

METHOD FOR PREPARING PEPTIDES BY ASSEMBLING MULTIPLE PEPTIDE FRAGMENTS

FIELD OF THE INVENTION

5 The present invention relates to a method for preparing peptides or polypeptides by assembling multiple peptide fragments, using native ligation of polypeptides. The invention also relates to the preparation of intermediates used in this method and said intermediates prepared by the method.

TECHNOLOGICAL BACKGROUND

10 The synthesis of polypeptides by conventional solid phase methods, amino acid by amino acid, is limited by low yields when the polypeptides synthesized are large in size. In order to overcome this limitation, it is known to assemble two polypeptides by chemical ligation in order to produce a longer polypeptide.

The total synthesis of polypeptides is increasingly useful for the preparation of proteins with well-defined structures. The chemical ligation methods provide a response to 15 this need, however they prove limited in their use and their industrial application.

Generally, in these methods, it is desired that the bond between the polypeptides assembled by ligation is native, i.e. corresponds to the natural structure of the polypeptides.

The main native ligation method currently existing is that of Kent and Dawson, described for example in the international applications WO 96/34878 and WO 98/28434. This 20 method is based on a chemoselective reaction between a (C-terminal) thioester peptide and a cysteinyl-peptide. Nevertheless, the main drawback of this method is the production of thioester peptides which requires complex chemical processes. In particular, according to an assembly method described by S. Kent (Kinetically Controlled Ligation (KCL)), it is necessary to synthesize the A-SAr, H-Cys-B-SAlk, and H-Cys-C fragments. However A-SAr type 25 fragments are difficult to produce and prove to be susceptible to hydrolysis. Furthermore, this method does not make it possible to go beyond the assembly of 3 fragments.

An alternative method is the so-called Staudinger ligation, described in the international applications WO 01/68565 and WO 01/87920. This comprises the reaction of a 30 phosphinothioester with an azide and the hydrolysis of the combined reagents to form an amide bond. However this method is difficult to apply on an industrial scale.

Another method, described in the international application WO 2007/037812, is based on the reaction of an α -keto acid with an alkoxyamine in a decarboxylative condensation reaction. However, the keto acids are molecules which are difficult to produce and to incorporate into peptides. Also, this third method is difficult to apply in peptide synthesis 35 laboratories that do not have the means for carrying out complex organic syntheses.

The publication by D. Bang, B.L. Pentelute and S.B.H. Kent, *Angew. Chem. Int. Ed.* 2006, 45, 3985–3988 proposes a synthesis route involving peptide-(thiophenylesters) with Cys-peptide-thioesters, and the publication by W. Hou et al., *Org Lett.*, (2010), 22 December 2010, proposes the formation of peptide-thioesters for the synthesis of proteins. However these methods cannot prevent competition between the reactions of the different thioesters, inevitably leading to mixtures that can be difficult to separate, therefore affecting the purity of the final product obtained, and to inevitable losses of yield.

Finally, the publication by O. Melnyk et al., *Org. Lett.*, 12(22), 5238-41 (2010) describes the native ligation of peptides by means of peptide-bis(sulphanylethyl)amino fragments. However this method has never to date been used for the synthesis of peptides with multiple fragments.

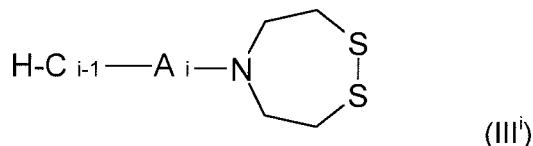
The transfer to the industrial scale of methods making it possible to implement peptide syntheses by total synthesis is a need that requires methods to be found which are simple, inexpensive, producing quality products of high purity which satisfy industrial health requirements.

For the abovementioned reasons, it has become essential to find a total synthesis method, which is coherent and can be used on an industrial scale, making it possible to synthesize a peptide chain formation of the desired length and nature. Particularly a method involving assembly from the N-terminal end towards the C-terminal end, which offers qualities of simplicity of production and purity of the peptides or polypeptides obtained.

DESCRIPTION OF THE INVENTION

It has been found, which forms the subject of the present invention, that assembling multiple peptide fragments in a one-pot method involving simple methods such as the formation of peptide-thioesters and native ligation, could lead to a total synthesis method, which is convergent, can be used on an industrial scale and corresponds to the required purity criteria.

According to the invention, the assembly method involves the synthesis of peptide-thioesters, their condensation reaction with a peptide (or polypeptide) bearing a bis(2-sulphanylethyl)amino function in the cyclic disulphide state, corresponding to the general formula:



in which

A_i represents a peptide fragment the C-terminal end of which bears a

cyclic bis(2-sulphanylethyl)amino  group hereafter called SEAoff

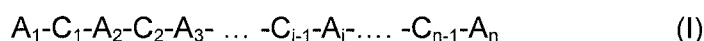
and C_{i-1} represents an amino acid residue bearing a thiol function,

as well as native chemical ligation in a reducing medium, at the C-terminal end, of the polypeptides obtained.

It is understood that the term SEAoff means non-reactive cyclic disulphide, as opposed to the reactive disulphide of structure $-N[(CH_2)_2-SH]_2$ hereafter called SEAon.

