007**, 848, 40-47

Examples

Materials and Methods

If not otherwise stated, all chemicals and solvents were of analytical grade, with the exception of example 1. Reagents used in example 1 were of preparative to analytical grade depending on particulate requirements and availability.
96 and 384-well filter plates having hydrophilic membrane filters with 0.45 µm average pore size were purchased from Pall GmbH (Dreieich/Germany). Top frits made from polyethylene with 10 µm average pore size were provided by Porex (Bautzen/Germany). General purpose microtiterplates for collection of fractions and analytical assays were ordered from Greiner Bio One GmbH (Frickenhausen/Germany). Analytical assays were read out using a Fluostar

Galaxy plate reader from BMG Labtech GmbH (Offenburg/Germany).
Column chromatography with antibody and purification of antibody fragments was conducted on a Waters HPLC system (Waters GmbH, Eschborn/Germany). Omnifit column housings (Diba Industries Ltd, Cambridge/United Kingdom) were were used for packing of columns. NHS-activated Sepharose 4 FF, rProtein A Sepharose FF and Superdex 70 chromatography media were bought from GE Healthcare (Uppsala/Sweden). Mabsorbent A2P HF was purchased from Prometic Life Sciences (Cambridge/United Kingdom).
Analytical chromatography of ligands was conducted on a Shimadzu HPLC system (Shimadzu Deutschland GmbH, Duisburg/Germany) including a diode array detector and single-quad mass spectrometer. The monolithic C18 reversed phase column was purchased from Merck KGaA (Darmstadt/Germany). Solvents used in analyses were of mass spectrometry grade.
Avastin (F. Hoffmann-La Roche, Switzerland) containing purified humanized antibody Bevacizumab (1) was used in experiments (referred to as antibody or whole IgG). Immobilized papain for preparation of antibody fragments was purchased from Thermo Scientific (Bonn/Germany).
Flowthrough from protein A chromatography (referred to as host cell proteins) was derived from the supernatant of antibody-producing CHO cell culture in serum-free medium.
Coomassie brilliant blue dye reagent for Bradford assay, the protein gel electrophoresis cell including power supplies, pre-cast gradient gels and colloidal Coomassie staining solution were purchased from Bio-Rad Laboratories GmbH (München/Germany).

Example 1

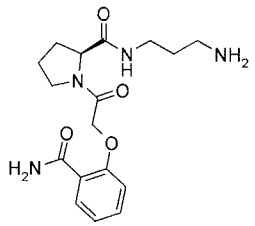
SYNTHESIS OF LIGANDS

General procedure for the synthesis of ligands A, B, D, E, and G:

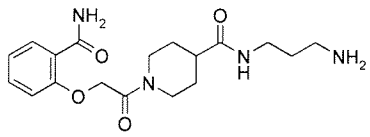
An amount of commercially available 1,3-diaminopropanetrityl-polystyrene resin or 1,3-diaminopropane-2'-chlorotrityl-polystyrene resin (prepared from commercially available 2-chlorotrityl chloride resin and excess 1,3-diaminopropane in NMP and subsequent washing and drying) corresponding to approx. 100 μmol was swollen in NMP, then the solvent was removed. The Fmoc amino acid (0.12-0.5 mmol) and an equimolar amount of HOAt were dissolved in NMP (1-3 mL), and DIC (equimolar amount relative to the Fmoc amino acid) was added. The mixture was added to the resin after 1-3 minutes, and the resin was shaken for several hours or overnight. Afterwards, the resin was washed (typically 3×DMF, 2×dichloromethane, 2×DMF, 3×dichloromethane). Fmoc deprotection was achieved by

reaction with 1-3 mL of 25% piperidine in DMF for 20-60 min, followed by washing as described above.

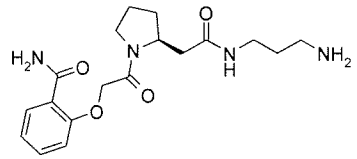
200-500 μmol of 2-carbamoylphenoxyacetic acid and an equimolar amount of HOAt were dissolved in NMP (1-3 mL), and DIC (equimolar amount relative to the carboxylic acid) was added. The mixture was added to the resin after 1-3 minutes, and the resin was shaken for several hours or overnight. Next, the resin was washed as described above, and the crude product was cleaved from the resin by treatment of the latter with a mixture of dichloromethane (85%), trifluoroacetic acid (10%) and triethylsilane (5%) (for trityl resins) or dichloromethane (50%), trifluoroacetic acid (40%) and triethylsilane (10%) (for 2-chlorotrityl resins) for 10 min up to several hours. The cleavage was repeated once, followed by washing of the resin with dichloromethane. The combined cleavage and washing solutions were evaporated with a stream of nitrogen and the remaining crude product was purified by reversed-phase preparative HPLC/MS. The collected fractions were evaporated to dryness with a stream of nitrogen, followed by drying in high vacuum.



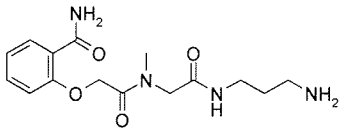
(*S*)-*N*-[(2-Carbamoylphenyloxy)acetyl]pyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide (Ligand A): Prepared from Fmoc-L-Proline following the general procedure. ESI-LCMS: 349 (M+1).



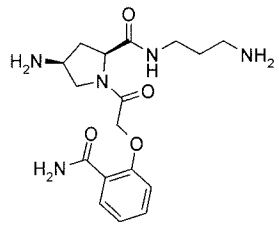
***N*-[(2-Carbamoylphenyloxy)acetyl]piperidine-4-carboxylic acid 3-aminoprop-1-yl amide (Ligand B):** Prepared from Fmoc-4-piperidinecarboxylic acid following the general procedure. ESI-LCMS: 363 (M+1).



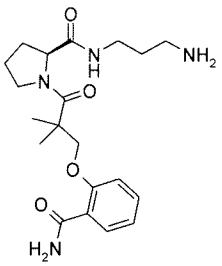
(*S*)-*N*-[(2-Carbamoylphenyloxy)acetyl]pyrrolidine-2-ylacetic acid 3-aminoprop-1-yl amide (Ligand D): Prepared from Fmoc-L-beta-homoproline following the general procedure. ESI-LCMS: 363 (M+1).



***N*-[(2-Carbamoylphenyloxy)acetyl]-*N*-methylglycine 3-aminoprop-1-yl amide (Ligand E):** Prepared from Fmoc-sarcosine following the general procedure. ESI-LCMS: 323 (M+1).



