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(54) Title: NEW CONJUGATED MOLECULES COMPRISING A PEPTIDE DERIVED FROM THE CD4 RECEPTOR COUPLED TO A POLYANIONIC POLYPEPTIDE FOR THE TREATMENT OF AIDS

(57) Abstract: This invention relates to a conjugated molecule comprising a peptide derived from the CD4 receptor coupled to an organic molecule by means of a linker as well as a process for its preparation. Said organic molecule comprises a 5 to 21 amino acid anionic polypeptide. Such a conjugated molecule can be used in antiviral treatment, namely in the treatment of AIDS.



NEW CONJUGATED MOLECULES COMPRISING A PEPTIDE DERIVED FROM
THE CD4 RECEPTOR COUPLED TO A POLYANIONIC POLYPEPTIDE FOR THE
TREATMENT OF AIDS

5 This invention relates to a conjugated molecule comprising a peptide derived from the CD4 receptor and an organic molecule, such as a polyanionic polypeptide. Such a conjugated molecule can be used in antiviral treatment, namely in the treatment of AIDS. This invention further relates to processes for the preparation of the conjugated molecule.

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Triple therapies combining nucleoside (NRTI), non-nucleoside (NNRTI) and/or protease inhibitors (PI) result in a reduction in viral charge beneath levels of detection in a large number of seropositive HIV patients. These therapies target reverse transcription and proteolysis at the same time. Their efficacy has led to a substantial decrease in the number of deaths resulting from HIV infection. Unfortunately, about 80 % of the patients show genotypes with antiviral resistance and, more worryingly, 45.5 % of viral populations are resistant to NRTI/PI combinations while 26 % are resistant to a combination of three anti-HIV classes (Tamalet et al., AIDS. 2003 Nov 7; 17(16):2383-8). This observation is particularly disturbing since the adverse effects of long-term triple therapy treatment (lipoatrophy, lipodystrophy, hypertriglyceridaemia, hypercholesterolaemia, neuropathy, etc.) found in 70 % of patients receiving the treatment result in poor compliance and “sudden” discontinuation of treatment which often leads to resistance. The development of less severe forms of treatment with fewer adverse effects and without cross-resistance is therefore a priority despite the large number of currently available medications on the market. With this in mind, it is essential to target HIV replication steps other than reverse transcription and proteolysis.

Entry of a virus into a cell is a crucial step in the viral infection cycle. This process is divided into two phases: first, the virus interacts with specific host receptors at the cell surface, followed by penetration of the viral genetic material into the target cell. With HIV, the molecular partners involved in the mechanisms of adhesion and entry are well established. The gp120 viral envelope glycoprotein essentially determines the virus/cell interaction complex, by binding to a transmembrane glycoprotein of the host cell, CD4.

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This interaction leads to a conformational change in gp120 which exposes a particular epitope, called CD4-induced (CD4i), thus creating a binding site for chemokine receptors (essentially CCR5 and CXCR4). CCR5 and CXCR4 therefore act as gp120 co-receptors at the cell surface. This second interaction leads to re-organization of the gp120/gp41 protein complex and initiation of cell/virus membrane fusion.

The cellular tropism of the HIV virus is defined by the type of co-receptor used. So-called X4 or « T-tropic » viruses tend to infect more specifically cell lines expressing CXCR4 at their surface, such as the T lymphocytes. So-called R5 or « M-tropic » viruses use co-receptor CCR5 and mainly infect macrophages and monocytes. The presence of R5 or X4 viruses is generally associated with quite distinct stages of AIDS development (asymptomatic phase for R5, appearance of X4 virus often linked to unfavourable evolution outcome of the disease, suggesting that use of co-receptor CXCR4 is an important factor in the pathogenesis of AIDS). As the structural determinants for recognition of CCR5 and CXCR4 are carried by gp120, the R5 and X4 viruses represent two separate targets.

It was shown in international patent application WO 2008/015273 that particular activated peptides derived from the CD4 receptor can react selectively with organic molecules affording chemically defined covalent conjugates. This activation requires the insertion of one and only one residue of the amino acid lysine in a defined position in the sequence of the peptide derived from the CD4 receptor. This insertion allows the coupling of organic compounds through linkers and chemistries after the miniCD4 synthesis and purification.

Numerous potential antiviral derivatives, consisting of conjugated molecules comprising a CD4 peptide specifically coupled to polyanionic heparan sulphate (HS) by means of a linker were disclosed in WO 2009/098147. These conjugates show potent antiviral activity. The use of HS was motivated by the presence of at least two different HS recognition sites in gp120, namely the V3 variable loop and the site induced by CD4 (CD4i): the CD4 moiety of the mCD4-HS is thought to trigger conformational changes in gp120 by direct interaction, thus resulting in exposure of the CD4i epitope, with which the covalently bound HS can then interact, thereby impairing HIV virus infection of X4 and R5 cell lines.

This approach consisting in inhibiting viral attachment to cells is therapeutically advantageous, since it directly targets the virus and not the cells themselves. It is therefore devoid of the cellular effects observed with medication which binds to co-receptors. In addition, in view of the preservation of the sites involved as a function of various viral tropisms, the compounds according to the invention should interact with the gp120 of different viral isolates. While it might be misleading to think that resistance will never arise, it can nevertheless be expected that it should occur at a much lower rate than with other treatments. Indeed, the CD4 site of gp120 has to remain intact in order to continue to bind to CD4, like the basic residues involved in binding to the polyanionic polysaccharide for interaction with the co-receptors. Mutation in any of these two sites should indeed result in a virus with reduced infectivity.

The present Inventors have now identified a new conjugated molecule capable of blocking the entry of the HIV virus into the cells. They show that the presence of heparan sulphate oligosaccharides in the mCD4 conjugated molecule is not absolutely required for inhibiting the virus entry into X4 or R5 cell lines and that very effective antiviral activity can also be obtained by replacing the previously used HS molecule by anionic polypeptides consisting of 5 to 21, advantageously 5 to 17, notably 5, 9, 13, 17 or 21, and preferably 13 amino acids. In a preferred embodiment, some amino acids are negatively charged, notably at least 1, advantageously at least 2, and preferably at least 3. In more preferred embodiments, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 amino acids are negatively charged (depending on the length of the anionic polypeptide). In the most preferred embodiment 9 amino acids are negatively charged when the anionic polypeptide consists of 13 amino acids. More importantly, they have shown that, surprisingly, these new mCD4 conjugated molecules (comprising the said anionic polypeptides) have a better antiviral activity against both R5 or X4 viruses than the conjugated molecules disclosed in the prior art.

According to a first aspect, the invention covers a conjugated molecule comprising a peptide derived from the CD4 receptor, said peptide being coupled to an organic molecule by means of a linker, wherein:

- the said peptide derived from the CD4 receptor comprises the following general sequence (I):

Xaa^f – P1 – Lys – Cys – P2 – Cys – P3 – Cys – Xaa^g – Xaa^h – Xaaⁱ – Xaa^j – Cys – Xaa^k
– Cys – Xaa^l – Xaa^m, (I)

in which:

- P1 represents 3 to 6 amino acid residues,
- 5 - P2 represents 2 to 4 amino acid residues,
- P3 represents 6 to 10 amino acid residues,
- Xaa^f represents N-acetylcysteine (Ac-Cys) or thiopropionic acid (TPA),
- Xaa^g represents Ala or Gln,
- Xaa^h represents Gly or (D)Asp or Ser,
- 10 - Xaaⁱ represents Ser or His or Asn,
- Xaa^j represents biphenylalanine (Bip), phenylalanine or [beta]-naphthylalanine,
- Xaa^k represents Thr or Ala,
- Xaa^l represents Gly, Val or Leu, and
- Xaa^m represents –NH₂ or –OH,
- 15 the amino acid residues in P1, P2 and P3 being natural or non-natural, identical or different, said residues of P1, P2 and P3 being all different from the Lys residue and P1, P2 and P3 having a sequence in common or not, and
 - the said organic molecule comprises
 - an anionic polypeptide consisting of 5 to 21, advantageously 5 to 17, and
 - 20 preferably 13 amino acid residues being natural or non-natural, identical or different, wherein at least 3, and notably 3 to 15, such as 3 to 13 amino acids are negatively charged,
 - a molecular group A-Z, wherein:
 - A comprises a group chosen between the groups of formula –
 - 25 CO(CH₂)₃NH-CO(CH₂)₂–, –CO(CH₂)_pNH-CO-(CH₂)_q–, –CO(CH₂-CH₂)-(O-CH₂-CH₂)_pNH-CO-(CH₂)_q–, –CO(CH₂)_pNH-CO-(CH₂-CH₂-O)_q-(CH₂-CH₂)– and –CO(CH₂-CH₂)-(O-CH₂-CH₂)_pNH-CO-(CH₂-CH₂-O)_q-(CH₂-CH₂)–, wherein p represents an integer comprised between 1 and 10 and q represents an integer comprised between 1 and 10, and
 - 30 wherein the first carbonyl group is coupled to the alpha NH₂ of N-terminal Serine, and advantageously A represents a group of formula –CO(CH₂)₃NH-CO(CH₂)₂– and

- Z represents an halogen atom, a thiol or a maleimide group, the said anionic polypeptide being linked to the linker by the said molecular group of formula A-Z, and the said linker being covalently bound at one of its extremity to the free amino group (-NH₂) of the amino acid residue Lys present in general sequence (I) of the said peptide derived from the CD4 receptor, and being covalently bound at its other extremity to the Z group of the said organic molecule.

Preferably, P3 comprises at least one basic amino acid, said basic amino acid being even more preferably arginine. The presence of basic residues in this portion of the CD4 receptor fragment contributes to its binding to the gp120 protein. The inventors therefore prefer to introduce at least one basic amino acid into P3, preferably arginine. This maintains thus a basic moiety which is not reactive at derivation at pH 7-8 but which has been found to be useful for the binding of miniCD4 peptide to the gp120 protein.

In this application, the terms “miniCD4 peptide”, “CD4 peptide” and “miniCD4” are used interchangeably to designate the peptide derived from the CD4 receptor comprising or consisting of general sequence (I) defined above.

As disclosed in WO 2009/098147, it is required that the miniCD4 peptide of the invention contains one and only one lysine (Lys) amino acid residue, and that said lysine is in the position as defined in general sequence (I). The Cys residues in general sequence (I) allow the formation of three disulphide bridges needed for folding back of miniCD4. Thiopropionic acid (TPA), when it is in the N-terminus position of the peptide of general sequence (I), makes it possible to reduce hindrance in N-ter and overcome the presence of an amine group. Thus, according to a preferred embodiment, Xaa^f represents TPA in general sequence (I).

Bip increases contact with glycoprotein gp120 in the cavity where the Phe 43 of CD4 receptor is lodged. Thus, in a preferred embodiment, Xaaⁱ represents Bip. Nevertheless, it may be advantageous to have Phe as Xaaⁱ in general sequence (I), since a structural analysis suggests that such a miniCD4 may mimic CD4 efficiently (Huang CC et al., Structure. 2005 May;13(5):755-68). Thus according to another preferred embodiment, Xaa^j represents Phe.

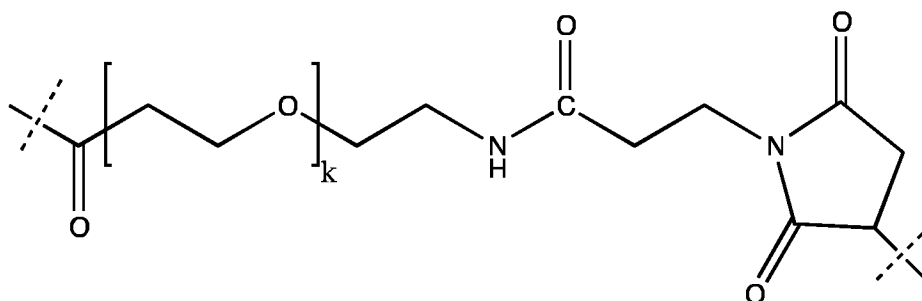
The peptide of general sequence (I) derived from the CD4 receptor forms an alpha helix structure followed by a beta sheet. The amino acids Xaa^g-Xaa^h-Xaaⁱ-Xaa^j-Cys-Xaa^k-Cys-Xaa^l participate in a major way to the binding to gp120. These peptides display an IC₅₀ (affinity for gp120) similar to those of sCD4 (soluble CD4).

- 5 The CD4 peptide of the invention can be prepared by conventional solid phase chemical synthesis techniques, for example according to the Fmoc solid phase peptide synthesis method ("Fmoc solid phase peptide synthesis, a practical approach", edited by W.C. Chan and P.D. White, Oxford University Press, 2000) and/or by genetic recombination.

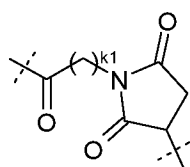
Preferably, the said CD4 peptide is chosen from the group consisting of sequences SEQ
10 ID No. 1 and SEQ ID No. 2. More preferably, the CD4 peptide of the invention has the sequence represented by SEQ ID No. 1.

The term "linker" refers in the present invention to a linker obtained by the coupling of
a bifunctional compound, as defined below, with a peptide derived from the CD4
receptor and the organic molecule. Thus, the length of the linker varies as a function of
15 the bifunctional compounds used.

In particular, the linker will be advantageously chosen among:

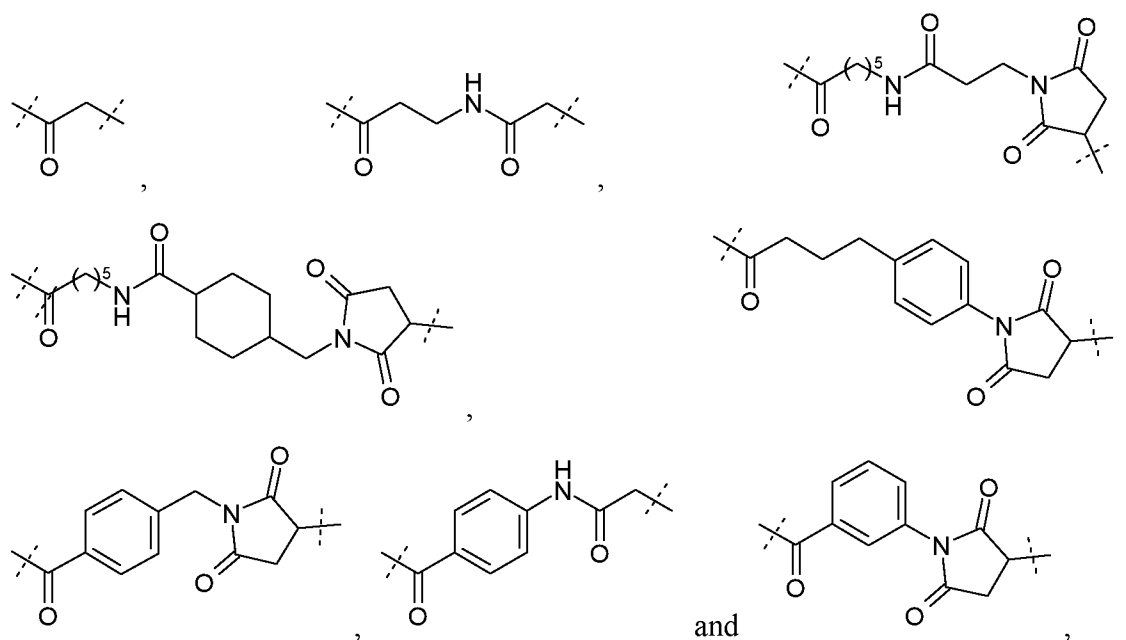


with k representing an integer comprised between 2 and 24, and being advantageously
2, 4, 8 or 12,

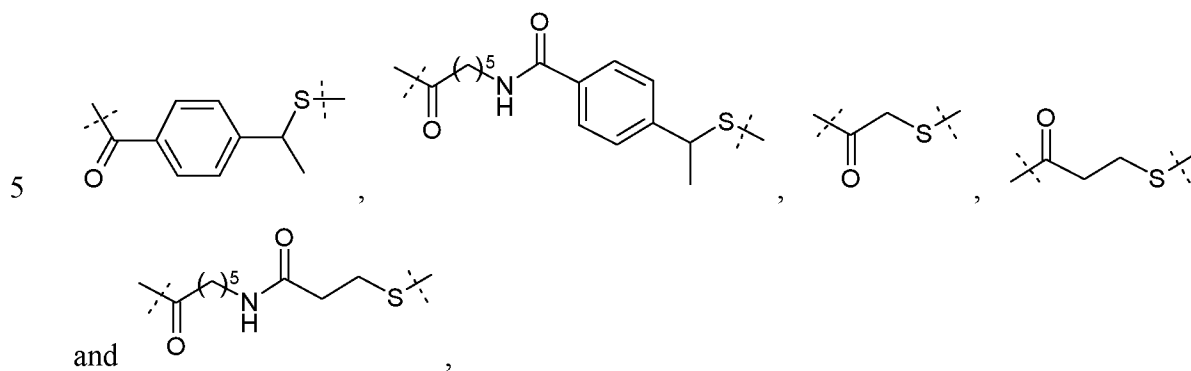


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with k1 representing an integer comprised between 1 and 10, thus
equal to 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, and advantageously equal to 1, 2, 3, 5 or 10,

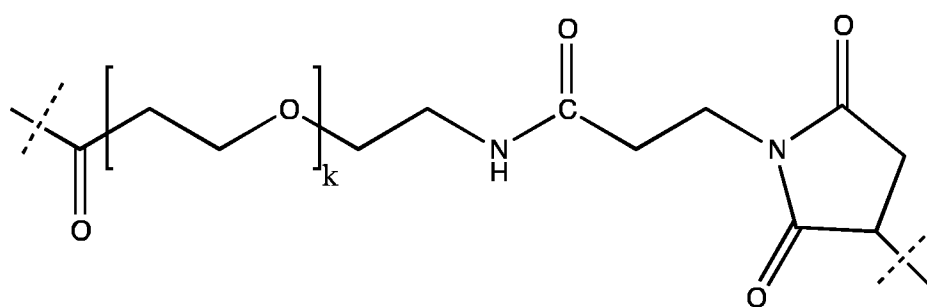


when Z represents a thiol group, and among:

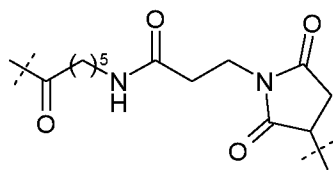


when Z represents a maleimide group or a halogen atom.