It is understood that the amino acid bearing a thiol function represented by C_{i-1} can be in particular chosen from cysteine, homocysteine, and that these amino acids bear a disulphide residue (SR') on the thiol of the amino acid, in the implementation of the reaction with the peptide-thioester.

According to the invention, the method leads to the preparation of a multiple peptide assembly comprising the amino acid residues bearing a thiol function, i.e. a peptide assembly of n fragments and of $n-1$ amino acids bearing a thiol function, represented by the formula:



in which $A_1, A_2, A_3, \dots, A_i, \dots, A_n$ are peptide fragments,
 $C_1, C_2, C_3, \dots, C_{i-1}, \dots, C_{n-1}$ are amino acid residues bearing a thiol function,
 n is comprised between 3 and 50,
preferably between 3 and 20,
or even more preferably between 3 and 10, and
 i is any integer comprised between 2 and n .

It is understood that, when $n = 3$, $C_{n-1}-A_n$ represents C_2-A_3 and $C_{i-1}-A_i$ represents C_1-A_2 .
In this case the peptide assembly of formula (I) is of structure: $A_1-C_1-A_2-C_2-A_3$.

The method according to the invention consists of the preparation of a peptide-thioester of formula:



in which A_1 is a peptide fragment and SR is an alkylated thioester residue, R being able to be an optionally substituted alkyl radical,

from a bis(2-sulphanylethyl)amino peptide A_1 -SEAoff in which SEAoff is defined as previously,

by the action of a thiol R-SH, in the presence of a cyclic disulphide reducing agent,
followed by condensation with a peptide fragment of structure:



in which C_1 , A_2 and SEAoff are defined as above and (SR') represents a disulphide residue on the thiol of the amino acid C_1 , in the presence of an aromatic thiol ArSH,

then conversion of the new peptide fragment obtained, of structure:



5 in which A_1 , C_1 , A_2 and SEAoff are as defined previously, to a peptide-thioester of formula:



in which A_1 , C_1 , A_2 and R are as defined previously, by the action of a thiol R-SH, in the presence of a cyclic disulphide reducing agent,

followed by condensation with a peptide fragment of structure:



in which SEAoff and R' and C_2 and A_3 are defined as above, in the presence of an aromatic thiol ArSH, in order to produce a peptide fragment, of structure:



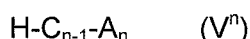
in which A_1 , C_1 , A_2 , C_2 , A_3 and SEAoff are as defined previously,

15 then reiteration of these 2 operations up to n-2 times, in order to obtain a peptide fragment of structure:



in which A_1 , A_2 , A_3 , ... A_i ..., A_{n-1} , C_1 , C_2 , C_3 ... C_{i-1} ... C_{n-2} and SEAoff are as defined previously, and

20 implementation of a native ligation reaction of this obtained peptide fragment (IV^m), with a peptide of formula:



in which C_{n-1} and A_n are as defined previously, by reduction in the presence of a cyclic disulphide reducing agent, in order to produce the multiple peptide assembly of general
25 formula (I).

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The invention is now described in more detail and non-limitatively in the description which follows.

30 By "peptides or polypeptides" is meant, in the context of the present application, a linear chain of amino acid residues (in a number greater than or equal to two) linked by peptide bonds. The "peptides or polypeptides" within the meaning of the present application can therefore for example be oligopeptides, peptides or proteins according to the conventionally accepted meaning of these words. The amino acid residues present in the polypeptides according to the invention can be chosen from the proteinogenic or non-

proteinogenic amino acid residues. Preferably, they are chosen from the twenty proteinogenic or amino acid residues.

Polypeptide notation is from the N-terminal end to the C-terminal end. The amino acid residues present along the polypeptide chain are denoted according to the usual one-letter or three-letter code. An amino acid residue is a polypeptide fragment of formula $\text{-NH-}(\text{CH-R})\text{-(C=O)-}$, in which R represents a side chain, which differs from one amino acid to another.

By "peptide fragment" is meant, in the context of the present application, a portion of polypeptide comprising at least one amino acid residue. A peptide fragment, within the meaning of the present application, can therefore be for example: a sequence of amino acid residues (such as -AHG- or -Ala-His-Gly-) if the peptide fragment comprises neither the N-terminal end nor the C-terminal end of the polypeptide; or a sequence of amino acid residues having a group at its N-terminal end (such as H-AHG- or H-Ala-His-Gly-) if the peptide fragment comprises the N-terminal end of the polypeptide; or a sequence of amino acid residues having a group at its C-terminal end (such as -AHG-OH or -Ala-His-Gly-OH) if the peptide fragment comprises the C-terminal end of the polypeptide.