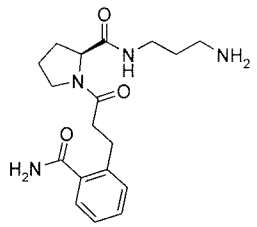
(*S*,*S*)-1-*N*-[(2-Carbamoylphenyloxy)acetyl]-4-aminopyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide (Ligand G): Prepared from (2*S*,4*S*)-Boc-4-amino-1-Fmoc-pyrrolidine-2-carboxylic acid following the general procedure. ESI-LCMS: 364 (M+1).



(*S*)-*N*-[3-(2-Carbamoylphenyloxy)-2,2-dimethylpropionyl]pyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide (Ligand C): An amount of commercially available 1,3-diaminopropanetrityl-polystyrene resin corresponding to approx. 100 μmol was swollen in

NMP, then the solvent was removed. Fmoc-Pro (0.5 mmol) and HOAt (0.5 mmol) were dissolved in NMP (approx. 1.5 mL) and reacted with DIC (0.5 mmol). After approx. 2 min the mixture was added to the resin and agitated overnight. After washing the resin (3×DMF, 2×dichloromethane, 2×DMF, 3×dichloromethane), Fmoc deprotection was effected by reaction with approx. 2 mL of 25% piperidine in DMF, followed by washing as described above.

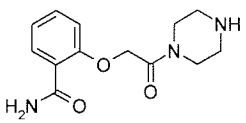
2,2-Dimethyl-3-bromopropionic acid (0.5 mmol) was dissolved in NMP (approx. 1.5 mL) and reacted with DIC (0.5 mmol) for approx. 1 min, whereupon the mixture was added to the resin. After approx. 1 h the solution was removed, the resin was washed (NMP) and the coupling step was repeated. After washing (NMP, dichloromethane), the resin was dried and transferred to a glass vial. Excess salicylic amide and Cs_2CO_3 were added along with a sufficient amount of water/DMSO (approx. 1:5 ratio), the glass vial was closed tightly, heated to 50 °C and shaken for three days. As LCMS of the crude cleavage product of a small sample amount of the resin indicated incomplete conversion, temperature was increased to 80 °C and shaking was continued for another day, whereupon the resin was washed and dried. Cleavage of the product was effected by twofold treatment with DCM (85%), trifluoroacetic acid (10%) and triethylsilane (5%), followed by washing with DCM. After removal of the cleavage reagents and solvent with a stream of nitrogen, the crude product was purified by reversed-phase preparative HPLC/MS. The collected fractions were evaporated to dryness with a stream of nitrogen, followed by drying in high vacuum. ESI-LCMS: 391 (M+1).

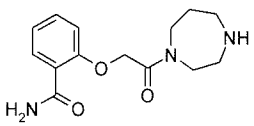


(*S*)-*N*-[3-(2-Carbamoylphenyl)propionyl]pyrrolidine-2-carboxylic acid 3-aminoprop-1-yl amide (Ligand F): 100 µmol of 1,3-diaminopropanetrityl resin (NovaBiochem) was loaded with Fmoc-Pro and Fmoc deprotected as described for Ligand C.
2'-Carboxyphenylpropionic acid (200 mg) was converted to the dimethyl ester by reaction with trimethyl orthoformate and methanol in the presence of strongly acidic ion exchange resin at 50 °C over night. The resin was removed by filtration, rinsed with methanol, and the remaining solution was concentrated to dryness. Cleavage of the aliphatic methyl ester was effected by enzymatic hydrolysis employing porcine pancreas lipase type II (125 mg, later on addition of a small additional portion) in phosphate buffer pH 7.4 (correction of the pH was

achieved by addition of aqueous NaOH) at 37 °C for three days, when LCMS indicated sufficient conversion, while the peak of the newly formed compound was assigned to 2-methoxycarbonylphenylproptionic acid, as the enzyme was expected to cleave exclusively the aliphatic ester moiety. Subsequently, the mixture was acidified with HCl (pH <2), extracted with EtOAc, washed (water), dried (Na_2SO_4) and concentrated to dryness.

An amount of the carboxylic acid corresponding to approx. 0.2 mmol and HOAt (0.2 mmol) were dissolved in NMP (approx. 1.5 mL) and reacted with DIC (0.2 mmol). After 1-2 min, the mixture was added to the Pro-resin synthesized before, and shaken overnight. On the next day, the resin was washed (3×DMF, 2×dichloromethane, 2×DMF, 3×dichloromethane). The resin was transferred to a glass vial, and methanol (1 mL), THF (1 mL) and aqueous NaOH (1 M, 1 mL) were added. The mixture was heated to 60 °C overnight, then the resin was washed (water, methanol, DMF, DCM, each several times) and dried. Next, the carboxylic acid was activated by reaction with excess HATU and DIPEA in NMP for 10-30 min, whereupon the solvent was removed and concentrated aqueous ammonia and THF were added and the mixture was agitated at ambient temperature until the conversion was completed. The resin was washed (3×DMF, 2×dichloromethane, 2×DMF, 3×dichloromethane), dried and the product was obtained by twofold cleavage with DCM (85%), trifluoroacetic acid (10%) and triethylsilane (5%), followed by washing with DCM. After removal of the cleavage reagents and solvent with a stream of nitrogen, the crude product was purified by reversed-phase preparative HPLC/MS. The collected fractions were evaporated to dryness with a stream of nitrogen, followed by drying in high vacuum. ESI-LCMS: 347 (M+1).

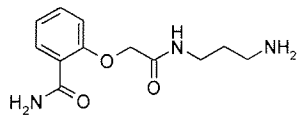




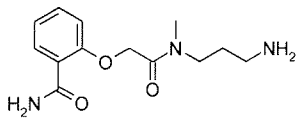
***N*-[(2-Carbamoylphenoxy)acetyl]piperazine (Ligand H) *and* *N*-[(2-carbamoylphenoxy)acetyl]homopiperazine (Ligand I), as trifluoroacetates:** 2-Carbamoylphenoxyacetic acid (0.5 mmol) and TBTU (0.5 mmol) were dissolved in DMF (2 mL) and treated with triethylamine (1 mmol). After 5 min, 0.6 mmol of the mono-protected secondary diamine (*N*-Boc-piperazine for Ligand H; *N*-Boc-homopiperazine for Ligand I) in DMF (least amount possible) was added and the mixture was agitated for 1 h. Subsequently, water and saturated aqueous Na_2CO_3 (2.5 mL each) were added and the mixture was extracted with ethyl acetate (5 mL, twice). The combined organic layers were washed twice with aqueous citric acid (10%) and saturated Na_2CO_3. After drying with Na_2SO_4 and filtration, the

solution was concentrated to dryness and the residue was purified by flash column chromatography on silica gel.