In a preferred embodiment, the linker will be chosen among



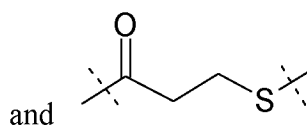
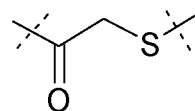
with k representing an integer comprised between 2 and 24, and being advantageously 2, 4, 8 or 12 and



, with k1 representing an integer comprised between 1 and 10, thus equal to 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10, and advantageously equal to 1, 2, 3, 5 or 10,

when Z represents a thiol group,

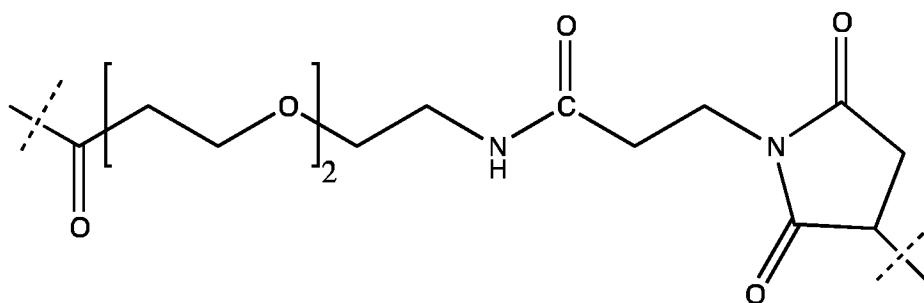
and among:



and , when Z represents a maleimide group or a halogen atom.

10 Such linkers correspond thus to the use of succinimidyl-6-[beta-maleimidopropionamido] hexanoate (SMPH), NHS-PEO_n-maleimide, with n representing an integer comprised between 2 and 24, and being advantageously 2, 4, 8 or 12, SATA (N-succinimidyl-S-acetylthioacetate) and SATP (N-succinimidyl-S-acetylthiopropionate), as bifunctional compound.

15 In another preferred embodiment, the linker is



The conjugated molecule of the invention comprises an anionic polypeptide, said anionic peptide consisting of 13 amino acids, wherein 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 amino acid residues are negatively charged. In a preferred embodiment, the anionic

polypeptide consists of 13 amino acids, wherein 9 amino acid residues are negatively charged.

Advantageously, the anionic peptide of the invention is linked to the A group of the compound of formula I by the N-terminal end. Advantageously, the bond formed
 5 between the said anionic peptide and the A group is a peptidic bond, and thus involves both the amino (NH₂) of the said peptide and the carboxylic group (COOH) on the A group.

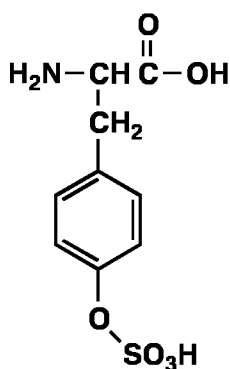
Without being bound by theory, it is thought that the said 13 amino acid-long anionic polypeptides impair the interaction between the CD4i exposed site and CXCR4 (or
 10 CCR5) chemokine receptors, thereby impairing the initiation of the cell / virus membrane fusion.

In the sense of the present invention, "amino acids" means all natural α -amino acid residues (for example alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine
 15 (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophane (Trp), tyrosine (Tyr) and valine (Val)) in D or L form, as well as non-natural amino acids (for example, β -alanine, allylglycine, *tert*-leucine, norleucine (Nle), 3-amino-adipic acid, 2-aminobenzoic acid, 3-aminobenzoic acid, 4-aminobenzoic acid, 2-aminobutanoic acid,
 20 4-amino-1-carboxymethyl piperidine, 1-amino-1-cyclobutanecarboxylic acid, 4-aminocyclohexanecarboxylic acid, 1-amino-1-cyclohexanecarboxylic acid, (1R,2R)-2-aminocyclohexanecarboxylic acid, (1R,2S)-2-aminocyclohexanecarboxylic acid, (1S,2R)-2-aminocyclohexanecarboxylic acid, (1S,2S)-2-aminocyclohexanecarboxylic acid, 3-aminocyclohexanecarboxylic acid, 4-aminocyclohexanecarboxylic acid,
 25 (1R,2R)-2-aminocyclopentanecarboxylic acid, (1R,2S)-2-aminocyclopentanecarboxylic acid, 1-amino-1-cyclopentanecarboxylic acid, 1-amino-1-cyclopropanecarboxylic acid, 4-(2-aminoethoxy)-benzoic acid, 3-aminomethylbenzoic acid, 4-aminomethylbenzoic acid, 2-aminobutanoic acid, 4-aminobutanoic acid, 6-aminohexanoic acid, 1-aminoindane-1-carboxylic acid, 4-aminomethyl-phenylacetic acid, 4-aminophenylacetic acid,
 30 acid, 3-amino-2-naphthoic acid, 4-aminophenylbutanoic acid, 4-amino-5-(3-indolyl)-pentanoic acid, (4R,5S)-4-amino-5-methylheptanoic acid, (R)-4-amino-5-

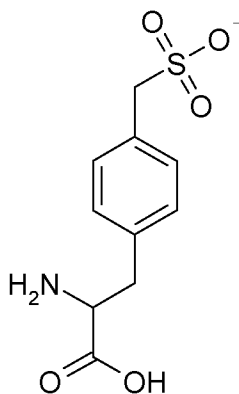
methylhexanoic acid, (R)-4-amino-6-methylthiohexanoic acid, (S)-4-amino-pentanoic acid, (R)-4-amino-5-phenylpentanoic acid, 4-aminophenylpropionic acid, (R)-4-aminopimeric acid, (4R,5R)-4-amino-5-hydroxyhexanoic acid, (R)-4-amino-5-hydroxypentanoic acid, (R)-4-amino-5-(p-hydroxyphenyl)-pentanoic acid, 8-aminooctanoic acid, (2S,4R)-4-amino-pyrrolidine-2-carboxylic acid, (2S,4S)-4-amino-pyrrolidine-2-carboxylic acid, azetidine-2-carboxylic acid, (2S,4R)-4-benzyl-pyrrolidine-2-carboxylic acid, (S)-4,8-diaminooctanoic acid, *tert*-butylglycine, γ -carboxyglutamate, β -cyclohexylalanine, citruline, 2,3-diamino propionic acid, hippuric acid, homocyclohexylalanine, moleucine, homophenylalanine, 4-hydroxyproline, indoline-2-carboxylic acid, isonipecotic acid, sulfotyrosine, aminosuberic acid, p-carboxymethyl phenylalanine, α -methyl-alanine, nicopetic acid, norvaline, octahydroindole-2-carboxylic acid, ornithine, penicillamine, phenylglycine (Phg), 4-phenyl-pyrrolidine-2-carboxylic acid, pipecolic acid, propargylglycine, 3-pyridinylalanine, 4-pyridinylalanine, 1-pyrrolidine-3-carboxylic acid, sarcosine, the statins, tetrahydroisoquinoline-1-carboxylic acid, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, tranexamic acid, 4,4-difluoro proline, 4-fluoro proline, alpha-(3,4-difluorobenzyl)-proline, gamma-(3,4-difluorobenzyl)-proline, alpha-(trifluoromethyl)phenylalanine, hexafluoroleucine, 5,5,5-trifluoroleucine, 6,6,6-trifluoronorleucine, 2-(trifluoromethyl)leucine, 2-(trifluoromethyl)norleucine, 4,4,4-trifluorovaline, 4,4,4,4',4',4'-hexafluorovaline, pentafluorophenylalanine, 2,3-difluorophenylalanine, 2,4-difluorophenylalanine, 2,5-difluorophenylalanine, 2,6-difluorophenylalanine, 3,4-difluorophenylalanine, 3,5-difluorophenylalanine, 3,3-difluoro-3-(4-fluorophenyl)alanine, 2,3-difluorophenylglycine, 2,4-difluorophenylglycine, 2,5-difluorophenylglycine, 3,4-difluorophenylglycine, 4,4-difluoroethylglycine, 4,4,4-trifluoroethylglycine and hexafluoronorleucine). The term also includes natural and non-natural amino acids carrying a conventional amino protecting group (for example, an acetyl group, *tert*-butyloxycarbonyl, benzyloxycarbonyl or 9-fluorenylmethylcarbonyl), as well as natural and non-natural amino acids protected at the carboxylic end (advantageously by a C₁-C₁₈ alkyl group, an ester, a phenyl amide or benzyl amide or an amide, which, respectively, give a carboxylic end of the following formula: -CO(C₁-C₁₈ alkyl), -COO(C₁-C₁₈ alkyl), -CONHphenyl, CONHbenzyl, or CONH₂).

As used herein, the term “anionic” designates a negatively charged molecule, which for example migrates to an anode in electrolysis. Also, a “negatively charged” amino acid designates an amino acid which carries a side-chain charge which is negative at a pH of 7.4. Negatively charged amino acid residues according to the invention thus include, for example, aspartic acid, tyrosine, sulfotyrosine, tyrosine sulfonate, aminosuberic acid, p-carboxymethyl phenylalanine, and glutamic acid residues.

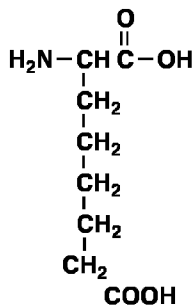
As used herein, sulfotyrosine (or 2-amino-3-(4-(sulfooxy)phenyl) propanoic acid) is a non-natural amino acid having the following formula:



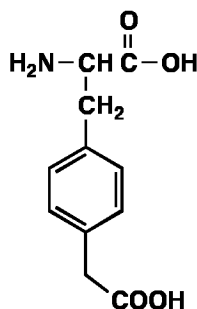
10 As used herein, the term “tyrosine sulfonate” (or “2-amino-3-(4-(sulfomethyl)phenyl) propanoic acid”) refers to a non natural amino acid having the following formula:



According to the present invention, aminosuberic acid is a non-natural amino acid having the following formula:



As used herein, p-carboxymethyl phenylalanine refers to a non-natural amino acid having the following formula:



- 5 In one embodiment of the invention, the said anionic polypeptide consists of 13 identical negatively charged amino acids. It can be, for example, the polypeptide consisting of 13 aspartic acid residues (SEQ ID NO: 9), 13 sulfotyrosine residues (SEQ ID NO: 10), 13 tyrosine sulfonate residues (SEQ ID NO: 11), 13 aminosuberic acid residues (SEQ ID NO: 12), 13 p-carboxymethyl phenylalanine residues (SEQ ID NO: 13), and 13 glutamic acid residues (SEQ ID NO: 3). In a preferred embodiment, said identical amino acid is glutamic acid E and the anionic polypeptide of the conjugated molecule of the invention is SEQ ID NO: 3.

- In another embodiment of the invention, the anionic peptide of the invention comprises negatively charged and uncharged amino acids. Preferably, the said anionic peptide comprises at least one negatively charged amino acid and at least another amino acid. Preferably, the said other amino acid is an amino acid carrying a polar uncharged side chain. Such amino acids include e.g. serine, threonine, asparagine, and glutamine. In a further preferred embodiment, the said other amino acid is serine or threonine. In a

still further preferred embodiment, the said other amino acid is serine. In another preferred embodiment, the negatively charged amino acid is aspartic acid.

In another further preferred embodiment, the said anionic polypeptide of the invention comprises at least two different negatively charged amino acids, in addition to the at least one other amino acid. In this case, said anionic polypeptide has for example
 5 the sequence $S-(X-D-X-S)_n$, such as $S-X-D-X-S-X-D-X-S-X-D-X-S$, where n is an integer comprised between 1 and 5, and preferably is 3, S represents serine, D represents aspartic acid, and X is selected from the group consisting of: tyrosine, sulfotyrosine, tyrosine sulfonate, aminosuberic acid, and p-carboxymethyl
 10 phenylalanine (SEQ ID NO: 19) and where the various X groups can be identical or different, preferably identical.

In this embodiment, said anionic polypeptide can have in particular any of the following sequences:

- $S-(Y_{SO_3}-D-Y_{SO_3}-S)_n$, such as $S-Y_{SO_3}-D-Y_{SO_3}-S-Y_{SO_3}-D-Y_{SO_3}-S-Y_{SO_3}-D-$
 15 $Y_{SO_3}-S$ (SEQ ID NO: 4), where Y_{SO_3} is sulfotyrosine;
- $S-(Y_{SN}-D-Y_{SN}-S)_n$, such as $S-Y_{SN}-D-Y_{SN}-S-Y_{SN}-D-Y_{SN}-S-Y_{SN}-D-Y_{SN}-S$
 (SEQ ID NO: 5), where Y_{SO_3} is tyrosine sulfonate;
- $S-(pF-D-pF-S)_n$, such as $S-pF-D-pF-S-pF-D-pF-S-pF-D-pF-S$ (SEQ ID
 NO: 6), where pF is p-carboxymethyl phenylalanine;
- 20 - $S-(Asu-D-Asu-S)_n$, such as $S-Asu-D-Asu-S-Asu-D-Asu-F-Asu-F-D-$
 $Asu-S$ (SEQ ID NO: 7), where Asu is aminosuberic acid; and
- $S-(Y-D-Y-S)_n$, such as $S-Y-D-Y-S-Y-D-Y-S-Y-D-Y-S$ (SEQ ID NO:
 8), where Y is tyrosine.

The anionic polypeptide can have also the following sequences:

- 25 - $S-Y_{SO_3}-D-Y_{SO_3}-S-Y_{SO_3}-D-Y-S-Y-D-Y-S$ (SEQ ID NO: 20), or
- $S-Y-D-Y-S-Y-D-Y_{SO_3}-S-Y_{SO_3}-D-Y_{SO_3}-S$ (SEQ ID NO: 21).

Most preferably according to the invention, X represents sulfotyrosine. Therefore, in the most preferred embodiment, the anionic polypeptide of the invention has the sequence: $S-(Y_{SO_3}-D-Y_{SO_3}-S)_n$, and in particular $S-Y_{SO_3}-D-Y_{SO_3}-S-Y_{SO_3}-D-Y_{SO_3}-$
 30 $S-Y_{SO_3}-D-Y_{SO_3}-S$ (SEQ ID NO: 4).