It is understood that each of the peptide fragments preferably comprises only acid residues chosen from the 20 proteinogenic or amino acid residues. However, according to a particular embodiment, the peptide fragments A_i in an internal position in the sequence can also comprise one or more non-proteinogenic amino acid residues, the one or more of the peptide fragments $A_2 \dots A_i \dots A_{n-1}$ being able to bear one or more modified amino acids, the modification being put into place prior to the implementation of the method. By way of non-limitative example, the modification of the amino acids can in particular be chosen from radicals chosen from carboxylic acid residues (biotin, acetyl group, aminooxyacetic residue, a fluorophore such as tetramethylrhodamine, a metal chelating agent such as 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), a lipid such as palmitic acid, a polymer such as for example alpha-methoxy-omega-carboxy poly(ethylene glycol) or others. Or also from post-translational modifications of proteinogenic amino acids known to a person skilled in the art (methylation, phosphorylation, acetylation, glycosylation, sulphation, hydroxylation, carboxymethylation, bearing appropriate protections for solid phase incorporation, etc.), or optionally a medicament (the protein then serving as vector).

A modification can be introduced simply by using a non-proteinogenic amino acid, in particular a proteinogenic amino acid derivative, in order to introduce the modification during the solid phase synthesis of the fragment considered. By way of example, it is possible to use an Fmoc-L-Lys(Biotin)-OH, i.e. a lysine bearing a biotin on its side chain, in order to carry out the synthesis of a fragment. This fragment is then involved in the assembly in a manner identical to that which uses proteinogenic amino acid fragments.

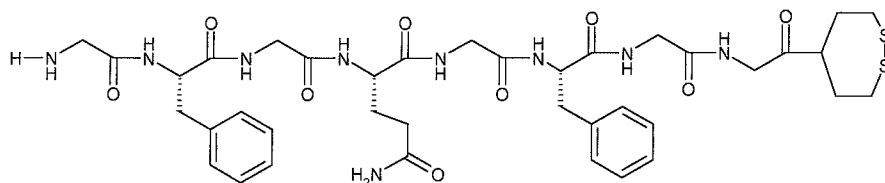
According to an embodiment of the invention, a peptide fragment A_i comprises between 2 and 300 amino acid residues, preferably between 5 and 100 amino acid residues, more particularly preferably between 8 and 50 amino acid residues.

The alkyl radical represented by R is an alkyl radical of 1 to 12 carbon atoms, linear or branched, optionally substituted by one or more groups chosen from the halogen atoms (F for example), or the CO_2H , SO_3H , CONH_2 , OH, SH, alkyloxy, alkylthio, mercaptoalkylthio radicals, an ester residue or a polyethylene glycol residue, or by an optionally substituted phenyl, or other organic groups which do not interfere with the reactions implemented. The thiol of general formula R-SH can be for example $\text{HSCH}_2\text{CH}_2\text{CO}_2\text{H}$, $\text{HSCH}_2\text{CH}_2\text{SO}_3\text{H}$, $\text{HSCH}_2\text{CH}_2\text{SH}$, $\text{HSCH}_2\text{CH}_2\text{SCH}_2\text{CH}_2\text{SH}$ or HSCH_2Ph .

The aromatic thiol is advantageously chosen from disulphide bond reducing compounds, preferably chosen from thiophenol and/or the derivatives obtained by substitution of the aromatic ring, for example 4-carboxymethylthiophenol, 4-mercaptophenylacetic acid, dithiothreitol, benzylmercaptan and mixtures thereof.

The SEAoff reducing agent can be chosen from the cyclic disulphide reducing agents. It can be in particular chosen from the phosphines (for example tris (2-carboxyethyl)phosphine), or any other cyclic disulphide reducing agent such as the thiols (for example dithiothreitol (DTT)).

An example of peptide A_1 -SEAoff can be represented by the formula:

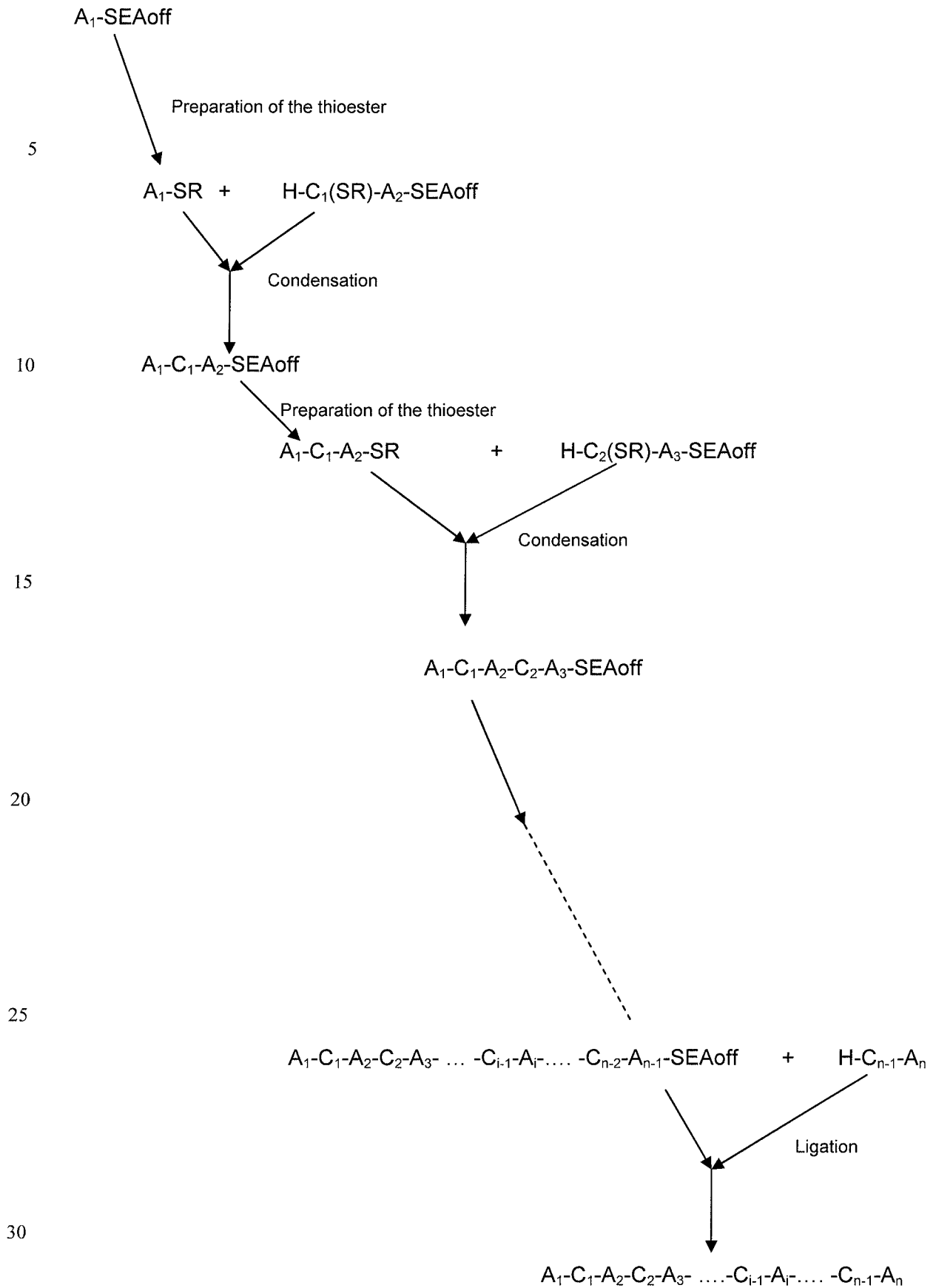


in this example, the peptide fragment A_1 is H-GFGQGFGG.

It is understood that the peptide fragment A_n may have been prepared beforehand by a sequence of operations as described above, according to the method of the invention.

According to the invention, the method for assembling multiple peptide fragments can be represented according to Diagram 1 below:

Diagram 1



All of the multiple peptide assembly preparation reactions have the great advantage of being able to be implemented in situ, in a "one pot" reaction, particularly when the number of peptide fragments n is equal to 3, without it being necessary to isolate the intermediates formed.

5 The method according to the invention can be carried out as described below, and as illustrated in the following examples.

The preparation of the peptide-thioesters of formula (II) is advantageously implemented by the action of an alkylthiol the alkyl part of which is optionally substituted on a bis(2-sulphanylethyl)amino peptide: A_1 -SEAoff, in the presence of a cyclic disulphide
10 reducing agent such as in particular a phosphine, for example tris(2-carboxyethyl)phosphine. The reaction is advantageously carried out under an inert atmosphere (under argon for example), at a pH comprised between 1 and 8, preferably at pH 4 and at a temperature comprised between 20 and 50°C. Advantageously 3-mercaptopropionic acid is used.

The condensation of the thioester of formula (II) with the peptide fragment of general
15 formula (III): $H-C_1(SR')-A_2$ -SEAoff, is advantageously carried out in the presence of an aromatic thiol (in particular 4-mercaptophenylacetic acid), by operating under an inert atmosphere (under argon for example), at a temperature comprised between 20 and 60°C. It is not necessary to isolate the obtained peptide fragment in order to use it in the following reaction.

20 The native ligation reaction of the peptide fragment (IV^m) ($A_1-C_1-A_2-C_2-A_3\ldots-C_{i-1}-A_i\ldots-C_{n-2}-A_{n-1}$ -SEAoff) with the peptide (V^n) ($H-C_{n-1}-A_n$) is implemented by the action of a cyclic disulphide reducing agent, which can preferably be a phosphine (for example tris(2-carboxyethyl)phosphine (TCEP)) a thiol compound such as 4-mercaptophenylacetic acid (MPAA), dithiothreitol (DTT), thiophenol (and its derivatives), an alkylthiol (in particular
25 benzylmercaptan) or also mixtures of several of these compounds, for example MPAA and TCEP, by operating under an inert atmosphere (under argon for example), at a temperature comprised between 20 and 60°C, preferably at a temperature of 37°C. The reaction is carried out in particular in aqueous medium, for example in a phosphate buffer. It is preferably carried out at a pH comprised between 5.5 and 8.5, more particularly preferably at a pH
30 comprised between 6 and 8 and ideally at a pH of approximately 7. The duration of the reaction depends on the reagents used, it is monitored and adjusted according to the results of liquid chromatography analysis. It is not necessary to isolate the obtained peptide fragment (IV^m) in order to use it in the native ligation reaction. It is understood that the reaction of the cyclic disulphide reducing agent can be implemented on the peptide fragment
35 of formula (IV^m) before or simultaneously with the reaction with the peptide of formula (V^n).