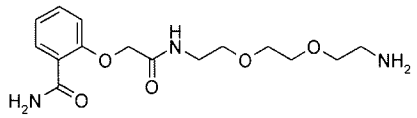
The Boc-protected compound was dissolved in DCM (2-3 mL) and treated with trifluoroacetic acid (0.5 mL). After 30 min, the solvent was removed with a stream of nitrogen. The residue was re-dissolved in DCM, evaporated and dried in high vacuum. ESI-LCMS: 264 (M+1; Ligand H); 278 (M+1; Ligand I).



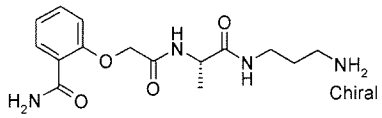
2-Carbamoylphenoxyacetic acid (3-amino-1-propyl)amide (Ligand J): 1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (2-carbamoylphenoxy)acetic acid was coupled employing a standard DIC/HOAt coupling protocol in NMP. After washing (DMF, DCM), the resin was treated twice with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 252 (M+1).



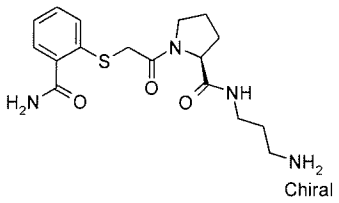
2-Carbamoylphenoxyacetic acid *N*-(3-amino-1-propyl)-*N*-methyl amide (Ligand K): 2-Chlorotrityl chloride polystyrene resin (0.1 mmol) was treated with 3-aminopropanol (1 mL) and NMP (1 mL) and agitated over night. After repeated washing with DMF and DCM, the resin was dried thoroughly. DIPEA (1 mmol) and methylsulfonyl chloride (0.5 mmol) in DCM (approx. 2 mL) were added and the mixture was agitated for 30 min, then the resin was washed repeatedly with DCM, followed by repetition of the mesilate formation step for 2 hours. Again, the resin was washed with DCM, then methylamine solution (8 M in EtOH, 1 mL) and DMSO (1 mL) were added and the resin was shaken over night. After thorough washing with DMF and DCM, (2-carbamoylphenoxy)acetic acid was coupled to the resin using TBTU/DIPEA in NMP (pre-activation 2 min, approx. 3-fold excess, reaction time 2 h). After final washing with DMF and DCM, the target compound was cleaved from the resin by twofold treatment with trifluoroacetic acid and triethylsilane (45% and 10% in DCM), followed by washing with DCM. After evaporation of the solvent, the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 266 (M+1).



2-Carbamoylphenoxyacetic acid (8-amino-3,6-dioxa-1-octyl)amide (Ligand L): 2-Chlorotrityl chloride polystyrene resin (0.1 mmol) was treated with 3,6-dioxaoctyl-1,8-diamine (1 mL) and NMP (1 mL) and agitated for 2 h. After repeated washing with DMF and DCM, the resin was dried. (2-Carbamoylphenoxy)acetic acid was coupled to the resin using a standard DIC/HOAt coupling protocol in NMP. After washing of the resin with DMF and DCM, the target compound was cleaved from the resin by twofold treatment with trifluoroacetic acid and triethylsilane (45% and 10% in DCM), followed by washing with DCM. After evaporation of the solvent, the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 326 (M+1).



(*S*)-*N*-[(2-Carbamoylphenoxy)acetyl]alanine 3-aminopropyl amide (Ligand M): 1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (*S*)-Fmoc-alanine was coupled to the support using a standard DIC/HOAt coupling protocol in NMP. After washing and deprotection with piperidine (25% in DMF), followed by thorough washing with DMF and DCM, (2-carbamoylphenoxy)acetic acid was coupled employing the same coupling protocol in NMP. After washing (DMF, DCM), the resin was treated twice with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 323 (M+1).

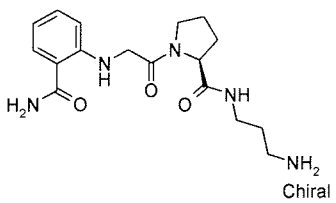


(*S*)-*N*-[(2-Carbamoylphenylthio)acetyl]proline 3-aminopropyl amide (Ligand N): Thiosalicylic acid (0.5 mmol) and Cs_2CO_3 (1.1 mmol) were suspended in acetone (2 mL) and

treated with bromoacetic acid *tert*-butyl ester (0.5 mmol) in acetone (0.5 mL), whereupon a thick white precipitate formed. The mixture was agitated for approx. 3 h, then the solvent was removed with a stream of nitrogen. Aqueous citric acid (10%) was added and the mixture was extracted twice with *tert*-butyl methyl ether. The combined organic layers were washed twice with water, then the solvent was removed and the residue was dried in high vacuum, furnishing (2-carboxyphenylthio)acetic acid *tert*-butyl ester as a colorless solid which was used for the next step without further purification.

To the residue, TBTU (0.5 mmol) in NMP (approx. 2 mL) and DIPEA (0.5 mmol) were added and the mixture was agitated for 5 min, whereupon concentrated aqueous ammonia (1 mL) was added. After agitating the mixture for 25 min, saturated aqueous $NaHCO_3$ (2 mL) was added and the mixture was extracted three times with *tert*-butyl methyl ether. The combined organic layers were washed with saturated $NaHCO_3$, twice with aqueous citric acid (10%) and twice with water. The solvent was removed, the residue was re-dissolved in DCM and evaporated again twice. The slightly moist residue was treated with trifluoroacetic acid (1 mL) for 12 min, then evaporated to dryness and dried in high vacuum, furnishing 42 mg (0.2 mmol, 40%) of (2-carbamoylphenylthio)acetic acid as a colorless solid.

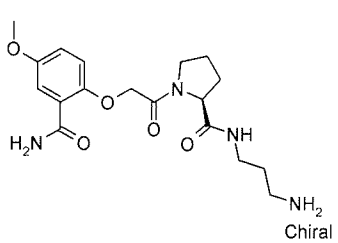
1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (*S*)-Fmoc-proline was coupled to the support using a standard DIC/HOAt coupling protocol in NMP. The resin was washed and deprotected with piperidine (25% in DMF), followed by thorough washing with DMF and DCM. To the carboxylic acid obtained before was added TBTU (1 equivalent) in NMP (adequate volume) and DIPEA (2 equivalents) and agitated for 2 min, whereupon the mixture was added to the resin. After the coupling was finished, the resin was washed (DMF, DCM) and the target compound was cleaved off by twofold treatment with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 365 (M+1).