In a particular embodiment, the conjugated molecule of the invention comprises a peptide derived from the CD4 receptor, said peptide being coupled to an organic molecule by means of a linker, wherein:

- the peptide derived from the CD4 receptor is chosen from the group consisting of sequences SEQ ID NO: 1 and SEQ ID NO: 2,
- the linker is $\text{CO}-(\text{CH}_2\text{O})_2\text{CH}_2\text{NHCO}(\text{CH}_2)_2$ -pyrrolidiny-2,5-dione,
- the organic molecule comprises an anionic polypeptide having a sequence selected from the group consisting of: $\text{S}-(\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S})_n$, $\text{S}-(\text{Y}_{\text{SN}}-\text{D}-\text{Y}_{\text{SN}}-\text{S})_n$, $\text{S}-(\text{pF}-\text{D}-\text{pF}-\text{S})_n$, $\text{S}-(\text{Asu}-\text{D}-\text{Asu}-\text{S})_n$, and $\text{S}-(\text{Y}-\text{D}-\text{Y}-\text{S})_n$; and in particular selected from the group consisting of: $\text{S}-\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S}-\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S}-\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S}$ (SEQ ID NO 4), $\text{S}-\text{Y}_{\text{SN}}-\text{D}-\text{Y}_{\text{SN}}-\text{S}-\text{Y}_{\text{SN}}-\text{D}-\text{Y}_{\text{SN}}-\text{S}-\text{Y}_{\text{SN}}-\text{D}-\text{Y}_{\text{SN}}-\text{S}$ (SEQ ID NO: 5), $\text{S}-\text{pF}-\text{D}-\text{pF}-\text{S}-\text{pF}-\text{D}-\text{pF}-\text{S}-\text{pF}-\text{D}-\text{pF}-\text{S}$ (SEQ ID NO: 6), $\text{S}-\text{Asu}-\text{D}-\text{Asu}-\text{S}-\text{Asu}-\text{D}-\text{Asu}-\text{S}-\text{Asu}-\text{D}-\text{Asu}-\text{S}$ (SEQ ID NO: 7), $\text{S}-\text{Y}-\text{D}-\text{Y}-\text{S}-\text{Y}-\text{D}-\text{Y}-\text{S}-\text{Y}-\text{D}-\text{Y}-\text{S}$ (SEQ ID NO: 8), said sequence being linked to the linker by a molecular group of formula A-Z, wherein A is $-\text{NHCO}(\text{CH}_2)_3\text{NH}-\text{CO}(\text{CH}_2)_2-$ and Z is a thiol group.

In a preferred embodiment, the conjugated molecule of the invention comprises the peptide derived from the CD4 receptor chosen from the group consisting of sequences SEQ ID No.1 and SEQ ID No.2, said peptide being coupled to an organic molecule by means of the linker $\text{CO}-(\text{CH}_2\text{O})_2\text{CH}_2\text{NHCO}(\text{CH}_2)_2$ pyrrolidiny-2,5-dione, and an anionic polypeptide having the sequence $\text{S}-(\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S})_n$, and in particular $\text{S}-\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S}-\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S}-\text{Y}_{\text{SO}_3}-\text{D}-\text{Y}_{\text{SO}_3}-\text{S}$ (SEQ ID NO 4), said sequence being linked to the linker by a molecular group of formula A-Z, wherein A is $-\text{NHCO}(\text{CH}_2)_3\text{NH}-\text{CO}(\text{CH}_2)_2-$ and Z is a thiol group.

The conjugates of the invention are capable of inhibiting HIV entry into the cell, by blocking the attachment of the virus on the cell membrane and thereby the virus entry. The conjugates of the invention can therefore be used in therapy. More preferably, the conjugates of the invention can be used for treating viral infections. Even more preferably, the said conjugates can be used for treating AIDS. Therefore, according to a second aspect, the invention covers a conjugated molecule as defined above for its use as medicament. The invention also relates to the conjugates as defined above for

treating viral infections, and, in particular, for the treatment of AIDS. The use of a conjugated molecule as defined above for the manufacture of a medicament for an antiviral treatment, and in particular for the treatment of AIDS, is also an object of the present invention.

- 5 According to a third aspect, the invention covers a pharmaceutical composition comprising a conjugated molecule as defined above and a pharmaceutically acceptable vehicle. This composition can be used as a medicament, preferably for the treatment of viral infections, and, more preferably, for the treatment of AIDS.

10 In the pharmaceutical compositions of the present invention for oral, intranasal, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active ingredient can be administered in unit forms for administration, mixed with conventional pharmaceutical carriers, to animals or to humans. Suitable unit forms for administration comprise the forms for oral administration, such as tablets, gelatin capsules, powders, granules and oral solutions or
15 suspensions, the forms for sublingual and buccal administration, the forms for subcutaneous, intramuscular, intravenous, intranasal or intraocular administration and the forms for rectal administration.

The pharmaceutical composition of the invention may contain, in addition to the carrier and conjugate of the invention, various diluents, fillers, salts, buffers, stabilizers,
20 solubilizers, and other materials well known in the art.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, buffers, salt solutions, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. The type of carrier can be selected based upon the intended route of
25 administration. In various embodiments, the carrier is suitable for intravenous, intraperitoneal, subcutaneous, intramuscular, topical, transdermal or oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of media and agents for pharmaceutically active substances is well
30 known in the art. A typical pharmaceutical composition for intravenous infusion could

be made up to contain 250 ml of sterile Ringer's solution, and 100 mg of the combination. Actual methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Science, 17th ed., Mack Publishing Company, Easton, Pa. (1985), and the 18th and 19th editions thereof, which are incorporated herein
5 by reference.

The conjugated molecule in the composition preferably is formulated in an effective amount. An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired result, such as induction of apoptosis in tumor
10 cells. A "therapeutically effective amount" means an amount sufficient to influence the therapeutic course of a particular disease state. A therapeutically effective amount is also one in which any toxic or detrimental effects of the agent are outweighed by the therapeutically beneficial effects.

Dosage regimens may be adjusted to provide the optimum response. For example, a
15 single bolus may be administered, several divided doses may be administered over time, or the dose may be proportionally reduced or increased. The compositions of the invention can be administered to a subject to affect viral infection in a subject. As used herein, the term "subject" is intended to include living organisms which is susceptible to viral infection, and specifically includes mammals, such as rabbits, dogs, cats, mice,
20 rats, monkey transgenic species thereof, and preferably humans.

For therapeutic applications, the conjugated molecule of the invention is administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form such as those discussed above, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by
25 intramuscular, intraperitoneal, intracerebrospinal, subcutaneous, intraarticular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

The invention also relates to an antiviral treatment method, preferably an anti-AIDS treatment method, comprising the administration to a patient in need thereof of a conjugated molecule according to the invention or the pharmaceutical composition
30 containing it.

In a fourth aspect, the invention provides a process for the preparation of a conjugated molecule as defined above, characterized in that the process comprises the following steps:

- 5 a. contacting the miniCD4 peptide of general sequence (I) as defined above with a bifunctional compound carrying two active groups, so that one of the two active groups forms a covalent bond with the free amino group ($-\text{NH}_2$) of the residue of the amino acid Lys present in general sequence (I), in order to obtain an activated peptide carrying the second active group of the bifunctional group, and
- 10 b. contacting the activated peptide obtained at step (a) with an organic molecule carrying a functional group as defined above carrying a thiol group for which the thiol group (SH) has been protected by a protective thiol group, so that the active group of the activated peptide forms a covalent bond with the functional group, protected or not, of the organic molecule, in order to obtain the conjugated molecule.

15

The compound obtained at step (a) will be called indifferently, in the present application, “activated peptide”, “activated miniCD4”, “activated CD4 peptide” or “activated miniCD4 peptide”.

20 The functional group according to the invention refers to a halogen atom, a maleimide, a thiol or a protective thiol group.

The term “protective thiol group”, as used in the present invention refers to a sulfur atom substituted by a S-protecting group in order to protect a thiol group against undesirable reactions during synthetic procedures. Commonly used S-protecting groups are disclosed in Greene, “Protective Groups In Organic Synthesis” (John Wiley & Sons, 25 New York (1981)). S-protecting groups comprise benzyl ethers, substituted or not, such as *p*-methoxybenzyl or *p*-nitrobenzyl, trityl ethers, thioethers, thioacetate or thioacetal. Advantageously, the protected thiol group is a thioacetyl.

When the organic compound carries a protective thiol group, said protective group will be deprotected before or during step (b), in order to recover a free thiol group and to 30 allow the coupling of this thiol with the active group of the activated peptide.

The characteristics of the peptide derived from CD4 receptor are the same as defined above. In particular, P3 comprises preferably at least one basic amino acid, said basic amino acid being even more preferably arginine. According to a preferred embodiment, Xaa^f represents TPA in general sequence (I). According to another preferred embodiment, Xaa^j represents Phe. Preferably, the sequence of the peptide derived from the CD4 receptor of general sequence (I) is chosen from the group consisting of sequences SEQ ID NO: 1 and SEQ ID NO: 2, advantageously SEQ ID NO: 1.

The term “bifunctional compound” in this patent application refers to any compound incorporating two active groups wherein one of the two active groups is capable of forming a covalent bond with the free amino group (–NH₂) of the residue of the amino acid Lys present in general sequence (I) and the other active group is capable of forming a covalent bond with the organic molecule.

The person skilled in the art knows well the bifunctional compounds which can be used within the framework of this invention. Namely, the bifunctional compound according to this invention can be chosen from the following non-limiting list: NHS-PEO_n-Maleimide where n is comprised between 2 and 24, advantageously n=2, 4, 8 or 12, Sulfo-KMUS (*N*-[*k*-maleimidoundecanoyloxy] sulfosuccinimide ester), LC-SMCC (succinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxy-[6-amidocaproate]), KMUA (*N*-*k*-maleimidoundecanoic acid), SMPB(succinimidyl 4-[*p*-maleimidophenyl] butyrate), Sulfo-SMPB (sulfosuccinimidyl 4-[*p*-maleimidophenyl]butyrate), Sulfo-SIAB (*N*-sulfosuccinimidyl[4-iodoacetyl]aminobenzoate), SIAB (*N*-succinimidyl [4-iodoacetyl]aminobenzoate), Sulfo-EMCS ([*N*-*e*-maleimidocaproyloxy] sulfosuccinimide ester), EMCA (*N*-*e*-maleimidocaproic acid), EMCS ([*N*-*e*-maleimidocaproyloxy] succinimide ester), SMCC (succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate), Sulfo-SMCC (sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate), MBS (*m*-maleimidobenzoyl-*N*-hydroxy succinimide ester), Sulfo-MBS(*m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester), GMBS (*N*-[*g*-maleimidobutyryloxy]succinimide ester), Sulfo-GMBS (*N*-[*g*-maleimidobutyryloxy] sulfosuccinimide ester), SPDP (*N*-succinimidyl-3-(2-pyridyldithio)propionate), SBAP (succinimidyl 3-[bromoacetamido]propionate), BMPS (*N*-[[*β*]-maleimidopropyl]succinimide ester), BMPA (*N*-[[*β*]-

maleimidopropionic acid), AMAS N-(α -maleimidoacetoxy) succinimide ester), SIA (*N*-succinimidyl iodoacetate), SMPH(succinimidyl-6-[β -maleimido propionamido]hexanoate), SATA (*N*-succinimidyl-S-acetylthioacetate) and SATP (*N*-succinimidyl-S-acetylthiopropionate).

- 5 According to the invention, NHS-PEO_n-Maleimide wherein n=2 is also called succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol]ester,
 NHS-PEO_n-Maleimide wherein n=4 is also called succinimidyl-[(*N*-maleimidopropionamido)-tetraethyleneglycol]ester,
 NHS-PEO_n-Maleimide wherein n=8 is also called succinimidyl-[(*N*-
 10 maleimidopropionamido)-octaethyleneglycol]ester,
 NHS-PEO_n-Maleimide wherein n=12 is also called succinimidyl-[(*N*-maleimidopropionamido)-dodecaethyleneglycol]ester.

- The active group capable of forming a covalent bond with the free amine group ($-\text{NH}_2$)
 15 of the residue of amino acid Lys present in general sequence (I) can be any active ester group.

- Preferably, the active group capable of forming a covalent bond with the free amine group ($-\text{NH}_2$) of the residue of amino acid Lys present in general sequence (I) is the active group *N*-hydroxysuccinimide ester (NHS) or *N*-hydroxy-4-sulfo-succinimide
 20 ester, and advantageously is the NHS active group. Even more preferably, the two active groups of the bifunctional compound are different (heterobifunctional group) and one of the two groups is the NHS active group or a *N*-hydroxy-4-sulfo-succinimide ester, and advantageously is the NHS active group.

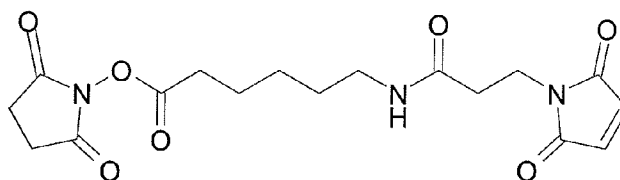
- Advantageously, the active group of the bifunctional compound, capable of forming a
 25 covalent group with the functional group of the organic molecule, is a halogen atom or a maleimide group when the functional group of the organic molecule is a thiol or a protective thiol group and is a thiol or a protective thiol group, as defined above, when the functional group of the organic molecule is a halogen atom or a maleimide group.

- According to a preferred embodiment, when the functional group of the organic
 30 molecule is a thiol group or a protective thiol group, the bifunctional compound is

chosen from the group consisting of succinimidyl-6-[beta-maleimidopropionamido] hexanoate (SMPH) and NHS-PEO_n-maleimide, n being comprised between 2 and 24, and advantageously is 2, 4, 8 or 12.

According to a particularly preferred embodiment, the bifunctional compound is SMPH.

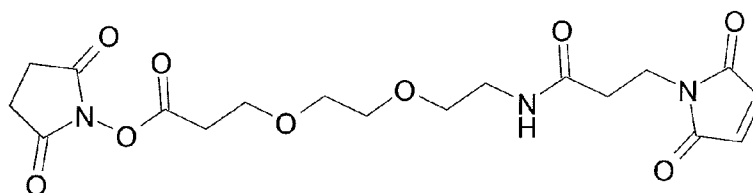
- 5 The molecular structure of SMPH is as follows:



SMPH

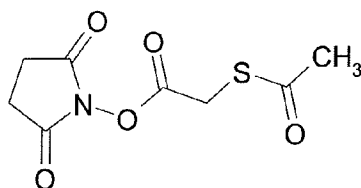
- According to yet another particularly preferred embodiment, the bifunctional compound is succinimidyl-[(N-maleimidopropionamido)-diethyleneglycol] ester, also called NHS-PEO₂-maleimide, succinimidyl-[(N-maleimidopropionamido)-tetraethyleneglycol] ester, also called NHS-PEO₄-maleimide, succinimidyl-[(N-maleimidopropionamido)-octaethyleneglycol] ester, also called NHS-PEO₈-maleimide, succinimidyl-[(N-maleimidopropionamido)-dodecaethyleneglycol] ester, also called NHS-PEO₁₂-maleimide, still more preferably the bifunctional compound is NHS-PEO₂-maleimide.
- 10

- 15 The molecular structure of NHS-PEO₂-maleimide is as follows:

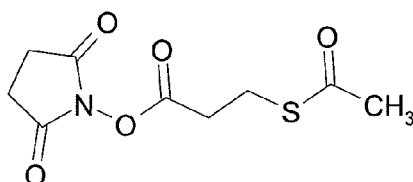


- According to another particularly preferred embodiment, when the functional group of the organic molecule is a halogen atom or a maleimide group, the bifunctional compound is chosen from the group consisting of N-succinimidyl-S-acetylthioacetate (SATA) and N-succinimidyl-S-acetylthiopropionate (SATP).
- 20

The molecular structure of SATA is as follows:



The molecular structure of SATP is as follows:



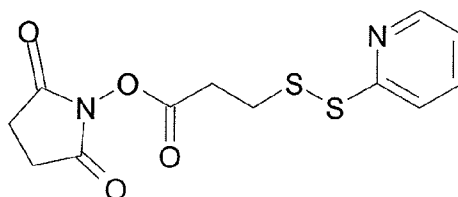
- 5 The bifunctional compounds can be obtained from PIERCE (Rockford, IL).

Preferably again, the process according to the invention includes a preliminary step for the preparation of the peptide derived from the CD4 receptor of general sequence (I), when Xaa^f represents TPA, said step consisting of contacting the peptide derived from the CD4 receptor of the following general sequence (III):

- 10 P1-Lys-Cys-P2-Cys-P3-Cys-Xaa^g-Xaa^h-Xaaⁱ-Xaa^j-Cys-Xaa^k-Cys-Xaa^l-Xaa^m, (III)

where P1 to P3 and Xaa^g to Xaa^m are as defined in general sequence (I), with N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) in order to incorporate TPA at the N-terminus of said peptide derived from the CD4 receptor of general sequence (III).