According to a particular embodiment, the peptide fragments of formula (IV^m) and (V^n) comprise one or more non-proteinogenic amino acid residues.

The amino acid residues of the polypeptides of formula (IV^m) and (Vⁿ) can optionally be protected by side-chain protective groups. The protection and removal of the protective radicals can be carried out according to known methods which do not alter the remainder of the molecule. More particularly according to the methods described by T. Greene and P. Wuts, *Protective groups in organic synthesis*, 2nd edition, John Wiley & Sons, Inc.

The preparation of the bis(2-sulphanylethyl)amino peptide: A₁-SEAoff can be carried out according to the method described by Melnyk, O. et al., Bis(2-sulphanylethyl)amino native peptide ligation., *Org. Lett.*, 12(22), 5238-41(2010). According to the procedure of this method, the coupling of an amino acid to a polymer resin support comprises bringing the polymer resin support into contact with an amino acid halide or with an amino acid and an activation agent, preferably chosen from PyBOP® (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), BOP, PyBroP® (bromo-tris-pyrrolidinophosphonium hexafluorophosphate), or also HATU (2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium) and more particularly preferably PyBroP. This procedure is described in more detail below, in the examples of the preparation of the starting peptide fragments.

In particular, the corresponding peptides-N(CH₂CH₂SH)₂ (SEAon peptides) are oxidized by the air, under stirring, in a 0.1M ammonium bicarbonate solution, at ambient temperature. Advantageously, they can also be obtained by oxidation in the presence of iodine or diamide, (CH₃)₂NCON=NCON(CH₃)₂.

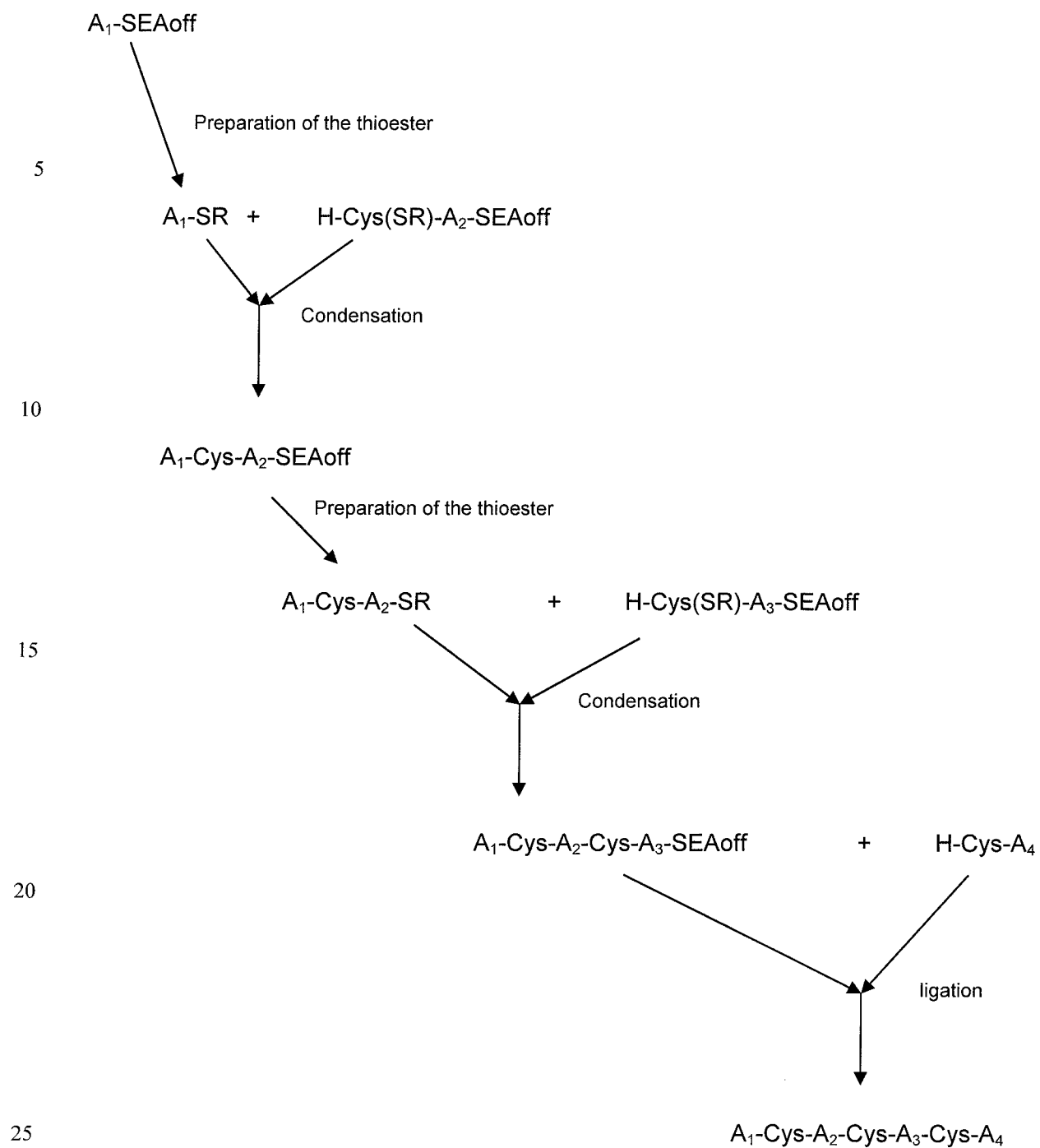
The polypeptide of formula (Vⁿ) can for example be obtained by a usual peptide synthesis method, in particular a solid phase synthesis method. It can also be obtained by means of native ligation as described in *Org. Lett.*, 12(22), 5238-41(2010).