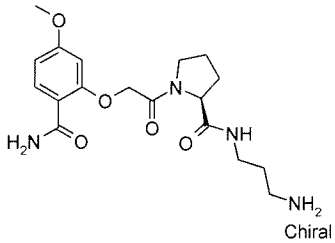


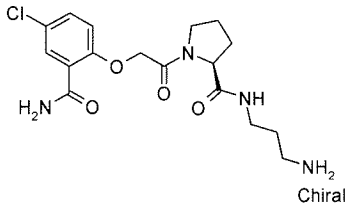
(*S*)-*N*-[(2-Carbamoylphenylamino)acetyl]proline 3-aminopropyl amide (Ligand O): To anthranilic amide (0.5 mmol), potassium carbonate (1 mmol) and bromoacetic acid *tert*-butyl

ester (0.5 mmol) was added DMSO (1 mL) and the mixture was heated to 100 °C for 90 min. Then water was added and the mixture was extracted twice with *tert*-butyl methyl ether. The combined organic layers were washed with aqueous citric acid (5%), saturated Na_2CO_3 and water (each twice). The solvent was removed and the moist residue was treated with trifluoroacetic acid (1 mL) for 5 min, then the acid was removed with a stream of nitrogen and the deprotection was repeated for 30 min. After removal of the acid, the residue was dried in high vacuum.

1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (*S*)-Fmoc-proline was coupled to the support using a standard DIC/HOAt coupling protocol in NMP. The resin was washed and deprotected with piperidine (25% in DMF), followed by thorough washing with DMF and DCM. To the carboxylic acid obtained before was added TBTU (0.3 mmol) in NMP (adequate volume) and DIPEA (1 mmol) and agitated for 2 min, whereupon the mixture was added to the resin. After 1 h the resin was washed (DMF, DCM) and the target compound was cleaved off by twofold treatment with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 348 (M+1).

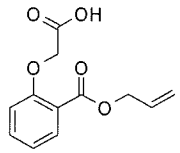




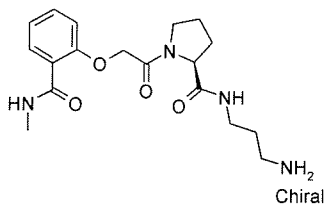


(*S*)-*N*-[(2-Carbamoyl-4-methoxyphenoxy)acetyl]proline 3-aminopropyl amide (Ligand P), (*S*)-*N*-[(2-carbamoyl-5-methoxyphenoxy)acetyl]proline 3-aminopropyl amide (Ligand Q) *and* (*S*)-*N*-[(2-carbamoyl-4-chlorophenoxy)acetyl]proline 3-aminopropyl amide (Ligand R): 1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (*S*)-Fmoc-proline was coupled to the support using a standard DIC/HOAt coupling protocol in NMP. The resin was washed and deprotected with piperidine (25% in DMF), followed by thorough washing with DMF and DCM. Then bromoacetic acid (0.5 mmol) was dissolved in NMP (1.5 mL) and treated with DIC (0.5 mmol). After 2 min the mixture was added to the resin and shaken for 30 min to 2 h. After washing with NMP and DCM (no DMF was used), the coupling step was repeated, followed by thorough washing as described before. Then the resin was transferred to a glass vial and Cs_2CO_3 (0.6 mmol) and 0.3 mmol of the respective phenol (2-hydroxy-5-methoxybenzamide

for Ligand P, 2-hydroxy-4-methoxybenzamide for Ligand Q, 2-hydroxy-5-chlorobenzamide for Ligand R) were added along with DMSO (3 mL) and water (1 mL). The mixture was shaken at 50 °C over night, then the resin was washed (water, methanol, DMF, DCM). The target compound was cleaved off by twofold treatment with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 379 (M+1; Ligand P); 379 (M+1; Ligand Q); 383, 385 (M+1; Ligand R).



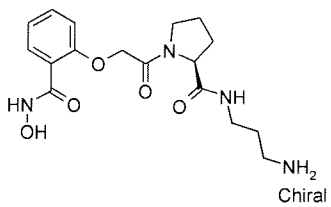
(2-Allyloxycarbonylphenoxy)acetic acid: Salicylic acid allyl ester (1 mmol) and Cs_2CO_3 (1 mmol) were suspended in acetone (2 mL) and treated with bromoacetic acid *tert*-butyl ester (1 mmol) in acetone (0.5 mL). The mixture was agitated over night, then it was heated to 40 °C for approx. 1 h. Then the solvent was removed, water was added and the mixture was extracted with *tert*-butyl methyl ether (3 times). The combined organic layers were washed with aqueous Na_2CO_3 and water (each twice), then the solvent was removed and the moist residue was treated with trifluoroacetic acid (2 mL) for 12 min. After evaporation of the acid, the residue was dried in high vacuum, furnishing a crystalline solid (227 mg, 0.96 mmol, 96%), which was used for the preparation of Ligand S and Ligand T without further purification.



(*S*)-*N*-{[2-(*N*-Methylcarbamoyl)phenoxy]acetyl}proline (3-amino-1-propyl)amide (Ligand S): 1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (*S*)-Fmoc-proline was coupled to the support using a standard DIC/HOAt coupling protocol in NMP. The resin was washed and deprotected with piperidine (25% in DMF), followed by thorough washing with DMF and DCM. (2-Allyloxycarbonylphenoxy)acetic acid (0.3 mmol) and TBTU (0.3 mmol) were dissolved in

NMP (approx. 1 mL) and DIPEA (0.6 mmol) was added. After 2 min the mixture was added to the resin. After shaking over night, the resin was washed (DMF, methanol, DCM).

The resin was swollen in DCM (1 mL) and treated with tetrakis(triphenylphosphine)-palladium-(0) (10 mg) in DCM (1 mL), followed by piperidine (200 µL). After shaking for 1 h, the resin was washed (DMF, methanol, DCM). Removal of the majority of bound piperidine was accomplished by treatment with acetic acid (0.3 mmol) which was preactivated with TBTU (0.25 mmol) and DIPEA (0.5 mmol) in NMP (sufficient volume) for 10 min, followed by washing (DMF, methanol, DCM). Then the resin was treated with TBTU (0.5 mmol) in NMP (sufficient volume) and DIPEA (1 mmol) for 5 min. Then the resin was filtered off, swollen in NMP and treated with methylamine solution (8 M in EtOH, 0.5 mL). The mixture was shaken for 5 min, then the resin was washed (DMF, methanol, DCM). The activation/coupling step was repeated with pre-swelling of the resin in DCM and subsequent swelling in NMP to ensure sufficient swelling. LCMS of the coupling product showed approx. 1/3 of piperidine amide, and 2/3 of the desired secondary amide. The target compound was cleaved off by twofold treatment of the resin with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 363 (M+1).