- 15 The molecular structure of SPDP is as follows:

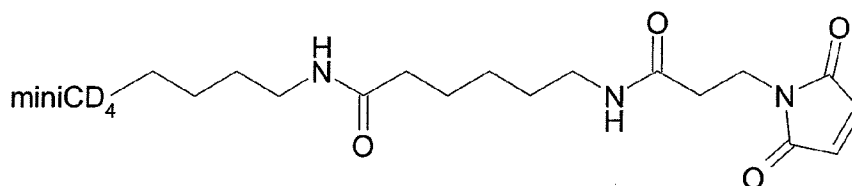


Moreover, as examples of active groups capable of coupling to an organic molecule by means of a covalent bond, the following groups can be cited: maleimide or bromoacetyl, S-S-pyridinium or thioacetyl.

When miniCD4 is activated by a protected thiol group (e.g. thioacetyl), it is possible to carry out coupling to an organic molecule which carries a maleimide group for example. This is possible when the functionalisation of the polyanionic polypeptide by a thiol group or protected thiol group, such as thioacetyl, poses a problem. This then called
 5 “reverse coupling”.

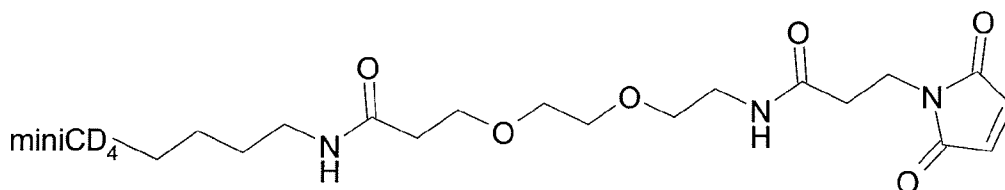
Preferably, the active group is the maleimide group.

The molecular structure of the activated peptide according to the invention whose active group is maleimide is the following when SMPH is the bifunctional compound used:



10 In this application, the term “SMPH activated miniCD4 peptide” refers to an activated peptide according to the invention whose amino acid Lys residue is covalently bound, advantageously by an amine bond, to a maleimide active group via a linker derived from SMPH.

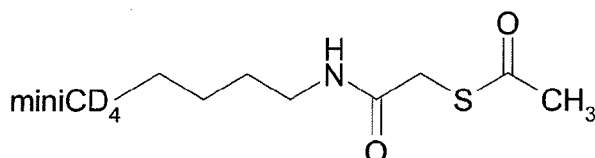
According to another advantageous embodiment, the molecular structure of the
 15 activated peptide according to the invention whose active group is the maleimide group is the following when NHS-PEO₂-maleimide is the bifunctional compound used:



In this application, the term “maleimide activated miniCD4 peptide via a PEO₂ linker” refers to an activated peptide according to the invention whose amino acid Lys residue
 20 is covalently bound, advantageously by an amine bond, to a maleimide active group via a PEO₂ linker.

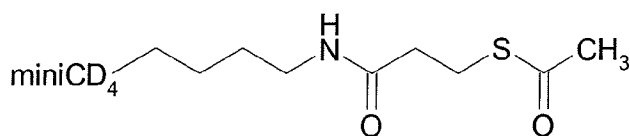
According to another preference, the active group is the thioacetyl group.

For example, the molecular structure of the activated peptide according to this invention whose active group is the thioacetyl group is the following when SATA is the bifunctional compound used:



5

Similarly, the molecular structure of the activated peptide according to this invention whose active group is the thioacetyl group is the following when SATP is the bifunctional compound used:

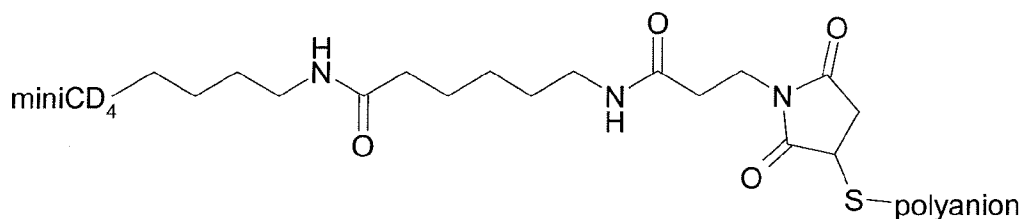


- 10 The thioacetyl group is a protected form of the thiol group. To deprotect the thiol group, we use hydroxylamine for example. This step is carried out simultaneously to coupling to the maleimide group carried by the organic molecule.

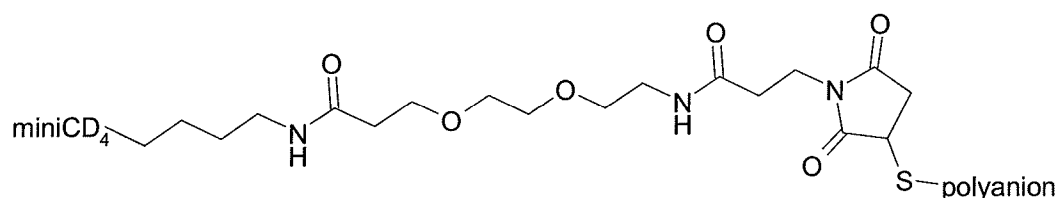
- In this application, the terms “SATA activated miniCD4 peptide” and “SATP activated miniCD4 peptide” refer to an activated peptide according to the invention whose amino acid Lys residue is covalently bound, advantageously by an amine bond, to a protected thiol group (e.g. thioacetyl) via a linker derived from SATA or SATP.
- 15

Thus, according to a particular embodiment, the active group of the activated peptide is the maleimide group and the organic molecule carries a thiol or thioacetyl group.

- The molecular structure of a conjugated molecule according to the invention, including a peptide derived from the CD4 receptor of general sequence (I) coupled to a modified anionic polypeptide (polyanion) carrying a thiol or protected thiol group, such as a thioacetyl group, is as follows when SMPH was used as bifunctional compound for the coupling:
- 20



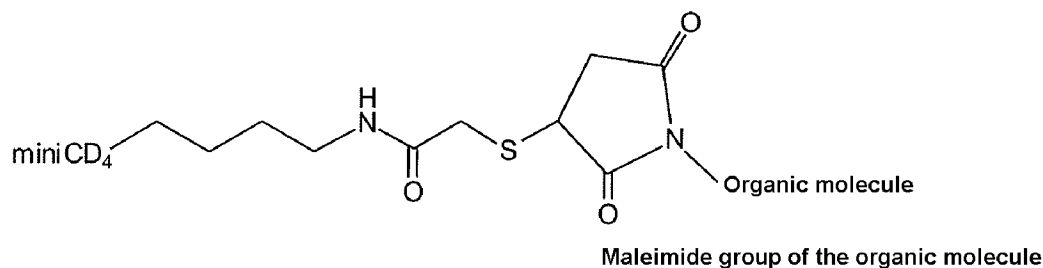
It can also be the following conjugated molecule when NHS-PEO₂-maleimide is the bifunctional compound used:



- 5 According to another embodiment, the conjugated molecule according to the invention comprises a peptide derived from the CD4 receptor comprising or consisting of general sequence (I), preferably sequence SEQ ID NO: 1, and an organic molecule carrying a maleimide or halogen group.

According to another particular embodiment, the active group of the activated peptide is
10 the thioacetyl group and the organic molecule carries a maleimide or halogen group.

For example, the molecular structure of such a conjugated molecule including a peptide derived from the CD4 receptor of general sequence (I) coupled to an organic molecule carrying a maleimide group is as follows when SATA is used for the coupling:



- 15 According to the invention, the anionic polypeptide (the organic molecule of the present invention) can be prepared by any convenient synthetic method known in the art. The

operating conditions for the processes according to the invention for preparation of the activated peptide and conjugated molecule are well known to the person skilled in the art as illustrated in the following examples.

5 The examples and figures below illustrate the invention but do not limit its scope in any way.

Figure legends

Figure 1 discloses the structure of the peptides of the invention.

Figure 2 represents the HPLC profile of the anionic polypeptide comprised in the conjugated molecules of the invention (from up to down: P3Y_{SO3}, P3Y, P3pF, P3Asu,
10 and E13).

Figure 3 represents the HPLC profile of the anionic polypeptide of the invention coupled to the S-acetylpropionate (SATP) group.

Figure 4 represents the HPLC profile of the conjugated peptides of the invention.

Figure 5 represents the results of the binding assays performed by Surface Plasmon
15 Resonance (SPR - Biacore) techniques, and showing the interaction between mAb17b (15 µg/ml) and surfaces coated with gp120 from X4 (MN) or R5 (YU2) virus, pretreated with 100nM of: mCD4 alone(A), mCD4-P3Y(B) or mCD4-P3YSO3(C). When surfaces are coated with mAb17b (D and G), the interaction of gp120 from X4 (MN) or gp120 from R5 (YU2) depends on the presence of mCD4 (which induce the
20 exposition of mAb17b epitope), but is fully inhibited when mCD4-P3Y or mCD4-P3YSO3 were used instead of mCD4. When surfaces are coated with sCD4 (E and H) the interactions are partially inhibited by mCD4 and fully inhibited by mCD4-P3Y or mCD4-P3YSO3. When surfaces are coated with heparin (F), the interaction is not
25 inhibited by mCD4 and partially inhibited by mCD4-P3Y and mCD4-P3YSO3, the later being more active.

Experimental examples

Example 1: Synthesis of the conjugated molecules of the invention

1.1. Synthesis of the peptides of formula H-γ-Abu-SXDXSXDXSXDXS-OH with X= YSO₃ (P3YSO₃), Y (P3Y), Asu (P3Asu), or pF (P3pF); H-γ-Abu-

SY_{SO3}DY_{SO3}SY_{SO3}DYSYDYS-OH (Nter3sulfates); H-γ-Abu-SYDYSDY_{SO3}SY_{SO3}DY_{SO3}S-OH (Cter3sulfates); and H-γ-Abu-(Glu)₁₃-OH (E13); where γ-Abu: NH₂-(CH₂)₃-CO

The following example describes the synthesis of modified peptides of formula H-γ-
 5 Abu-SXDXSXDXSXDXS-OH with X= YSO₃ (P3YSO₃), Y (P3Y), Asu (P3Asu), or
 pF (P3pF); H-γ-Abu-SY_{SO3}DY_{SO3}SY_{SO3}DYSYDYS-OH (Nter3sulfates); H-γ-Abu-
 SYDYSDY_{SO3}SY_{SO3}DY_{SO3}S-OH (Cter3sulfates); H-γ-Abu-(Glu)₁₃-OH (E13); where
 γ-Abu: NH₂-(CH₂)₃-CO. In this formula, the anionic peptide of the invention (except
 peptide E13) corresponds to SXDXSXDXSXDXS-OH (SEQ ID NO: 19), with X as
 10 above (identical or different).

The resulting modified peptides are:

P3YSO₃ (SEQ ID NO: 14): NH₂-(CH₂)₃-CO-S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S

P3Y (SEQ ID NO: 15): NH₂-(CH₂)₃-CO-S-Y-D-Y-S-Y-D-Y-S-Y-D-Y-S

15 P3pF (SEQ ID NO: 16): NH₂-(CH₂)₃-CO-S-pF-D-pF-S-pF-D-pF-S-pF-D-pF-S

P3Asu (SEQ ID NO: 17): NH₂-(CH₂)₃-CO-S-Asu-D-Asu-S-Asu-D-Asu-F-Asu-F-D-Asu-S

E13 (SEQ ID NO: 18): NH₂-(CH₂)₃-CO-EEEEEEEEEEEEEE

Nter3sulfates (SEQ ID NO: 20): NH₂-(CH₂)₃-CO-S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y-S-Y-D-
 20 Y-S

Cter3sulfates (SEQ ID NO: 21): NH₂-(CH₂)₃-CO-S-Y-D-Y-S-Y-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S

Reagents

Resins were purchased from RAPP Polymere GmbH. Fmoc AAs, HATU, NMP, DMF,
 25 TFA were from Applied Biosystems and Piperidin from Sigma. Fmoc-Tyr
 (SO₃.NnBu₄)-OH and Fmoc-γ-Aminobutyric-OH (γ-Abu) were from Novabiochem,
 (S)-Fmoc-2-amino-octanedioic acid-8-ter-butyl ester (Asu) from Polypeptides and Fmoc
 -L-4 (O-tButylcarboxymethyl)-Phe-OH (pF) from Anaspec. HPLC grade triethylamine
 acetate buffer was from GlenResearch. N-succinimidyl-S-acetylthiopropionate (SATP)
 30 was from Pierce.

General protocol for peptide synthesis

Peptides were synthesized on H-Ser(tBu)-2-ClTrt-PS-resin (100 μ moles; 0.78 mmole/g) on an Applied 433 peptide synthesizer. E13 peptide was synthesized on Fmoc-Glu(tBu)-PHB-PS-resin (100 μ moles; 0.61 mmole/g). Chain elongation was performed using 10 equivalents of Fmoc amino acids and HATU/DIEA activation. Peptides were released from the resin by TFA/TIS/H₂O (95/2,5/2,5) treatment for 1 h 30 at room temperature, except for sulphated peptides which was done at 4°C (ice bath). The crude peptides were isolated by cold diethyl ether precipitation, solubilised in water by adding 3 % NH₄OH, except the sulphated peptide that was rapidly dissolved in 100 mM ammonium hydrogen carbonate buffer. After lyophilisation, the crude peptides were purified by C18 RP-HPLC using 50 mM aqueous NEt₃-AcOH (100 mM for the sulphated and E13 peptides) and CH₃CN as eluents. Purified peptides was checked by mass spectrometry (Waters ionspray Q-TOF–micro) and quantified by amino acids analyses (Hitachi L-8800 apparatus). Peptides purity was controlled by analytical C18 RP-HPLC using linear gradient of CH₃CN in 50 mM aqueous NEt₃-AcOH over 20 min (Waters Symetry C18-300Å, 3.5 μ m, 2.1x100 mm column, 0.35 ml/min flow rate).

Table 1: analysis of peptide purity by HPLC

Peptide	Overall yield (%)	Formula	Expected mass (monoisotopic)	Found	HPLC retention time (min)
P3YSO3	4	C ₈₂ H ₉₈ N ₁₄ O ₄₉ S ₆	2253.3835 [M-H] ⁻	2253.3164	8.3 (10-30% over 20min)
P3Y	8	C ₈₂ H ₉₈ N ₁₄ O ₃₁	1775.6601 [M+H] ⁺	1775.6370	11.7
P3pF	14	C ₉₄ H ₁₁₀ N ₁₄ O ₃₇	2027.7235 [M+H] ⁺	2027.7490	6.2
P3Asu	10	C ₇₆ H ₁₂₂ N ₁₄ O ₃₇	1823.8174 [M+H] ⁺	1823.8474	16.5 (0-10% over 20 min)
E13	73	C ₆₉ H ₁₀₀ N ₁₄ O ₄₁	1781.6170 [M+H] ⁺	1781.6299	12.1
Nter3 sulfates	41	C ₈₂ H ₉₈ N ₁₄ O ₄₀ S ₃	2015.9495 M (average)	2015.6544	12.242 (5-25% over 20min)
Cter3 sulfates	46	C ₈₂ H ₉₈ N ₁₄ O ₄₀ S ₃	2015.9495 M (average)	2015.6346	12.454 (5-25% over 20min)

1.2. S-acetylthiopropionate peptides (SATP peptides)

The peptides were dissolved in 100 mM sodium phosphate buffer pH 8.2 (1 mM final concentration). S-acetylthiopropionate group was introduced via stepwise addition of 10 equivalents of SATP (0.26 M in DMSO) over a 40 min period. After 1h30, S-acetylthiopropionate peptides were purified by C18 RP-HPLC using linear gradient of CH₃CN in 50 mM aqueous NEt₃-AcOH over 20 min (C18-300Å, 5 µm, 10x250 mm column, 6 ml/min flow rate). SATP derived peptides purity was controlled by analytical C18 RP-HPLC using linear gradient of CH₃CN in 50 mM aqueous NEt₃-AcOH over 20 min (Waters Symetry C18-300Å, 3.5 µm, 2.1x100 mm column, 0.35 ml/min flow rate).