According to preferred embodiments of the present invention, a peptide assembly of 3 or 4 fragments is prepared, of structures A₁-C₁-A₂-C₂-A₃ or A₁-C₁-A₂-C₂-A₃-C₃-A₄.

According to another preferred embodiment of the invention, the C_i amino acids bearing a thiol function are cysteine Cys residues.

In this case, according to a preferred embodiment of the invention, a peptide assembly of structure A₁-Cys-A₂-Cys-A₃ or A₁-Cys-A₂-Cys-A₃-Cys-A₄ is prepared.

According to these preferred embodiments, the peptide assembly preparation diagram can be represented according to Diagram 2 below:

Diagram 2

The present invention also relates to the preparation of the peptide-thioesters of formula (II) from bis(2-sulphanylethyl)amino peptide: A₁-SEAoff according to the method described above, by the action of a thiol R-SH, in the presence of a cyclic disulphide reducing agent. The invention also relates to the peptide-thioesters thus obtained.

5 The polypeptides of formula (I) obtained according to the invention can be used to produce a polypeptide library, for example for screening purposes.

They can also be used for the manufacture of pharmaceutical compositions, in combination with one or more compatible and pharmaceutically acceptable adjuvants or vehicles. By way of examples of pharmaceutical compositions that can be obtained
10 according to the invention, there can be mentioned medicaments that can be used for humans and for animals, and vaccine preparations.

They can also be used for producing diagnostic kits.

According to another subject of the present invention, the invention also relates to a method for manufacturing a pharmaceutical composition comprising:

- 15 – the manufacture of a polypeptide or peptide according to the method for manufacturing a polypeptide of formula (I) described previously, and
– the pharmaceutical compositions comprising a polypeptide or peptide thus prepared, in the pure state or in combination with one or more compatible and pharmaceutically acceptable adjuvants.

20 The invention also relates to a method for manufacturing a diagnostic device comprising:

- the manufacture of a polypeptide or peptide according to the method for manufacturing a polypeptide of formula (I) described previously, and
– the formulation or forming of this polypeptide in a form suitable for use for a
25 diagnostic device.

The method according to the present invention is particularly useful as it makes it possible to synthesize, in total synthesis, a peptide chain formation of the desired length and nature, and comprising amino acid units with thiol substitution of the cysteine (Cys) or homocysteine type, distributed along the chain.

30 It has the advantage of being capable of being used on an industrial scale, due to the convergent synthesis which it involves, and this type of synthesis is particularly sought after with respect to the industrialization of methods since it provides advantageous yields, and in particular little loss in yield of products already developed, the synthesis cost of which is already high.

35 According to the present invention the SEA fragments allow the synthesis of proteins from the N-terminal end to the C-terminal end by successive ligation of peptide fragments.

Assembling from the N-terminal end to the C-terminal end offers a considerable advantage, compared with the reverse strategy of assembling from the C-terminal end to the N-terminal end, in fact, in this case the method induces self-purification of the peptide

fragments which is very important in industrial synthesis.

According to another advantage the method of the invention makes it possible to use proteinogenic amino acids for the synthesis of the peptide fragments. It is therefore unnecessary to resort to the manufacture of amino acid derivatives (such as keto acids for example), which would make the synthesis considerably more difficult.

The following examples illustrate the present invention.

Example 1

One-pot assembly of 3 fragments

1- Synthesis of the **peptide 1** (**H-ILKEPVHGA-S(CH₂)₂COOH**), equivalent of **A₁-SR** with R = alkyl, by conversion of **A₁-SEAoff** in the presence of an alkylthiol R-SH, R = -(CH₂)₂COOH and a cyclic disulphide reducing agent.

The precursor peptide H-ILKEPVHGA-SEAoff was prepared as described by Ollivier N., Dheur J., Mhidia R., Blanpain A., and Melnyk O., Bis(2-sulphanylethyl)amino native peptide ligation., Org. Lett. 12(22), 5238-41 (2010).

Tris(2-carboxyethyl)phosphine hydrochloride, TCEP,HCl (921.2 mg) is dissolved in 0.2 M phosphate buffer pH = 7.3 (40 mL). 3-Mercaptopropionic acid (MPA) (2 mL) is added to this solution. The pH of the solution is readjusted to 4 with 5 N soda (approximately 3 mL).

The peptide H-ILKEPVHGA-SEAoff (40.31 mg) is dissolved in the preceding solution (40 mL). The reaction medium is placed under an inert atmosphere by means of 3 vacuum/argon cycles, and placed in a bath at 37°C.