(*S*)-*N*-{[2-(*N*-Hydroxycarbamoyl)phenoxy]acetyl}proline (3-amino-1-propyl)amide (Ligand T): 1,3-Diaminopropane immobilized to trityl polystyrene resin (0.1 mmol) was pre-swollen in DCM and NMP, then (*S*)-Fmoc-proline was coupled to the support using a standard DIC/HOAt coupling protocol in NMP. The resin was washed and deprotected with piperidine (25% in DMF), followed by thorough washing with DMF and DCM. (2-Allyloxycarbonylphenoxy)acetic acid (0.3 mmol) and TBTU (0.3 mmol) were dissolved in NMP (approx. 1 mL) and DIPEA (0.6 mmol) was added. After 2 min the mixture was added to the resin. After shaking over night, the resin was washed (DMF, methanol, DCM).
The resin was swollen in DCM (1 mL) and treated with tetrakis(triphenylphosphine)-palladium-(0) (10 mg) in DCM (1 mL), followed by piperidine (200 µL). After shaking for 1 h, the resin was washed with triethylamine (5% in DMF), acetic acid (5% in DMF), triethylamine (5% in DMF), acetic acid (5% in DMF), methanol and DCM (3 times each).

Then TBTU (0.5 mmol) in NMP (sufficient volume) was added, followed by DIPEA (1 mmol). After shaking for 5 min, the resin was filtered off and NMP (sufficient volume) and hydroxylamine (50% in water, 0.2 mL) were added. The mixture was shaken for 20 min, then the resin was washed (DMF, methanol, DCM). The reaction was repeated with HATU instead of TBTU and with the coupling time extended over night. A third activation/coupling step was carried out, this time with thorough pre-swelling of the resin with DCM prior to addition of NMP, and using TBTU as coupling reagent. Both preactivation and coupling times were 10 min. Subsequently the resin was washed (DMF, methanol, DCM) and the target compound was cleaved off by twofold treatment of the resin with trifluoroacetic acid and triethylsilane (10% and 5% in DCM), followed by washing with DCM. The solvent was removed with a stream of nitrogen and the residue was purified by preparative reversed-phase HPLC/MS. ESI-LCMS: 365 (M+1).

Example 2: Immobilization of Ligands

Experimental

Dried ligands were redissolved in DMSO at an approximate concentration of 100 mM based on gravimetric analyses. Precise concentrations of stock solutions were determined by the o-phthaldialdehyde assay (2). Dye-ligand molecules were quantitated by measuring absorbance at 340 nm. Measurements were calibrated with 1-(3-aminopropyl)imidazole.

In coupling reactions, one volume of ligand dissolved at an approximate concentration of 15 to 20 mM in 90% DMSO and 10% *N*-Methyl-2-pyrrolidone containing 1 M *N,N*-Diisopropylethylamine was added to one volume of settled NHS-activated Sepharose 4 FF (GE Healthcare) equilibrated with isopropanol. Reactions were conducted for 2 h at 25 °C while shaking vigorously. The supernatant of reactions was withdrawn and resins were washed twice with appropriate solvent. Remaining active groups on resins were blocked with 1 M Ethanolamine for 1 h at 25 °C. Final resins were washed and stored in 20% ethanol at 4 °C until used in subsequent experiments. Reaction yields were determined based on mass balances after analysis of ligand concentrations in all fractions.

Summary and Results

Ligands A to T from example 1 were immobilized on NHS-activated Sepharose 4 FF for subsequent chromatography and batch adsorption experiments. Coupling was achieved by formation of an amide bond between the amino group of the aminopropyl linker on ligands and the NHS-activated carboxylic group of the pre-activated resin.

The table below gives the results for coupling reactions with ligands from example 1. On

average, an immobilized ligand density of 14.0 µmol per ml of resin was obtained. Reaction yields averaged at 68%.
As the concentrations of the secondary amines Ligand H and Ligand I were indeterminable by the o-phthaldialdehyde assay, no coupling yields could be assessed for those two ligands.

Ligands A to G from example 1 were immobilized on NHS-activated Sepharose 4 FF for subsequent chromatography and batch adsorption experiments. Coupling was achieved by formation of an amide bond between the amino group of the aminopropyl linker on ligands and the NHS-activated carboxylic group of the pre-activated resin.
The table below gives the results for coupling reactions with ligands from example 1. On average, an immobilized ligand density of 11.9 µmol per ml of resin was obtained. Reaction yields averaged at 60%.

Ligand	Initial (µmol per ml gel)	Supernatant (µmol per ml gel)	Immobilized (µmol per ml gel)	Yield (%)
A	17.0	5.8	11.2	66
B	14.6	2.8	11.8	81
C	13.3	2.7	10.6	80
D	19.4	5.6	13.8	71
E	18.3	4.4	13.9	76
F	20.6	8.1	12.5	61
G	17.0	7.4	9.6	56
H	-	-	-	-
I	-	-	-	-
J	24.8	9.1	15.7	63
K	25.5	8.8	16.6	65
L	24.1	9.6	14.5	60
M	26.6	10.1	16.4	62
N	29.3	8.7	20.6	70
O	21.9	7.7	14.1	65
P	13.7	1.8	11.9	87
Q	18.8	5.2	13.6	72
R	16.9	3.5	13.3	79
S	25.2	10.2	15.0	59
T	26.6	9.7	16.9	64
		Averages	14.0	68

Ligand	Initial (µmol per ml gel)	Supernatant (µmol per ml gel)	Immobilized (µmol per ml gel)	Yield (%)
A	17.0	5.8	11.2	56
B	14.6	2.8	11.8	59
C	13.3	2.7	10.6	53
D	19.4	5.6	13.8	69
E	18.3	4.4	13.9	70
F	20.6	8.1	12.5	63
G	17	7.4	9.6	48
		Averages	11.9	60

Example 3: Chromatographic Evaluation of Ligands (from Example 1)

Experimental

Resins were assessed in packed mode by microtiter plate chromatography. For each column, approximately 30 µl of resin were transferred to a 384-well filter plate and sealed with appropriate top frits. Columns with rProtein A Sepharose FF and Mabsorbent A2P HF were included as controls.