10 Table 2: analysis of the purity of the S-acetylthiopropionate peptides

Peptide-SATP	Yield (%)	Formula	Expected mass (monoisotopic)	Found	HPLC retention time (min) (5-25% over 20min)
P3YSO3-SATP	60	C ₈₇ H ₁₀₄ N ₁₄ O ₅₁ S ₇	2383.3942 [M-H] ⁻	2383.4316	11.6
P3Y-SATP	43	C ₈₇ H ₁₀₄ N ₁₄ O ₃₃ S ₁	1903.6533 [M-H] ⁻	1903.6781	16.0
P3pF-SATP	50	C ₉₉ H ₁₁₆ N ₁₄ O ₃₉ S ₁	2155.7167 [M-H] ⁻	2155.7869	9.3
P3Asu-SATP	36	C ₈₁ H ₁₂₈ N ₁₄ O ₃₉ S ₁	1953.8262 [M+H] ⁺	1953.7822	15.2*
E13-SATP	34	C ₇₄ H ₁₀₆ N ₁₄ O ₄₃ S ₁	1909.6181 [M-H] ⁻	1909.6188	6.3
Nter3 sulfates-SATP	52	C ₈₇ H ₁₀₄ N ₁₄ O ₄₂ S ₄	2146.1159 M (average)	2146.1746	11.0 (10-30% over 20min)
Cter3 sulfates-SATP	57	C ₈₇ H ₁₀₄ N ₁₄ O ₄₂ S ₄	2146.1159 M (average)	2146.1357	11.3 (10-30% over 20min)

*10-30% linear gradient of CH₃CN in 0.08% aqueous TFA over 20 min.

1.3. Maleimide activated miniCD4 (mCD4-Mal)

The mini-CD4 peptide of the invention is obtained as disclosed in WO 2009/098147 and Nature Chemical Biology, 2009, 5(10), 743-748.

15 Mini-CD4-PEO2-maleimide = mini-CD4 + linker

A solution of 10 mg of mCD4 (MW: 2897; 3.4 μ moles) in 1 ml of H₂O was diluted in 1 ml of phosphate buffer 0.1 M pH 8. 4.5 mg of NHS- PEO₂-Maleimide (MW: 325; 13.8 μ moles; 4 equiv) were added to this cloudy solution in 20 μ l of DMSO with stirring. After 10 minutes, 85% (HPLC) of the starting materials was converted into maleimide derivative. Because of the low stability of the maleimide group at pH 8, the coupling reaction was directly loaded onto a SepaK C18 column calibrated with 10% CH₃CN in aqueous TFA 0.08%. The maleimide derivative was eluted with 50% CH₃CN. After freeze drying, the compound was then purified on a semi preparative column.

1.4. mCD4-peptide conjugates

SATP Peptides were dissolved in 100 mM sodium phosphate buffer pH 7.2 (1 mM final concentration). 100 equivalents of 0.5 M NH₂OH, HCl in 100 mM sodium phosphate buffer (pH adjusted to 7.2 by 4N NaOH) was added. Deprotection of the thiol function was monitored by HPLC. After 30 min, 0.3 equivalent of mCD4-Mal in H₂O (1.5 mM) was added. After 30 min, mCD4-peptide conjugates were purified by C18 RP-HPLC using linear gradient of CH₃CN in 50 mM aqueous NEt₃-AcOH over 20 min (C18-300Å, 5 μ m, 10x250 mm column, 6 ml/min flow rate). mCD4-peptide conjugates were controlled by analytical C18 RP-HPLC using linear gradient of CH₃CN in 50 mM aqueous NEt₃-AcOH over 20 min (Waters Symetry C18-300Å, 3.5 μ m, 2.1x100 mm column, 0.35 ml/min flow rate), negative mode mass spectrometry and quantified by amino acids analysis.

Table 3: HPLC analysis of the mCD4-peptide conjugates

Conjugates	Yield (%)	Formula	Expected mass (average)	Found	HPLC retention time (min) (20-40% over 20min)
mCD4-P3YSO3	47	C ₂₂₁ H ₃₁₄ N ₅₄ O ₈₈ S ₁₃	5552.0933	5551.5127	11.5
mCD4-P3Y	38	C ₂₂₁ H ₃₁₄ N ₅₄ O ₇₀ S ₇	5071.7081	5071.5005	12.8
mCD4-P3pF	67	C ₂₃₃ H ₃₂₆ N ₅₄ O ₇₆ S ₇	5323.9318	5323.5850	10.7
mCD4-P3Asu	23	C ₂₁₅ H ₃₃₈ N ₅₄ O ₇₆ S ₇	5119.8291	5119.5283	10.8
mCD4-E13	58	C ₂₀₈ H ₃₁₆ N ₅₄ O ₈₀ S ₇	5077.5750	5077.1021	11.4
mCD4-Nter3 sulfates	36	C ₂₂₁ H ₃₁₄ N ₅₄ O ₇₉ S ₁₀	5311.9007	5311.5166	13.9 (20-35% over 20min)
mCD4-Cter3 sulfates	43	C ₂₂₁ H ₃₁₄ N ₅₄ O ₇₉ S ₁₀	5311.9007	5311.4155	14.0 (20-35% over 20min)

Synthesis of the control peptide mCD4-HS12 was performed as described in WO/2009/098147 and Nature Chemical Biology, 2009, 5(10), 743-748.

5

Example 2: Biacore evaluation

2.1. Protocol

Two different gp120 were used, originating from a type X4 isolate (gp120 MN) and a type R5 isolate (gp120 YU2). The capacity of different molecules to inhibit gp120 interactions with various receptors or co-receptors is measured by surface plasmonic resonance (Biacore).

To do this, proteins (in this case gp120 envelop proteins) can be immobilized on the surface of a biosensor (Sensorchip CM 4 Biacore) in accordance with the described procedure (Vivès et al. *J. Biol. Chem.* 279, 54327-54333, 2005). When injected on a surface coated with MN(X4) or YU2(R5) gp120 envelops, mCD4 binds and unmasks the CD4i epitope on gp120, responsible to envelop binding of mAb17b (figure 5A) (mAb17b antibody recognizes the epitope induced by CD4 and mimics co-receptor CCR5 or CXCR4). When mCD4 or the compound to be tested is injected onto these

surfaces, the interaction signal between gp120 and mAb17b is measured as a function of time (figures 5 A to C).

In another set of experiments, mAb17b are coated on the surface, and gp120 alone (grey line) or pre-incubated with mCD4, or the compound to be tested are injected onto these surfaces (figure 5D and 5G). The interaction signal between gp120 and mAb17b is measured as a function of time.

In another set of experiments, sCD4 is coated on the surface, and gp120 alone (grey line) or pre-incubated with the compounds to be tested are injected onto these surfaces (figure 5E and 5H). The interaction signal between gp120 and sCD4 is measured as a function of time.

In another set of experiments, heparin is coated on the surface, and gp120 alone (grey line) or pre-incubated with the compounds to be tested are injected onto these surfaces (figure 5F). The interaction signal between gp120 and heparin is measured as a function of time.

The difference between the light line and the heavy lines shows the inhibitory capacity of the tested compound vis-à-vis interactions between gp120 and the molecule immobilized on the biosensor.

2.2. Results

mCD4-P3Y_{SO3} fully inhibits mAb17b binding on gp120/mCD4 complex.

When injected on gp120 surface coated with MN(X4) or YU2(R5) envelops, mCD4 binds and unmask the CD4i epitope on gp120, responsible to envelop binding of mAb17b (figure 5A). The same experiments performed with mCD4-P3Y and mCD4-P3Y_{SO3} (figures 5B and 5C respectively) show that mCD4-P3Y partially inhibits mAb17b binding to MN(X4) (figure 5B), whereas mCD4-P3Y_{SO3} fully inhibits mAb17b on MN(X4) and YU2(R5) (figure 5C).

mCD4-P3Y_{SO3} fully inhibits MN(X4) envelop binding on mAb17b and CD4 and partially on Heparin.

When injected on mAb 17b surface, MN envelop does not bind to 17b surface (CD4i epitope is masked, light line, figure 5D). This binding occurs when gp120 envelop is in complex with mCD4. When injected in complex with mCD4-P3Y_{SO3}, the binding is fully inhibited (figure 5D). MN envelop binds to CD4 surface. When injected in complex with mCD4-P3Y_{SO3}, the binding is fully inhibited (figure 5E), whereas MN binding to heparin is only partially inhibited by mCD4-P3Y_{SO3} (figure 5F).

mCD4-P3Y_{SO3} fully inhibits YU2(R5) envelop binding on mAb17b and CD4.

When injected on mAb17b surface, YU2 envelop partially binds to 17b surface. This binding is enhanced when mCD4/YU2 complex is injected. This binding is fully inhibited by mCD4-P3Y_{SO3} (figure 5G). YU2 envelop binds to CD4 surface. When injected in complex with mCD4-P3Y_{SO3}, the binding is fully inhibited (figure 5H). As YU2 envelop does not bind to heparin surface, the experiment on heparin surface was not performed.

Example 3: Antiviral activity of the peptides of the invention on the X4-tropic HIV-1-LAI and R5-tropic HIV-1/Ba-L strains

3.1. Protocol

The antiviral experiment have been performed as described in WO/2009/098147 and Nature Chemical Biology, 2009, 5(10), 743-748.

Briefly, the X4-tropic HIV-1-LAI (Barre-Sinoussi, Science 220, 868-71, 1983) or the R5-tropic HIV-1/Ba-L (Gartner et al, Science 233, 215-9, 1986) strains were amplified and titrated *in vitro* on Phytohemagglutinin-P (PHA-P)-activated peripheral blood mononuclear cells (PBMC). Tissue culture infectious doses were calculated using Kärber's formula (Kärber, Arch. Exp. Path. Pharmac. 162, 480-483, 1931). For the antiviral assay, PHA-P-activated PBMC were pre-treated for 30 minutes with six concentrations of each drug (1:5 dilutions between 0,5 μ M and 160 pM) and infected with one hundred 50% tissue culture infectious doses (TCID₅₀) of either the X4-tropic LAI or R5-tropic Ba-L strain. Drugs were maintained throughout the culture, and cell supernatants were collected at day 7 post-infection and stored at -20°C. Azidothymidine (AZT) was used in these experiments as an internal control. Viral replication was

measured by quantifying reverse transcriptase (RT) activity in cell culture supernatants using the RetroSys HIV RT kit (Innovagen). In parallel, cytotoxicity of the samples was evaluated in uninfected PHA-P-activated PBMC by a methyltetrazolium salt (MTT) assay on day 7. Experiments were performed in triplicate and 50, 70 and 90 % effective
5 doses and cytotoxic doses were calculated using SoftMaxPro software.

PHA-P-activated PBMC were treated with each of the drug under investigation (1:5 dilutions between 0.5 μ M and 160 pM) and infected with 100 TCID₅₀ of either HIV-1-LAI (X4 tropic) or /Ba-L (R5 tropic) strain. Molecules and viruses were maintained throughout the culture, and cell supernatants were collected at day 7 post-infection from
10 which reverse transcriptase activity was quantified. Experiments were performed in triplicate and 50, 70 and 90% effective doses (ED), in nM (\pm S.D.) were calculated using SoftMaxPro software. None of these molecules showed cytotoxicity up to 1 μ M.

3.2. Results

When used alone, none of the anionic peptides demonstrated antiviral activity at the
15 highest concentration tested (500 nM). However, when conjugated to mCD4, they displayed inhibitory activity against the LAI and/or Ba-L strain, with effective doses giving 50% inhibition (ED₅₀) as low as 0.5 nM for mCD4-P3YSO₃, which compares well to 1.4 nM for mCD4-HS₁₂ (see table 4 below).

Table 4: anti-viral activity of the conjugated peptides of the invention (effective dose (ED, mean of triplicate determination), in nM (\pm s.d.), required to inhibit 50, 70 and 90 % of HIV-1 replication)

		VIH-1-LAI(X4)		VIH-1/Ba-L(R5)	
		Average	\pm S.D *	Average	\pm S.D
P3YSO3	ED50	>500	—	>500	—
	ED70	>500	—	>500	—
	ED90	>500	—	>500	—
P3Y	ED50	>500	—	>500	—
	ED70	>500	—	>500	—
	ED90	>500	—	>500	—
P3pF	ED50	>500	—	>500	—
	ED70	>500	—	>500	—
	ED90	>500	—	>500	—
P3Asu	ED50	>500	—	>500	—
	ED70	>500	—	>500	—
	ED90	>500	—	>500	—
E13	ED50	>500	—	>500	—
	ED70	>500	—	>500	—
	ED90	>500	—	>500	—
mCD4	ED50	201	161	>500	—
	ED70	242	169	>500	—
	ED90	317	187	>500	—
mCD4P3YSO3	ED50	0,5	0,2	1,3	1,1
	ED70	0,6	0,3	3,1	2,7
	ED90	1,1	0,8	20	9,2
mCD4 P3Y	ED50	98	36	454	104
	ED70	124	74	498	11
	ED90	160	125	>500	—
mCD4 P3pF	ED50	8,2	6,5	245	196
	ED70	11	7,4	>500	—
	ED90	20	11	>500	—
mCD4P3Asu	ED50	15	5	499	4,3
	ED70	20	7	>500	—
	ED90	31	14	>500	—
mCD4 E13	ED50	30	25	435	159
	ED70	36	31	451	121
	ED90	48	41	>500	—
mCD4-PEO2-HP12L	ED50	1,4	1,2	18	9,6
	ED70	1,7	1,6	215	231
	ED90	3,5	5,3	>500	—
AZT	ED50	11	6	8,7	7,0
	ED70	21	15	16	11
	ED90	60	53	38	28
*SD: standard deviation					

None of these molecules showed cytotoxicity up to the highest tested dose (0.5 μ M).

Example 4: Antiviral activity of mCD4-P3YSO₃ on the 92UG029, SF162, 92US723, 96USHIPS4, 92HT599 and 98IN017 strains

4.1. Protocol

- 5 The antiviral assay was extended to a series of more clinically relevant primary strains, including 92UG029, SF162, 92US723, 96USHIPS4, 92HT599 and 98IN017.

Phytohemagglutinin (PHA)-P-activated PBMCs were infected either with the reference lymphotropic HIV-1/LAI strain (Barre-Sinoussi, et al., 1983) or with the reference macrophage-tropic HIV-1/Ba-L strain (Gartner, et al., 1986). These viruses were
10 amplified in vitro with PHA-P-activated blood mononuclear cells. Viral stocks (including clinical isolates) were titrated using PHA-P-activated PBMCs, and 50% tissue culture infectious doses (TCID₅₀) were calculated using Kärber's formula (Kärber, 1931). Viruses (125 TCID₅₀) were incubated for 30 min with five
15 concentrations (1:5 dilutions between 500 nM and 320 pM) of each of the molecules to be tested and added to 150 000 PBMCs (m.o.i. \sim 0.001). Cell supernatants were collected at day 7 post-infection and stored at -20°C. In some cases, the compounds were added to the cells prior to viral challenge. Viral replication was measured by
20 quantifying reverse transcriptase (RT) activity in the cell culture supernatants using the Lenti RT Activity Kit (Cavisi) and AZT was used as reference anti-HIV-1 molecule. In parallel, cytotoxicity was evaluated on day 7 in uninfected PHA-P-activated PBMC using a colorimetric methyl-tetrazolium salt (MTS/PMS) assay (Promega). Experiments were performed in triplicate and 50, 70 and 90% effective doses (ED) were calculated using SoftMaxPro software.

4.2. Results

- 25 As shown in Table 5 below, mCD4-P3YSO₃ displayed a high level of antiviral activity, characterized by ED₅₀ in the range of 0.2 to 1.2 nM for five of them, and 29 nM for HIV-1 98IN017, a clade C virus. As for the LAI and Ba-L strains, the mCD4 or P3YSO₃ were only poorly active or inactive, further supporting the very strong

synergistic effect induced by the coupling strategy. None of the molecules showed cytotoxicity at up to 1 μ M.