After 22 hours, the reaction medium is transferred to a separating funnel. An aqueous solution containing 7% trifluoroacetic acid (TFA) (20 drops) is added. 4 extractions with diethyl ether are carried out. An aqueous solution containing 7% TFA (10 mL) is added and 3 new extractions with diethyl ether are carried out.

The aqueous phase is degassed for 13 minutes by bubbling argon through, before being purified in RP-HPLC (Nucleosil C18 column 120Å, 5 µm, buffer A 100% water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0 to 10% of buffer B over 5 minutes then 10 to 100% of buffer B over 150 minutes, flow rate 6 mL/minute, 215 nm). 18.7 mg of pure peptide 1 (H-ILKEPVHGA-S(CH₂)₂COOH) is obtained (Yield = 47.4%) C₄₇H₇₈N₁₂O₁₃S₁ MALDI-TOF [M+H]⁺ calculated (monoisotopic mass) 1051.56; observed 1051.70.

2- Synthesis of peptide 2 H-C(StBu)HHLEPGG-SEAoff, equivalent of H-C₁(SR)-A₂-SEAoff, with R = tBu

Standard synthesis of a SEAoff peptide on a 0.25 mmol scale, according to:

Ollivier N., Dheur J., Mhidia R., Blanpain A., and Melnyk O., Bis(2-sulphanylethyl)amino native peptide ligation., Org. Lett. 12(22), 5238-41 (2010).

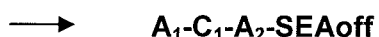
The disulphide residue -S-t.Bu is coupled using Fmoc-Cys(St.Bu)-OH, in a manner analogous to the method described in Example 2 below.

The final deprotection and cleavage of the peptide from the resin are carried out with TFA/TIS/DMS/H₂O (90/2.5/2.5/5/2.5 by volume, 25 mL) over 1 hour 30 minutes. The peptide is precipitated from a cold diethyl ether/heptane mixture (1/1 by volume), dissolved in a minimum amount of water and lyophilized. 132.6 mg of crude peptide are obtained (Yield = 38%). C₄₃H₆₉N₁₃O₁₀S₄ MALDI-TOF [M+H]⁺ calculated (monoisotopic mass) 1056.42; observed 1056.10.

Part of peptide 2 is then oxidized before purification. For this, fragment 2 (122.6 mg) and I₂ (222.52 mg, 10 eq.) are dissolved in AcOH/water 4/1 (12.5 ml). After stirring for 10 minutes, the diluted reaction medium (total volume = 37 mL) is transferred to a separating funnel containing diethyl ether (75 mL). 3 extractions with diethyl ether are carried out (3 x 75 mL). The aqueous phase is frozen and lyophilized in order to produce 94.3 mg of crude peptide.

After RP-HPLC purification (Nucleosil C18 column, 120Å, 5 µm, 215 nm, buffer A 100% water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0 to 10% of buffer B over 5 minutes, then 10 to 100% of buffer B over 150 minutes, flow rate 6 mL/minute) 40.52 mg of pure peptide is obtained (Yield = 33%) C₄₃H₆₇N₁₃O₁₀S₄ MALDI-TOF [M+H]⁺ calculated 1054.4 (monoisotopic mass); observed 1054.4.

3) A₁-SR (peptide 1) (R = CH₂CH₂CO₂H) + H-C₁(StBu)-A₂-SEAoff (peptide 2)



4-mercaptophenylacetic acid (MPAA, 33.64 mg, 0.2 mmol, aromatic thiol) is dissolved in 0.1 M phosphate buffer pH = 7.3 (1 mL). A 5 N soda solution (80 µL) is added to adjust the pH of the solution to 7.64. Peptide 1 (10.40 mg, 7.5 µmol) and peptide 2 (10.42 mg, 7.5 µmol) are dissolved together in the preceding solution (533 µL). The reaction medium is placed in a temperature-controlled bath at 37°C under an inert atmosphere.

The ligation is monitored by RP-HPLC on an Xbridge BEH C18 column (4.6 x 250 mm, 300 Å, 5 µm) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B over 30 minutes). For this, an aliquot (5 µL) of reaction medium is acidified with a solution of 10% TFA in water, extracted with diethyl ether to remove the MPAA and MPA before analysis.

4- Synthesis of peptide 3 H-CILKEPVHGV-NH₂, equivalent of H-C₂-A₃

The preparation of the peptide H-C₂-A₃ is described in the publication by: Ollivier N., Dheur J., Mhidia R., Blanpain A., and Melnyk O., Bis(2-sulphanylethyl)amino native peptide ligation., Org. Lett. 12(22), 5238-41 (2010).

5- Protocol for one-pot ligation of three fragments.

Synthesis of peptide 4 H-ILKEPVHGACHHLEPGGCILKEPVHGV-NH₂, equivalent of A₁-C₁-A₂-C₂-A₃

Preparation of peptide 4 from A₁-C₁-A₂-SEAoff and peptide 3 (H-C₂-A₃)

A₁-C₁-A₂-SEAoff converted to A₁-C₁-A₂-SEAon by reduction in the presence of TCEP (tris(2-carboxyethyl)phosphine)

After reacting for 5 hours 30 minutes, TCEP,HCl (tris(2-carboxyethyl)phosphine hydrochloride) (57.34 mg, 0.2 mmol) is dissolved in a 0.1 M phosphate buffer pH = 7.3 (1 mL). A 5 N soda solution (140 µL) is added to adjust the pH of the solution to 7.33.