Columns were equilibrated with 6.7 column volumes (cv) of phosphate buffered saline (0.15 M NaCl, 20 mM sodium phosphate, pH 7.3; PBS) prior to injection. Either 3.3 cv of whole IgG dissolved at 0.75 mg ml^{-1}, Fab or Fc fragments dissolved at 0.25 mg ml^{-1} (all three in PBS) or host cell proteins were injected onto columns. Antibody fragments of Bevacizumab (Fab and Fc) were prepared by cleavage of whole IgG with papain protease (4). Digestion of antibody with papain was performed according to the manufacturer's instructions. Unbound protein was washed from columns with 5 cv of PBS prior to elution with 5 cv of glycine buffer at pH 2.5. Volumes transferred were spun through columns at 50 g for 1 – 2 min. During injection of samples, acceleration was reduced to 10 g and centrifugation time increased to 5 – 10 min. Effluent fractions were collected in 384-well plates and concentrations of protein analyzed by Bradford assay. Protein masses m_i, m_{ft} and m_e (see below) were calculated as the product of fraction volume and measured protein concentration.

Summary and Results

Binding and elution of Bevacizumab whole IgG and related Ranibizumab by resins from example 2 and in some cases of Bevacizumab Fab and Fc fragments was demonstrated

applying non-optimized operating conditions. Selectivity of ligands towards the antibody was demonstrated by low binding of host cell proteins. Results for commercial resins rProtein A Sepharose FF (rProtein A) and Mabsorbent A2P (A2P) were included for comparison.

The fraction of 'bound' protein was calculated as the difference between the total mass of protein injected m_i and the mass of protein detected in the flowthrough m_{ft}, divided by the total mass of protein injected.

$$bound = \frac{m_i - m_{ft}}{m_i}$$

The 'yield' of protein was calculated as the mass of protein eluted m_e, divided by the mass of protein injected m_i.

$$yield = \frac{m_e}{m_i}$$

The 'selectivity' of resins was calculated as the fraction of bound antibody B_{Ab}, divided by the fraction of bound host cell proteins B_{HCP}.

$$selectivity = \frac{B_{Ab}}{B_{HCP}}$$

The fraction of 'bound' protein and the 'yield' of protein were calculated for each resin based on data for Bevacizumab whole IgG. In the case of Ranibizumab, Bevacizumab fragments and host cell proteins, only the fraction of 'bound' protein is given. Selectivity of resins was calculated from data for Bevacizumab whole IgG and host cell proteins. Due to limited experimental precision, selectivity was truncated beyond values of 10.

Chromatography results for resins from example 2 and reference resins are given in the table below. With the exception of resins C, F, N, S and T, all resins were capable of binding Bevacizumab whole IgG completely or to at least 95%. Binding by resins C and F was lower with 72% and 15%, respectively. A similar result was obtained regarding the binding of Ranibizumab. Except that binding by resins C and F was even lower if not negligible. Bound Bevacizumab whole IgG was further elutable from resins at an acidic pH of 2.5. Binding by residual resins was between 15% and 88%. A subset of ligands was further evaluated in respect to Ranibizumab binding. Also here, ligands A, B, D, E and G showed efficient binding, whereas ligands C and F showed low binding.

Dominant binding of the Bevacizumab Fab fragment and Ranibizumab compared to little binding of the Bevacizumab Fc fragment by resins A and B suggests binding of affinity ligands on the Fab domain of Bevacizumab. With the exception of Mabsorbent A2P HF, all resins (including rProtein A Sepharose FF) showed little to now binding of host cell proteins, resulting in high selectivity for both antibodies (Bevacizumab and Ranibizumab).

Ligand	Bevacizumab IgG Bound (%)	Bevacizumab IgG Yield (%)	Bevacizumab Fab Bound (%)	Bevacizumab Fc Bound (%)	Ranibizumab Bound (%)	Host Cell Proteins Bound (%)	Selectivity
A	100	103	100	7	100	11	8.8
B	100	99	100	6	100	10	9.8
C	72	58	-	-	12	6	≥ 10
D	100	96	-	-	100	2	≥ 10
E	100	98	-	-	94	0	≥ 10
F	15	14	-	-	4	0	≥ 10
G	100	105	-	-	94	10	≥ 10
H	97	103	-	-	-	19	5.1
I	95	102	100	0	-	11	8.9
J	99	96	-	-	-	12	8.3
K	100	93	-	-	-	15	6.8
L	100	92	58	3	-	8	≥10
M	95	108	84	2	-	9	≥10
N	88	103	-	-	-	13	7.0
O	100	93	-	-	-	16	6.3
P	99	95	-	-	-	13	7.7
Q	99	96	100	0	-	8	≥10
R	96	104	-	-	-	21	4.7
S	77	94	-	-	-	2	≥ 10
T	62	102	-	-	-	0	≥ 10
rProtein A	100	101	0	100	-	0	≥ 10
A2P	100	89	100	100	-	100	1.0

	Bevacizumab				Ranibizumab	Host Cell Proteins	
	IgG		Fab	Fc			
Ligand	Bound (%)	Yield (%)	Bound (%)	Bound (%)	Bound (%)	Bound (%)	Selectivity
A	100	103	100	7	100	11.3	8.8
B	100	99	100	6	100	10.2	9.8
C	72	58	-	-	12	6.2	≥ 10
D	100	96	-	-	100	2.4	≥ 10
E	100	98	-	-	94	0	≥ 10
F	15	14	-	-	4	0	≥ 10
G	100	105	-	-	94	9.6	≥ 10
rProtein A	100	101	0	100	-	0	≥ 10
A2P	100	89	100	100	-	100	1

Example 4: Isotherms and Time-Scales of Binding

Experimental

Parameters of Langmuir isotherms for purified antibody were determined by batch adsorption experiments in 96-well microtiter plates. In each well, 10 µl of sorbent slurry (50% v/v) were mixed with 100 µl of protein solution. Initial concentrations of antibody were varied from 0.05 – 5 mg ml^{-1} Bevacizumab in phosphate-buffered saline (0.15 M NaCl, 20 mM phosphate buffer, pH 7.3; PBS). Reactions were agitated vigorously at 25 °C for at least 3 h. Supernatants were sampled afterwards and concentrations of the antibody were analyzed by Bradford assay.

Uptake kinetics were characterized similarly by batch adsorption in 96-well filter plates. Again, 10 µl of sorbent slurry (50% v/v) were mixed with 100 µl of protein solution. However, a fixed initial concentration of 0.75 mg ml^{-1} Bevacizumab in PBS was used. Reactions were agitated vigorously at 25 °C for up to 80 min. Supernatants were separated rapidly by spinning through filters after 2.5, 5, 10, 20, 40 and 80 minutes and sampled for analysis. Concentrations of the antibody were analyzed by Bradford assay.