Table 5: anti-HIV-1 activity of AZT, mCD4-P3YSO₃, P3YSO₃ and mCD4 against clinical HIV-1 isolates (effective dose (ED, mean of triplicate determination), in nM (\pm s.d.), required to inhibit 50, 70 and 90 % of HIV-1 replication)

Viral strain: Clade-tropism		92UG029 A-X4	SF162 B-R5	92US723 B-R5/X4	96USHIPS4 B-R5/X4	92HT599 B-X4	98IN017 C-X4
AZT	ED ₅₀	7 \pm 0	8 \pm 7	8 \pm 0.1	19 \pm 9	9 \pm 4	8 \pm 3
	ED ₇₀	16 \pm 3	13 \pm 8	17 \pm 1	27 \pm 11	22 \pm 5	19 \pm 5
	ED ₉₀	61 \pm 17	31 \pm 3	59 \pm 19	56 \pm 15	110 \pm 13	108 \pm 25
mCD4- P3YSO₃	ED ₅₀	0.2 \pm 0.0	0.3 \pm 0.2	0.3 \pm 0.1	1.2 \pm 1	0.5 \pm 0.2	29 \pm 18
	ED ₇₀	0.3 \pm 0.1	0.4 \pm 0.3	0.35 \pm 0.2	1.6 \pm 1.2	1.3 \pm 0.9	147 \pm 9
	ED ₉₀	0.8 \pm 0.3	0.9 \pm 0.2	0.45 \pm 0.2	3 \pm 1.4	3.5 \pm 0.0	> 500
P3YSO₃	ED ₅₀	> 500	> 500	> 500	> 500	> 500	> 500
	ED ₇₀	> 500	> 500	> 500	> 500	> 500	> 500
	ED ₉₀	> 500	> 500	> 500	> 500	> 500	> 500
mCD4	ED ₅₀	403 \pm 76	245 \pm 155	23 \pm 1	> 500	355 \pm 155	> 500
	ED ₇₀	> 500	352 \pm 105	34 \pm 10	> 500	> 500	> 500
	ED ₉₀	> 500	> 500	52 \pm 22	> 500	> 500	> 500

We also observed that mCD4-P3YSO₃ does not need to be preincubated with the virus to be active. Indeed, addition of the molecule either to the cells, prior to the viral challenge or to the virus prior to the cell infection return, identical results (cf. table 7).

Table 7: Anti-HIV-1 activity of AZT, mCD4-P3YSO₃, P3YSO₃ and mCD4 against LAI HIV-1 (effective dose (ED, mean of triplicate determinations), in nM (\pm s.d.) required to inhibit 50, 70 and 90 % of HIV-1 replication, when the compounds were preincubated either with the cells or with the viruses)

		Pre-treated cells	Pre-treated viruses
AZT	ED ₅₀	16.5 \pm 12	20 \pm 12
	ED ₇₀	33 \pm 18	38 \pm 18
	ED ₉₀	96 \pm 11	111 \pm 40
mCD4- P3YSO₃	ED ₅₀	0.5 \pm 0.2	0.5 \pm 0.3
	ED ₇₀	0.6 \pm 0.2	0.7 \pm 0.3
	ED ₉₀	1 \pm 0.2	0.9 \pm 0.2
P3YSO₃	ED ₅₀	> 500	> 500
	ED ₇₀	> 500	> 500
	ED ₉₀	> 500	> 500
mCD4	ED ₅₀	310 \pm 190	406 \pm 94
	ED ₇₀	> 500	474 \pm 27
	ED ₉₀	> 500	> 500

5

Example 5: Antiviral activity of mCD4-P3YSO₃ *in cellulo* on SHIVsf162p3 cells

5.1. Protocol

TZN-bl cells that express Luciferase under the control of the HIV LTR were used. These cells express high levels of CD4 and CCR5 and can be easily infected with HIV-1/-2 and SIV. Upon infection of these cells, viral Tat will induce transcription of Luciferase. The Luc signal is proportional to the amount of infection. We add a serial dilution of the compound to be tested (mCD4, mCD4-HS12 or mCD4-P3YSO₃) to cells and subsequently add virus. The obtained Luc signal is read out after 48h.

The test was carried out in duplicate.

5.2. Results

The results obtained are presented in the table 6 below.

15

	EC50 (nM)	
	1 st experiment	2 nd experiment
mCD4	4617	6126
mCD4-HS12	781	631
mCD4-P3Y _{SO3}	34	37

Conclusions

This study thus shows that relatively small synthetic molecules, comprising a 3 kDa CD4 mimetic linked to an anionic polypeptide can efficiently mimic several large gp120 ligands, including CD4 and coreceptor binding site recognizing mAbs. Remarkably, the conjugates described in the present invention neutralize both R5- and X4-tropic HIV-1, a significant advantage since the efficacy of CCR5-specific antagonists could be jeopardized by the emergence of viral strains that utilize CXCR4, for which no inhibitors are yet available.

Surprisingly, the peptides of the invention displayed a better antiviral activity than the molecules of the prior art (mCD4-HS12) both on X4 and R5 virus strains and can thus enhance the inhibition of the HIV virus entry into host cells.

The conjugated molecule mCD4-P3Y_{SO3} has far better inhibitory effect than any other tested compounds (ED50 of 0.5 and 1.3 against respectively X4 and R5).

Abbreviations:

Fmoc: 9-fluorenylmethyloxycarbonyl

DMF: Dimethylformamide

HATU: hexafluorophosphate N-oxide of N[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridine-1-ylmethylene]-N-methylmethanaminium

DIEA: diisopropylethylamine

SPDP: N-succinimidyl-3(2-pyridyldithio)propionate

TFA: trifluoroacetic acid

EDT: ethanedithiol

TIS: triisopropylsilane

- DTT: 1,4-dithiothreitol
MPLC: medium pressure liquid chromatography
ES⁺MS: electrospray mass spectrometry, positive mode
GSH: reduced glutathion
- 5 GSSG: oxidised glutathion
HPLC: high-performance liquid chromatography
RP-HPLC: reverse phase high-performance liquid chromatography
SMPH: succinimidyl-6-[β -maleimidopropionamido]hexanoate
SATP: N-succinimidyl-S-acetylthiopropionate
- 10 RT: room temperature
Rt: retention time
Cbz: benzyloxycarbonyl
pMBn: *p*-methoxybenzyl
Bn: benzyl
- 15 Ac: acetyl
Me: methyl
Et: ethyl
eq: equivalent
HRMS: high resolution mass spectrum
- 20 ESI: electrospray ionisation
LC-ESI-TOF-MS: Liquid chromatography/electrospray ionization Time-of-Flight mass spectrometry
LCMS: Liquid chromatography/Mass spectrometry
DMSO: Dimethylsulfoxide

CLAIMS

1. Conjugated molecule comprising a peptide derived from the CD4 receptor, said peptide being coupled to an organic molecule by means of a linker, wherein:

- 5 ▪ the said peptide derived from the CD4 receptor comprises the following general sequence (I):

$\text{Xaa}^f - \text{P1} - \text{Lys} - \text{Cys} - \text{P2} - \text{Cys} - \text{P3} - \text{Cys} - \text{Xaa}^g - \text{Xaa}^h - \text{Xaa}^i - \text{Xaa}^j - \text{Cys} - \text{Xaa}^k - \text{Cys} - \text{Xaa}^l - \text{Xaa}^m$, (I)

in which:

- 10 - P1 represents 3 to 6 amino acid residues,
 - P2 represents 2 to 4 amino acid residues,
 - P3 represents 6 to 10 amino acid residues,
 - Xaa^f represents N-acetylcysteine (Ac-Cys) or thiopropionic acid (TPA),
 - Xaa^g represents Ala or Gln,
 15 - Xaa^h represents Gly or (D)Asp or Ser,
 - Xaa^i represents Ser or His or Asn,
 - Xaa^j represents biphenylalanine (Bip), phenylalanine or [beta]-naphthylalanine,
 - Xaa^k represents Thr or Ala,
 - Xaa^l represents Gly, Val or Leu, and
 20 - Xaa^m represents $-\text{NH}_2$ or $-\text{OH}$,

the amino acid residues in P1, P2 and P3 being natural or non-natural, identical or different, said residues of P1, P2 and P3 being all different from the Lys residue and P1, P2 and P3 having a sequence in common or not, and

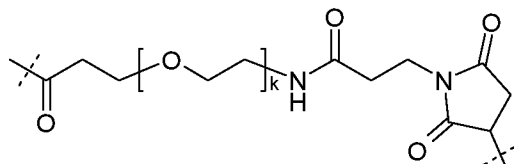
- the said organic molecule comprises
- 25 - an anionic polypeptide consisting of 5 to 21 amino acid residues being natural or non-natural, identical or different, wherein at least 3 amino acids are negatively charged,
- a molecular group A-Z, wherein:
- 30 ○ A comprises a group chosen between the groups of formula $-\text{CO}(\text{CH}_2)_3\text{NH}-\text{CO}(\text{CH}_2)_2-$, $-\text{CO}(\text{CH}_2)_p\text{NH}-\text{CO}-(\text{CH}_2)_q-$, $-\text{CO}(\text{CH}_2-\text{CH}_2)-(\text{O}-\text{CH}_2-\text{CH}_2)_p\text{NH}-\text{CO}-(\text{CH}_2)_q-$, $-\text{CO}(\text{CH}_2)_p\text{NH}-\text{CO}-(\text{CH}_2-\text{CH}_2-\text{O})_q-(\text{CH}_2-\text{CH}_2)-$ and $-\text{CO}(\text{CH}_2-\text{CH}_2)-(\text{O}-\text{CH}_2-\text{CH}_2)_p\text{NH}-\text{CO}-(\text{CH}_2-\text{CH}_2-$

5 $O)_q-(CH_2-CH_2)-$, wherein p represents an integer comprised between 1 and 10 and q represents an integer comprised between 1 and 10, , and wherein the first carbonyl group is coupled to the alpha NH_2 of N-terminal serine, and advantageously A represents a group of formula $-CO(CH_2)_3NH-CO(CH_2)_2-$, and

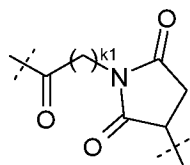
○ Z represents an halogen atom, a thiol or a maleimide group, the said anionic polypeptide being linked to the linker by the said molecular group of formula A-Z, and the said linker being covalently bound at one of its extremity to the free amino group ($-NH_2$) of the amino acid residue Lys present in general sequence (I)
10 of the said peptide derived from the CD4 receptor, and being covalently bound at its other extremity to the Z group of the said organic molecule.

2. Conjugated molecule according to claim 1, wherein the sequence of the peptide derived from the CD4 receptor of general sequence (I) is chosen from the group consisting of sequences SEQ ID NO: 1 and SEQ ID NO: 2.

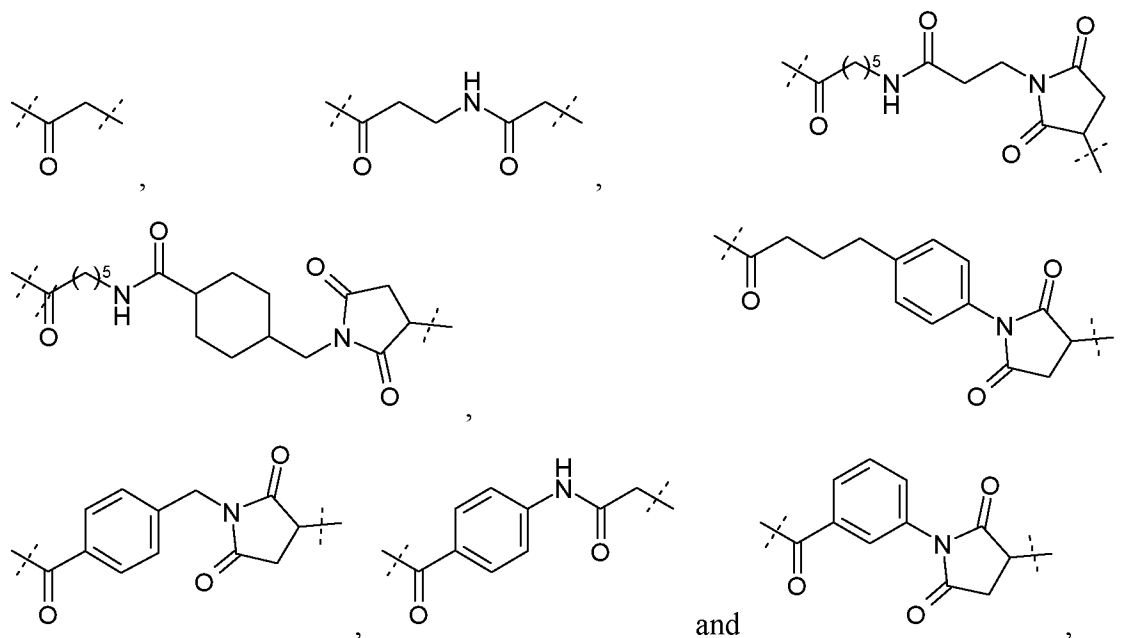
15 3. Conjugated molecule according to claim 1 or 2, wherein the linker is chosen from the group consisting of: $CO-(CH_2O)_2CH_2NHCO(CH_2)_2$ pyrrolidinyl-2,5-dione



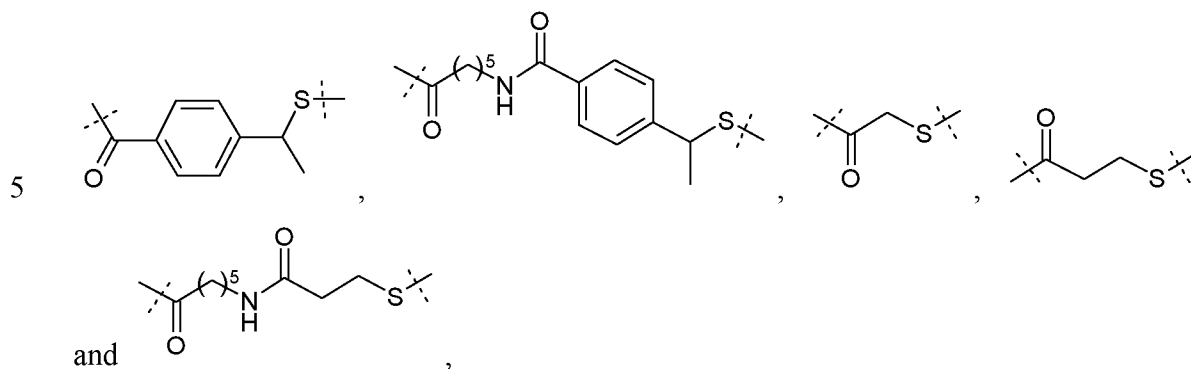
, with k representing an integer comprised between 2 and 24,



with k1 representing an integer equal to 1, 2, 3, 5 and 10,

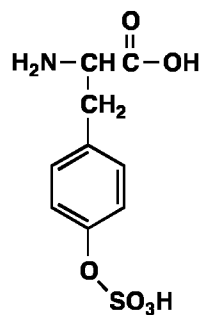


when Z represents a thiol group, and among:

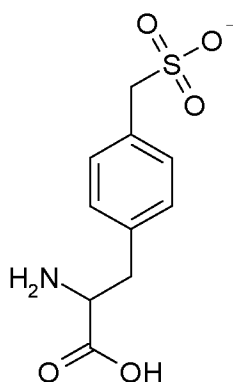


when Z represents a maleimide group or a halogen atom.

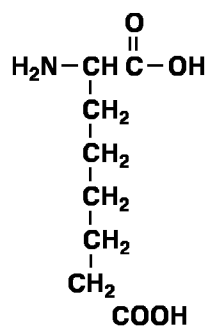
4. Conjugated molecule according to any one of claims 1 to 3, wherein the negatively charged amino acids of the anionic polypeptide are chosen among aspartic acid, tyrosine, sulfotyrosine, tyrosine sulfonate, aminosuberic acid, p-carboxymethyl phenylalanine, and glutamic acid,
- 10
- wherein said sulfotyrosine has the formula:



- wherein said tyrosine sulfonate has the formula:



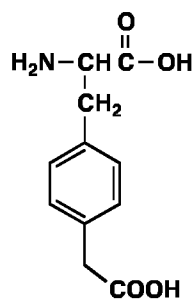
- wherein said aminosuberic acid has the formula:



5

and

- wherein said p-carboxymethyl phenylalanine has the formula:

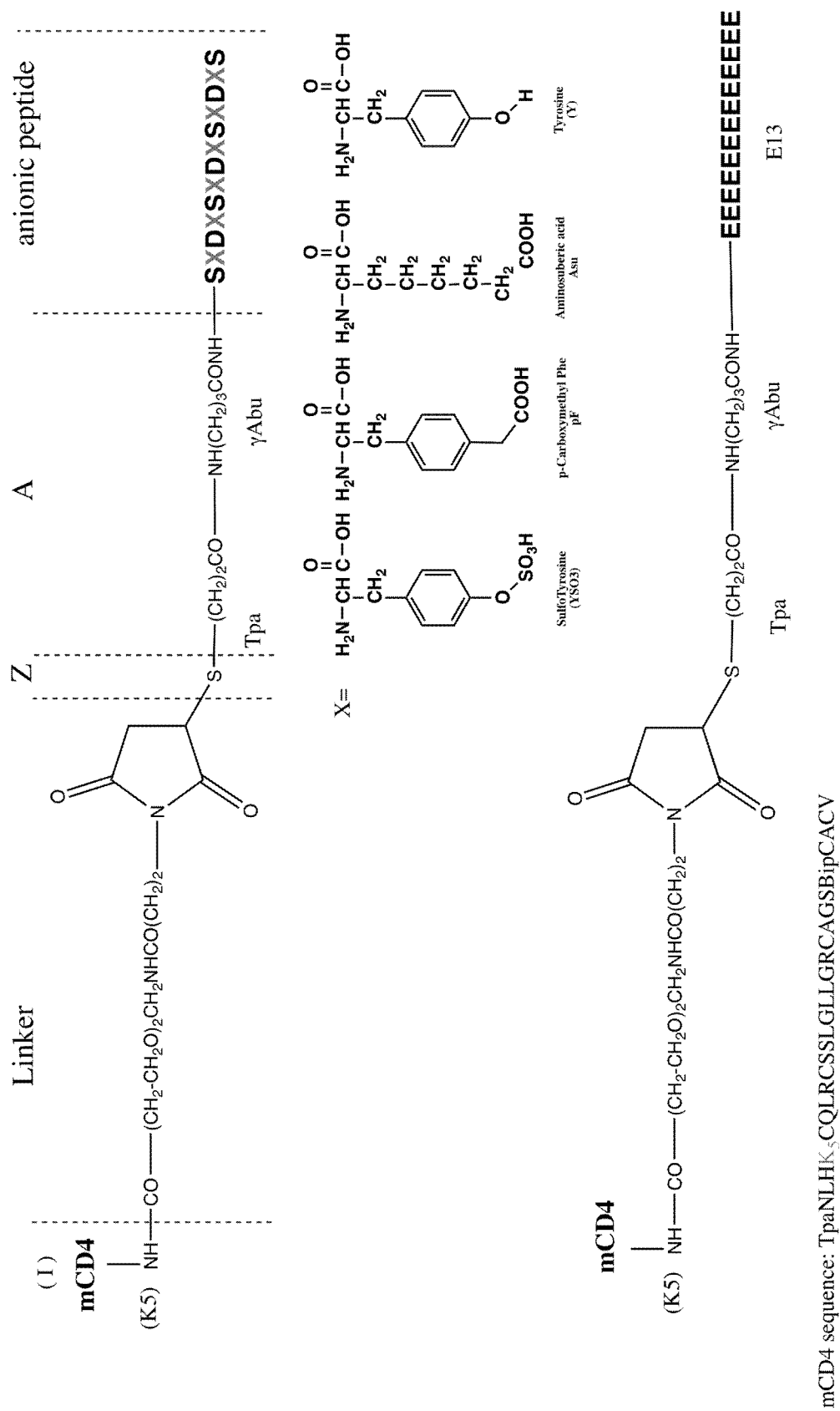


5. Conjugated molecule according to any one of claims 1 to 4, wherein the anionic polypeptide consists of 13 amino acids.
6. Conjugated molecule according to any one of claims 1 to 5, wherein the anionic polypeptide consists of identical amino acids.
- 5 7. Conjugated molecule according to claim 6, wherein said identical amino acid is glutamic acid (E).
8. Conjugated molecule according to any one of claims 1 to 5, wherein the anionic polypeptide comprises at least two different amino acids.
9. Conjugated molecule according to claim 8, wherein the anionic polypeptide
10 comprises at least aspartic acid (D) and serine (S).
10. Conjugated molecule according to claims 8 and 9, wherein said anionic polypeptide has a sequence of S-(X-D-X-S)_n, such as S-X-D-X-S-X-D-X-S-X-D-X-S (SEQ ID NO: 19), where n represents an integer comprised between 1 and 5, S represents serine and D aspartic acid, and where X is selected from the group consisting of: tyrosine,
15 sulfotyrosine, tyrosine sulfonate, aminosuberic acid, and p-carboxymethyl phenylalanine, and is preferably sulfotyrosine.
11. Conjugated molecule according to claim 10, wherein said anionic polypeptide has a sequence which is selected in the group consisting of: S-(Y-D-Y-S)_n, S-(Y_{SO3}-D-Y_{SO3}-S)_n, S-(Y_{SN}-D-Y_{SN}-S)_n, S-(pF-D-pF-S)_n, and S-(Asu-D-Asu-S)_n, where n represents an
20 integer comprised between 1 and 5, S represents serine, D represents aspartic acid, Y represents tyrosine, Y_{SO3} represents sulfotyrosine, Y_{SN} represents tyrosine sulfonate, pF represents p-carboxymethyl phenylalanine and Asu represents aminosuberic acid.
12. Conjugated molecule according to claim 11, wherein said anionic polypeptide has a sequence which is selected in the group consisting of: S-Y-D-Y-S-Y-D-Y-S-Y-D-Y-S (SEQ ID NO: 8), S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S (SEQ ID NO: 4), S-Y_{SN}-D-Y_{SN}-S-Y_{SN}-D-Y_{SN}-S-Y_{SN}-D-Y_{SN}-S (SEQ ID NO: 5), S-pF-D-pF-S-pF-D-pF-S-pF-D-pF-S (SEQ ID NO: 6), S-Asu-D-Asu-S-Asu-D-Asu-F-Asu-F-D-Asu-S (SEQ ID NO: 7), S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y-S-Y-D-Y-S (SEQ ID NO: 20), and S-Y-D-Y-S-Y-

D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S (SEQ ID NO: 21), where S represents serine, D represents aspartic acid, Y represents tyrosine, Y_{SO3} represents sulfotyrosine, Y_{SN} represents tyrosine sulfonate, pF represents p-carboxymethyl phenylalanine and Asu represents aminosuberic acid.

- 5 13. Conjugated molecule according to any one of claims 1 to 12, wherein:
- the peptide derived from the CD4 receptor is chosen from the group consisting of sequences SEQ ID NO: 1 and SEQ ID NO: 2,
 - the linker is CO-(CH₂O)₂CH₂NHCO(CH₂)₂pyrrolidiny-2,5-dione,
 - the organic molecule comprises an anionic polypeptide having the following
- 10 sequence S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S-Y_{SO3}-D-Y_{SO3}-S as defined in claim 12, which is linked to the linker by a molecular group of formula A-Z, wherein A is -NHCO(CH₂)₃NH-CO(CH₂)₂- and Z is a thiol group.
14. Conjugated molecule according to any one of claims 1 to 13, for its use as medicament.
- 15 15. Conjugated molecule according to any one of claims 1 to 13, for its use for the treatment of AIDS.
16. Pharmaceutical composition comprising a conjugated molecule according to any one of claims 1 to 13 and a pharmaceutically acceptable vehicle.
17. Process for the preparation of a conjugated molecule according to any one of
- 20 claims 1 to 13, characterized in that the process comprises the following steps:
- a. contacting the peptide derived from the CD4 receptor of general sequence (I) as defined in claim 1 with a bifunctional compound carrying two active groups, so that one of the two active groups forms a covalent bond with the free amino group (-NH₂) of the residue of the amino acid Lys present in general sequence
- 25 (I), in order to obtain an activated peptide carrying the second active group of the bifunctional group and
- b. contacting the activated peptide obtained at step (a) with an organic molecule carrying a functional group as defined in claim 1 or with an organic molecule corresponding to the organic molecule carrying a thiol group defined in claim 1

for which the thiol group (SH) has been protected by a protective thiol group, so that the active group of the activated peptide forms a covalent bond with the functional group, protected or not, of the organic molecule, in order to obtain the conjugated molecule.



mCD4 sequence: TpaNLHK₅QQLRCSSLLGLRGCAgSBipCACV

Tpa: Thiopropionic acid

Bip: Biphenylalanine

γ Abu: γ aminobutyric acid

Figure 1

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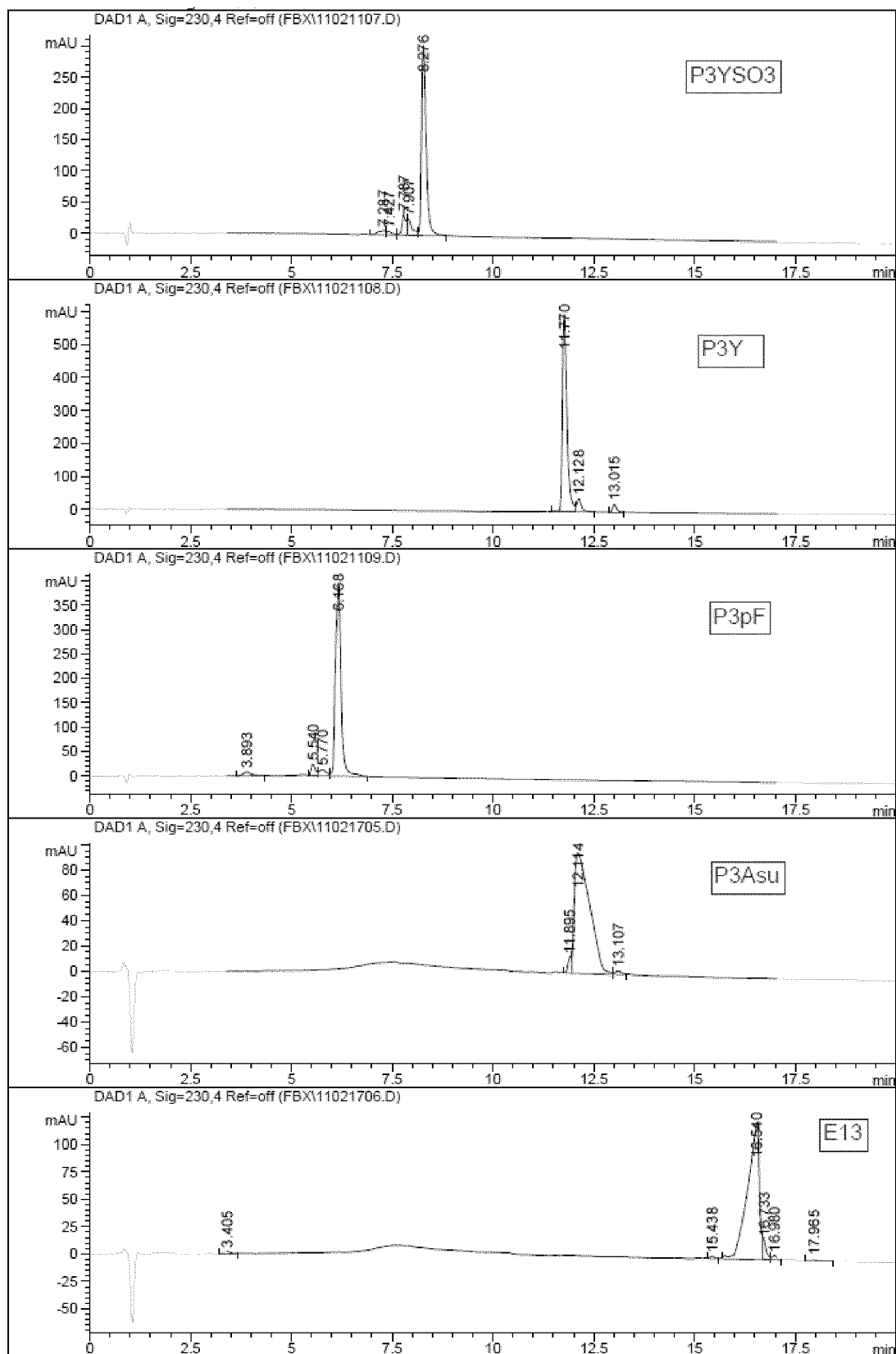


Figure 2

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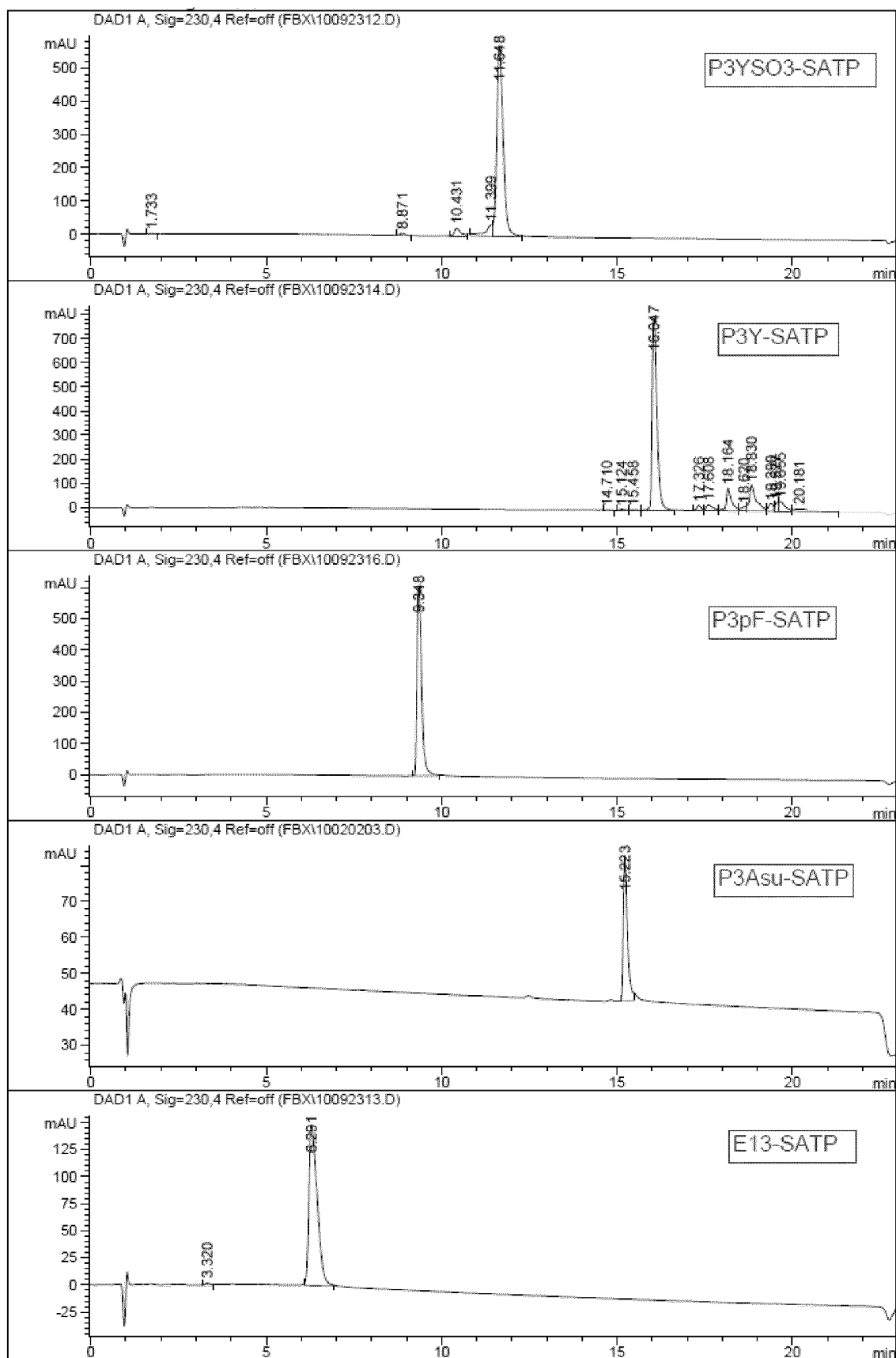