Summary and Results

Resins A and B (from example 2) were characterized with respect to their affinity and maximum capacity towards purified antibody and the time-scale required for binding. Commercial resins rProtein A Sepharose FF (rProtein A) and Mabsorbent A2P (A2P) were

included for comparison.

Parameters of Langmuir isotherms, i.e. dissociation constants K_d and maximum capacities q_m, were determined from the measured concentrations in supernatants. Parameters were estimated by numerical fitting of the model equation derived by Chase (5).

Uptake kinetics were characterized by a time-scale of binding $t_{0.8}$, which was defined as the time after which 80% of the equilibrium saturation of resin with antibody had occurred. For determination of $t_{0.8}$, measured concentrations in the supernatant were interpolated by fitting of double-exponentials (6) and values of $t_{0.8}$ were read out from graphs.

Parameters of Langmuir isotherms and time-scales of binding for resins A and B, including reference resins, are reported in the table below. Highest affinity was observed for rProtein A (4.7 μg ml^{-1}). Identical dissociation constants of resins B and A2P (49 μg ml^{-1}) were lower by one order of magnitude. The dissociation constant of resin A was similar with 71 μg ml^{-1}. Concerning maximum capacity, resins A, B and A2P were similar with values around 65 mg per ml of resin. The maximum capacity of rProtein A was significantly lower with 41 mg per ml of resin. Uptake of antibody was fastest with resins A and B (5.3 min), followed by rProtein A (8.9 min) and A2P (9.2 min).

Ligand	K_d (μg ml^{-1})	q_m (mg per ml resin)	$t_{0.8}$ (min)
A	71	65	5.3
B	49	67	5.3
rProtein A	4.7	41	8.9
A2P	49	68	9.2

Example 5: Dynamic Binding Capacity

Experimental

Dynamic binding capacities were determined by column chromatography with purified antibody. Resins were packed into analytical columns of 25 mm length and 3 mm inner diameter. Columns were fed with 1 mg ml^{-1} Bevacizumab in phosphate-buffered saline (150 mM NaCl, 20 mM phosphate buffer, pH 7.3) at constant flow rate, while the concentration of antibody in effluents was monitored online via absorbance at 280 nm. Antibody was loaded until effluent concentration reached at least 10% of the feed concentration. Dynamic binding capacities were determined at flow rates of 50, 100 and 200 cm h^{-1}, corresponding to 3, 1.5 or 0.75 min empty column residence time. Equilibrium capacities were determined at the lowest flow rate of 50 cm h^{-1} by loading until complete breakthrough of antibody. Bound antibody

was stripped from columns by washing with sodium citrate at pH 3.0 followed by glycine hydrochloride at pH 2.5.

Summary and Results

Dynamic (binding) capacities for purified antibody in buffer as a function of flow rate were determined for resin A (from example 2) and rProtein A Sepharose 4 FF (rProtein A).

'Dynamic capacity' was defined as the amount of antibody that can be injected onto a column at constant flow rate until the effluent concentration reaches 10% of the feed concentration. It was calculated as the time $t_{0.1}$ after which 10% breakthrough occurred, multiplied by the flow rate F and feed concentration c_f.

$$dynamic\ capacity = t_{0.1} \cdot F \cdot c_f$$

Equilibrium capacity is defined as the maximum amount of antibody that binds to a column for a given feed stream concentration. It was calculated by evaluation of the following integral

$$equilibrium\ capacity = \int_0^\infty \left[c_f - c(t)\right] \cdot F dt$$

containing the feed concentration c_f, effluent concentration over time $c(t)$ and flow rate F.

The integral was evaluated numerically. Integration was limited to the time after which complete breakthrough had occurred. Calculations of dynamic capacity and equilibrium capacity were both corrected for the hold-up of columns and the chromatography system. Capacities were normalized by the volume of resin in columns

Dynamic binding capacities for resin A and rProtein A Sepharose 4 FF as a function of flow rate and equilibrium binding capacities for a feed concentration of 1 mg ml^{-1} antibody are given in the table below. Empty column residence times are indicated by values in brackets.

Comparison of dynamic capacities revealed that both resins perform similarly at high flow rates with rProtein A providing slightly higher capacity at 200 cm h^{-1}. At the lowest flow rate, however, resin A provided 27% higher capacity compared to rProtein A. Equilibrium capacity of resin A was even higher by 49%, confirming the results from example 4.

Ligand	Dynamic Capacity (mg per ml of resin)			Equilibrium Capacity (mg per ml of resin)
	50 cm h^{-1} (3 min)	100 cm h^{-1} (1.5 min)	200 cm h^{-1} (0.75 min)	
A	36.2	23.3	10.8	59.8
rProtein A	28.4	20.5	13.3	40.2

Example 6: Alkaline Stability

Ligands from example 1 were tested for their alkaline stability over a period of 8 days. Ligands in solution were either treated with 0.1 M or 0.5 M sodium hydroxide at room temperature. Hydrolysis of ligands was monitored by LC-MS analysis.

At 0.1 M NaOH, half-lifes of ligands varied between 30 and 130 h with the exception of ligands F and C. Ligand C was so stable that no reduction in the concentration occurred, while an extended half-life of 537 h was observed at 0.5 M sodium hydroxide. Regarding ligand F, neither a reduction in the concentration at 0.1 M nor 0.5 M sodium hydroxide was observed.

Example 7: Purification of Antibody from Cell Culture Supernatant

Experimental

Suitability of resins for the purification of antibody from cell culture supernatant was assessed in packed mode by microtiter plate chromatography similar to example 3. Bevacizumab spiked into host cell proteins at a concentration of 0.05 mg ml^{-1} was used as feed. In addition, chromatography runs with host cell proteins and pure antibody alone were conducted. A total of 25 column volumes was injected per run. Columns were equilibrated and washed with PBS before and after injection. Bound protein was eluted with sodium citrate at pH 3.0. Protein concentrations in feeds and column effluents were analyzed by Bradford assay.

Samples from feeds and eluate fractions after chromatography on resin A (see example 2) and rProtein A Sepharose FF were analyzed by reducing SDS-PAGE according to the manufacturer's instructions. Identical volumes of sample were loaded onto gels. A protein marker was included for approximate determination of molecular weights. Resulting gels were stained with colloidal Coomassie according to the manufacturers instructions and digitalized using a standard document scanner.

Summary and Results

Antibody was purified from cell culture supernatant by chromatography on resins A and B from example 2. Chromatography on commercial resins rProtein A Sepharose FF (rProtein A) and Mabsorbent A2P (A2P) was included for comparison. Three chromatography runs under identical conditions were conducted on each resin, either injecting the antibody, host cell proteins or a mixture of the latter.