Figure 3

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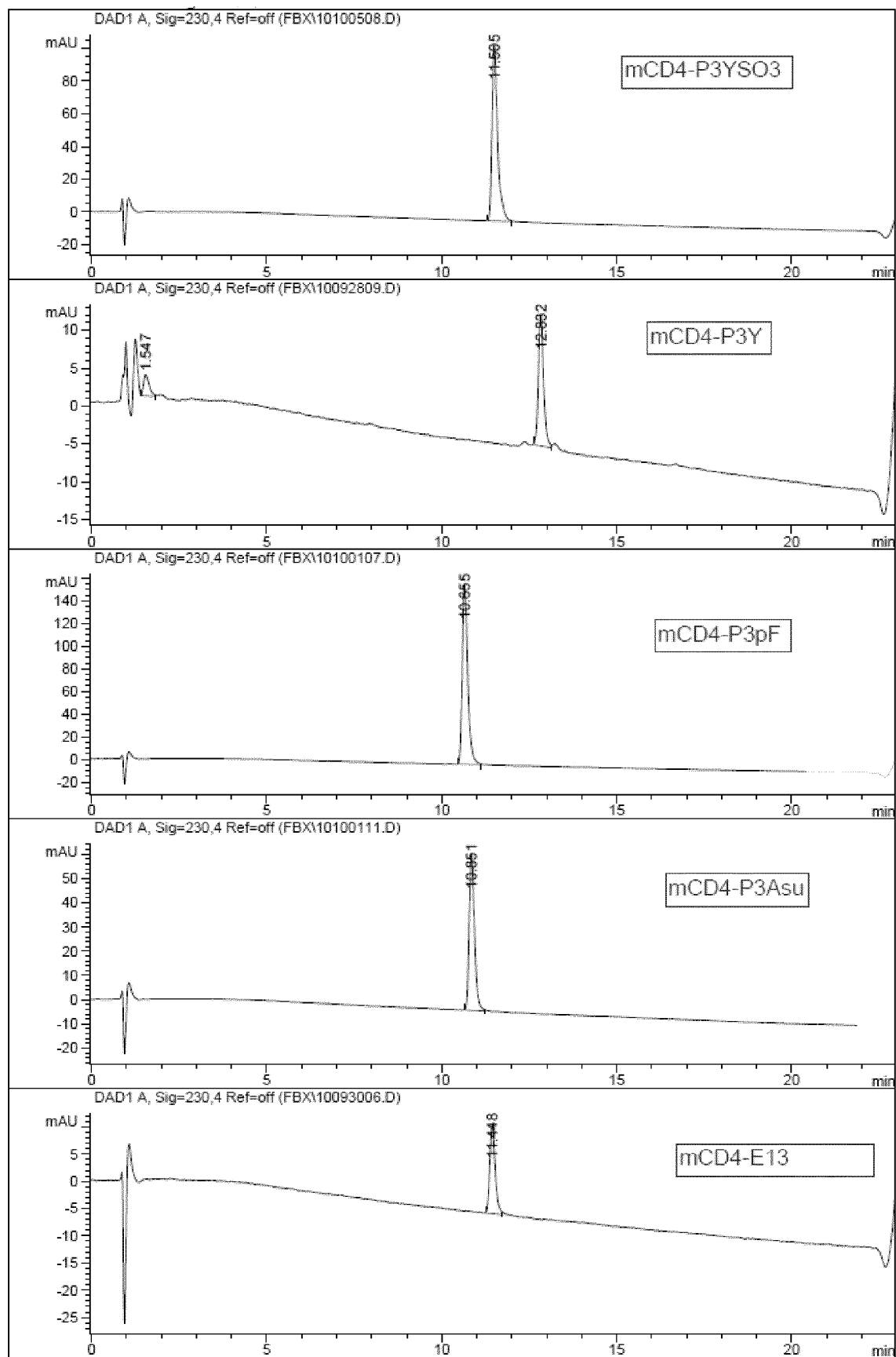
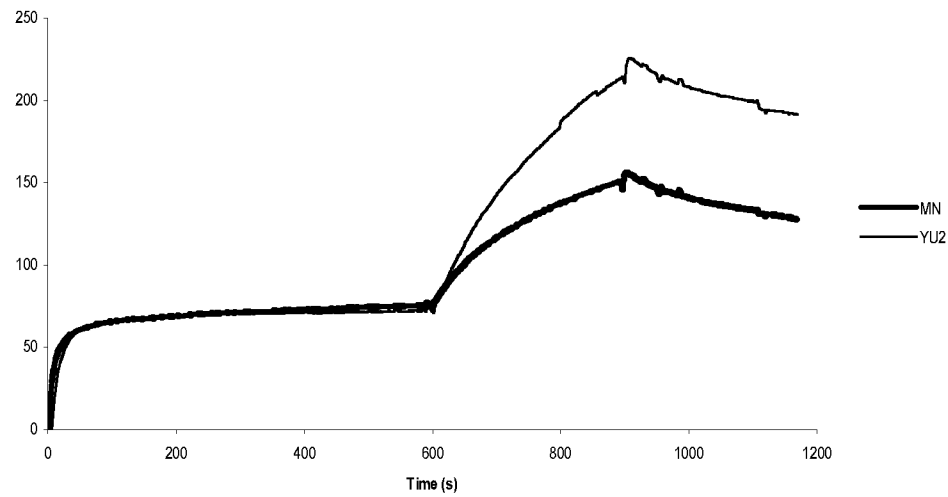


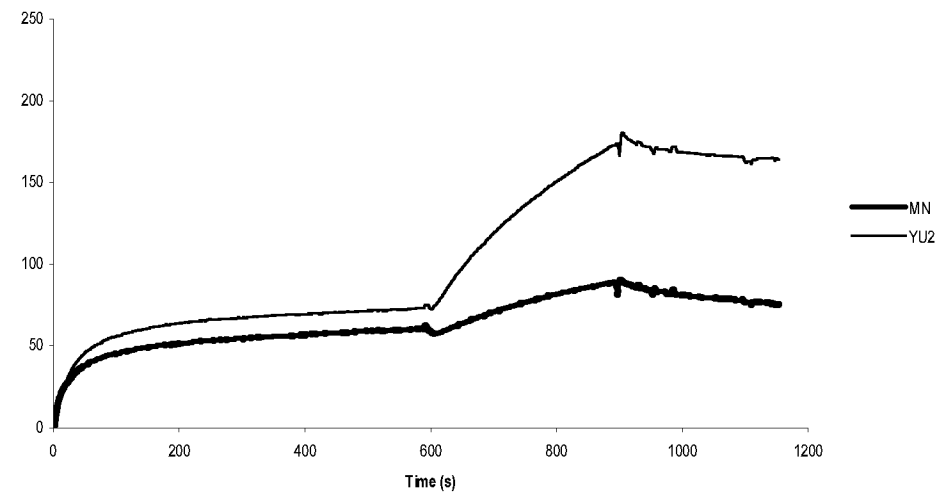
Figure 4

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A



B



C

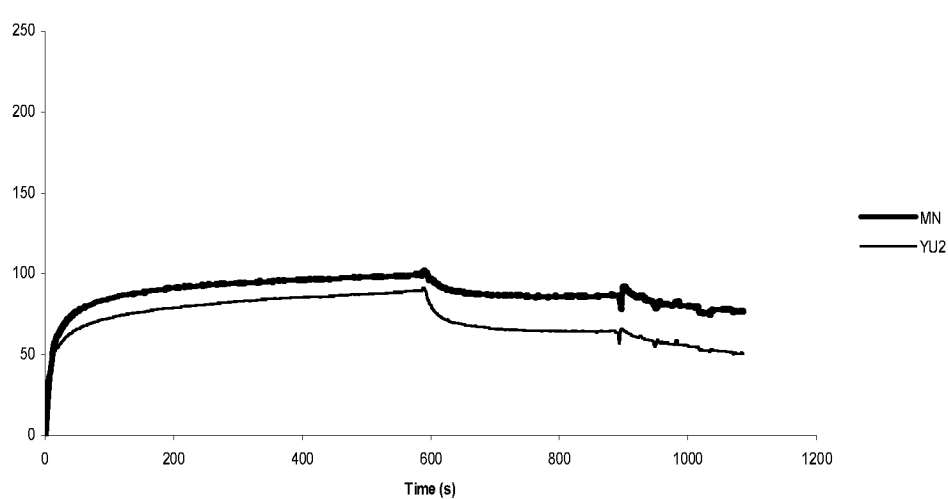


Figure 5

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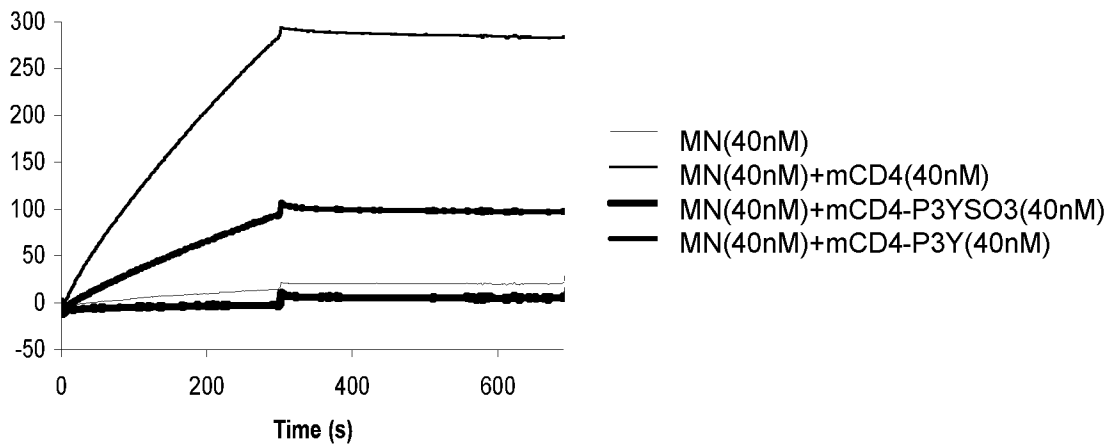
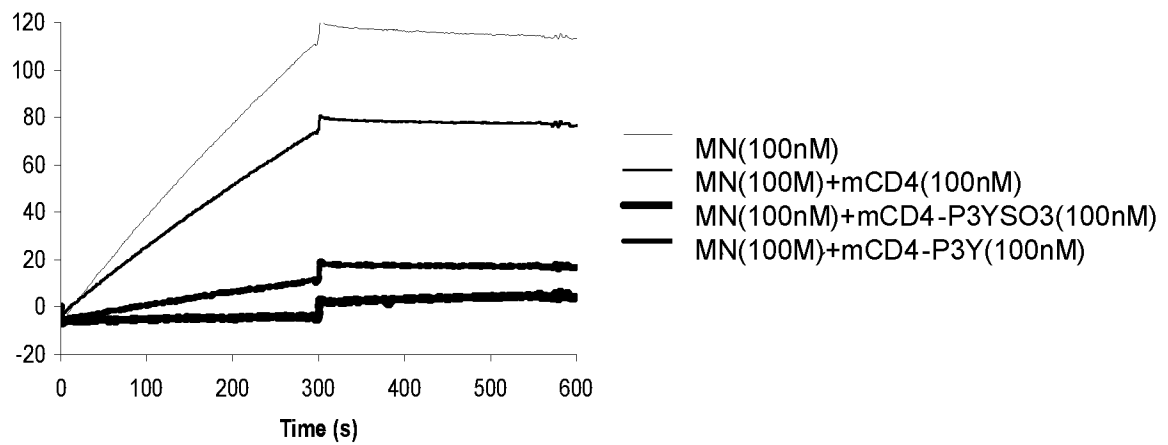
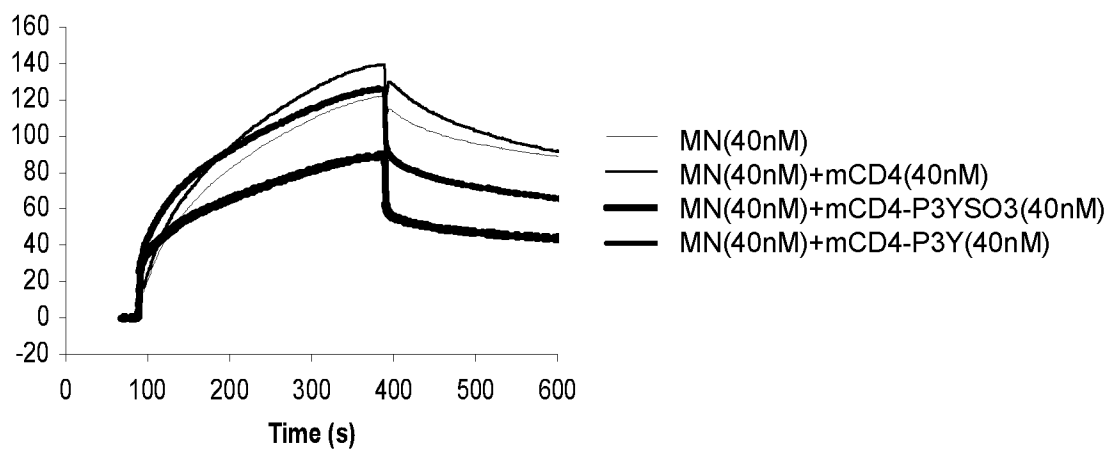
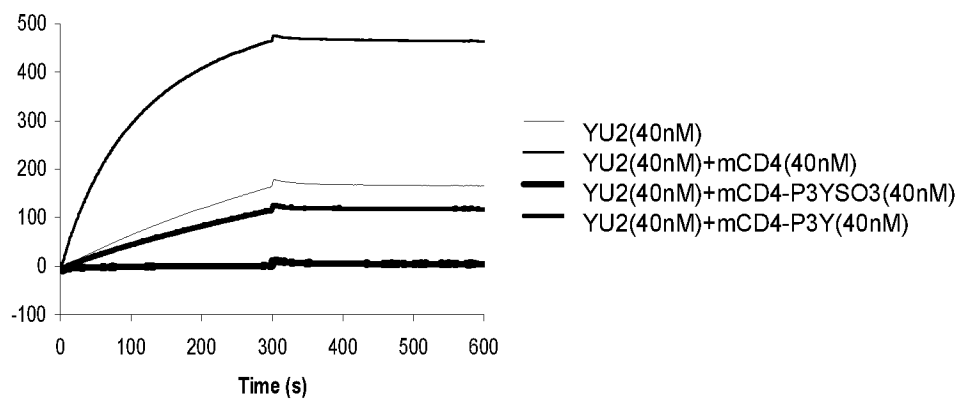
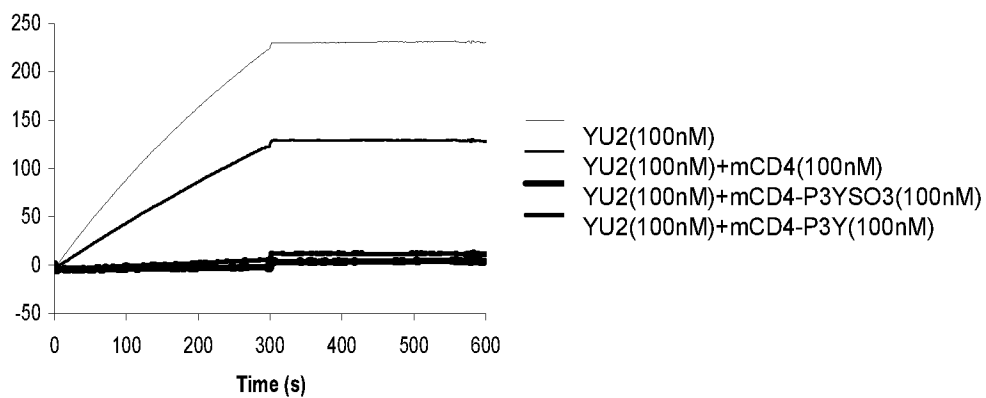
D**E****F**

Figure 5 (continued)

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G**H****Figure 5 (continued)**

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/054674

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 C07K14/705 A61P31/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/015273 A1 (PASTEUR INSTITUT [FR]; CENTRE NAT RECH SCIENT [FR]; BALEUX FRANCOISE []) 7 February 2008 (2008-02-07) claim 22	1-17
A	EP 2 087 911 A1 (PASTEUR INSTITUT [FR]; COMMISSARIAT ENERGIE ATOMIQUE [FR]) 12 August 2009 (2009-08-12) claims 1,7,8	1-17

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search

26 April 2012

Date of mailing of the international search report

22/05/2012

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Authorized officer

Schleifenbaum, A

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/054674

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FRANÇOISE BALEUX ET AL: "A synthetic CD4-heparan sulfate glycoconjugate inhibits CCR5 and CXCR4 HIV-1 attachment and entry", NATURE CHEMICAL BIOLOGY, vol. 5, no. 10, 1 October 2009 (2009-10-01), pages 743-748, XP055003411, ISSN: 1552-4450, DOI: 10.1038/nchembio.207 Scheme 1 -----	1-17
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/054674

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