Operation 'yield' after chromatography of the mixture was calculated from the mass of total protein $m_{mix,e}$ recovered upon elution after injection of the mixture, the mass recovered after injection of host cell proteins alone $m_{HCP,e}$ and the mass of antibody injected $m_{Ab,i}$.

$$yield = \frac{m_{mix,e} - m_{HCP,e}}{m_{Ab,i}}$$

The 'purity' of antibody after chromatography of the mixture was calculated from the mass of total protein recovered upon elution $m_{mix,e}$ after injection of the mixture and masses $m_{Ab,e}$ and $m_{HCP,e}$ recovered after injection of antibody or host cell proteins, respectively.

$$purity = \frac{m_{mix,e} - m_{HCP,e}}{m_{Ab,e} - m_{HCP,e}}$$

Purity and yield obtained after chromatography on resins A and B from example 2 and reference resins are given in the table below.

Except for A2P, yields were close or identical to 100% within experimental precision. Recovery from A2P (12%) was low due to moderate acidic pH of 3.0 upon elution. Purity was highest after chromatography on rProtein A (100%), closely followed by chromatography on resins A (100%) and B (96%). Purity was lowest after chromatography on A2P with only 32%.

Purity of eluates was further checked by SDS-PAGE of respective samples. Results for the injected samples (antibody, mixture and host cell proteins) are given in lanes A, B and C. Results for eluate fractions from chromatography on resin A and rProtein A after injection of the mixture are given in lanes D. Their banding pattern is almost indistinguishable. Eluate fractions from resin A and rProtein A after injection of host cell proteins are given in lanes E. In both cases, the amount of protein was below the limit of detection since host cell proteins did not bind to resins.

Ligand	Yield (%)	Purity (%)
A	100	100
B	97	96
rProtein A	106	100
A2P	12	32

Documents cited in the examples:

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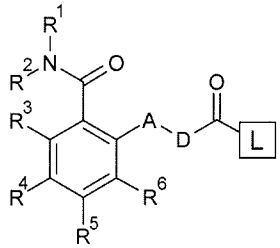
Claims

1. Use, for affinity purification of a protein, of a compound according to the general formula (I),



(I)

wherein

A is selected from the group: –O-;-S-; $>CR^7R^8$; and NR^9;
D is a linear or branched C_1 to C_6 alkyl group, which may comprise a cycloalkyl unit, wherein 1 or more C-atoms which are not connected to each other may be replaced by O or S, and wherein one or more C-atoms in the alkyl group may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I;
L is the linking point via which the compound is attached to a support matrix, L optionally comprising a spacer group –Z-;
R^1, R^2 are identical or different and are independently of each other selected from the group: H; -OH; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit; wherein in the alkyl 1 C-atom may be replaced by –O-, or a terminal C-atom may be replaced by –OH; wherein N may be part of a heterocycle having 3 to 7 members; and wherein one or more C-atoms in the alkyl may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I;
R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; -F; -Cl; -Br; -I; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein in the alkyl 1 C-atom may be replaced by –O-, or a terminal C-atom may be replaced by –OH; and wherein one or more C-atoms in

each R^3, R^4, R^5 and R^6 may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I; and $-NR^{10}R^{11}$;
R^7, R^8 are identical or different and are selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, wherein the alkyl group may optionally be substituted;
R^9 is selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein the alkyl group may optionally be substituted;
R^{10}, R^{11} can be identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein the alkyl group may optionally be substituted.

2. The use according to claim 1, wherein

A is selected from: –O-;-S-; $-CH_2-$; -NH; $N(CH_3)$;
D is a linear or branched C_1 to C_6 alkyl group, wherein one or more C atoms not connected to each other may be replaced by –O-, and wherein one or more C-atoms may be substituted by one or more F atoms;
R^1, R^2 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit; wherein N may be part of a heterocycle having 3 to 5 members; and wherein one or more C-atoms in the alkyl may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I;
R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; -F; Cl; -Br; -I; linear and branched C_1 to C_4 alkyl, which may comprise a cycloalkyl unit, and wherein in the alkyl 1 C-atom may be replaced by –O-, and wherein one or more C-atoms in each R^3, R^4, R^5 and R^6 may be substituted by one or more atoms independently from each other selected from the group F, Cl, Br, I.

3. The use according to claim 1, wherein

A is –O-;
D is a linear or branched C_1 to C_6 alkyl group;
R^1, R^2 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl;
R^3, R^4, R^5 and R^6 are identical or different and are independently of each other selected from the group: H; linear and branched C_1 to C_4 alkyl, wherein in the alkyl 1 C-atom may be replaced by –O-.

4. The use according to any of claims 1 to 3 wherein the protein is an antibody, preferably an IgG type antibody.

5. The use according to claim 4, wherein the IgG type antibody is a monoclonal IgG antibody or a fragment thereof, preferably a Fab fragment or a part of the Fab fragment of an IgG.

6. The method according to claim 4 or 5, wherein the antibody is an antibody of the Bevacizumab type or an antibody of the Ranibizumab type.

7. The method according to claim 6, wherein the antibody comprises in its light chain a sequence as defined by SEQ ID NO: 1 and in its heavy chain a sequence as defined by SEQ ID NO: 2.

8. A method according to any of claims 1 to 7, wherein the compound according to formula (I) is attached to a support matrix via group L, optionally containing a spacer group Z.

9. A matrix for affinity chromatography (affinity matrix) for protein separation, comprising a support matrix comprising a support material and at least one compound as specified in any of claims 1 to 3 attached thereto via group L, optionally containing a spacer group Z.

10. The matrix according to claim 9, wherein the support matrix comprises a material selected from carbohydrates, preferably agarose, cellulose, dextran, starch, alginate and carrageenan; synthetic polymers, preferably polystyrene, styrene-divinylbenzene copolymers, polymethacrylates, polyvinyl alcohol, polyamides and perfluorocarbons; inorganic materials, preferably glass, silica and metal oxides; and composite materials.

11. The matrix according to claim 9 or 10, wherein the ligand is attached via a spacer Z.

12. A method of synthesis of an affinity matrix for protein separation according to claims 9 to 11, wherein a compound according to formula (I) as specified in claims 1 to 3 is attached to a support matrix.

13. A compound of formula (I) as defined in any of claims 1 to 3.

14. A method for affinity purification, preferably affinity chromatography, of a protein, wherein a protein to be purified is contacted with an affintiy matrix as defined in claims 9 to 11.