Peptide 3 (16.17 mg, 11.2 µmol) is dissolved in the preceding solution (533 µL) then added to the preceding reaction medium which is again placed under an inert atmosphere at 37°C.

The ligation is monitored by RP-HPLC on an Xbridge BEH C18 column (4.6 x 250 mm, 300 Å, 5 µm) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B over 30 minutes). For this, an aliquot (5 µL) of reaction medium is acidified with a 10% TFA solution in water, extracted with diethyl ether to remove the MPAA (4-mercaptophenylacetic acid) before analysis.

When the reaction is complete, the reaction medium is diluted with water containing 0.05% TFA (3 mL) and a 10% TFA solution in water (15 drops) is added. After 5 extractions with diethyl ether (5 x 8 mL), the aqueous phase is degassed for 10 minutes by bubbling argon through. After purification by RP-HPLC on a Nucleosil C18 column 120Å, 5 µm (215 nm, flow rate 6 mL/minute, buffer A: water containing 0.05% TFA, buffer B: CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0-10% of buffer B over 5 minutes, then 10-100% of buffer B over 150 minutes) 16.2 mg of pure peptide 4 is obtained (59.2%).

Example 2

1-Synthesis of K1(fragment 1)-MPA

a-Synthesis of K1(fragment 1)-SEAoff



25

The SEA resin (0.5 mmol, 0.175 mmol/g) is conditioned in dichloromethane (DCM). Fmoc-Lys(Boc)-OH (2.342 g, 5 mmol) is dissolved in DCM and a few drops of dimethylformamide (DMF) in order to aid solubilization, then the solution is added to the resin. PyBrop (2.331 g, 5 mmol) is dissolved in a minimum amount of DCM then added to the resin. Diisopropylethylamine (DIEA) (2.613 mL, 15 mmol) is then added to the resin and cleavage takes 2 hours (total volume < 5 mL). The resin is then washed for 3 x 2 minutes with DCM. The resin is then treated with 10% Ac₂O/5% DIEA/DCM (10 mL, 2 minutes) then (10 mL, 20 minutes). The resin is then washed for 5 x 2 minutes with DCM.

30

Fragment 1 is assembled on a portion of the preceding resin (0.25 mmol, 0.175 mmol/g) with a peptide synthesizer (CEM μ Waves, Saclay, France) without using microwaves, using the Fmoc/*tert*-butyl strategy. The coupling is carried out with the amino acids (0.2 M, 4 eq), the activator HBTU (0.5 M, 3.6 eq.) and the base DIEA (2 M, 8 eq). The final deprotection and cleavage of the peptide from the resin are carried out with TFA/TIS/DMS/thioanisole/H₂O (90/2.5/2.5/2.5/2.5 by volume, 25 mL) for 2 hours 30 minutes. The peptide is precipitated from a cold diethyl ether/heptane mixture (1/1 by volume), dissolved in a minimum amount of water and lyophilized. 288 mg of crude peptide is obtained (Yield = 32%). C₁₂₀H₂₁₆N₃₆O₃₁S₄ LC-MS [M+H]⁺ calculated (average mass) 2788.5; observed 2788.1.

A portion of fragment 1 is then oxidized before purification. For this, fragment 1 (95.7 mg) is dissolved in AcOH/water 1/4 (47.9 mL). 197 mM I₂ in DMSO (270 μ L, 50 μ mol, 2 eq.) is added to the preceding solution. After stirring for 30 seconds, 64.8 mM dithiothreitol (DTT, 823 μ L, 50 μ mol, 2 eq) is added to consume the remaining I₂ and injected directly into RP-HPLC.

After RP-HPLC purification (Nucleosil C18 column 120Å, 5 μ m, buffer A 100% water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0 to 20% of buffer B in 5 minutes, then 20 to 40% in 40 minutes, flow rate 6 mL/minute, 215 nm) 25.8 mg of pure peptide is obtained (Yield = 27%) C₁₂₀H₂₁₄N₃₆O₃₁S₄ MALDI-TOF [M+H]⁺ calculated (monoisotopic mass) 2784.52; observed 2784.6.

b-Synthesis of K1(fragment 1)-MPA



Tris(2-carboxyethyl)phosphine hydrochloride (TCEP,HCl, 229.63 mg, 0.8 mmol) is solubilized in 0.2 M sodium phosphate buffer pH = 7.3 (10 mL). Mercaptopropionic acid (MPA, 0.5 mL) is added. NaOH 5M (340 μ L) is added to adjust the pH to 3.98.

Fragment 1 K1-SEAoff (24.67 mg, 6.9 μ mol) is dissolved in the preceding solution (10.3 mL). The reaction medium is heated to 37°C under an argon atmosphere. The progress of the reaction is monitored by RP-HPLC on an Xbridge BEH C18 column (215 nm, 1 mL/minute, 30°C, eluent A water containing 0.05% trifluoroacetic acid (TFA), eluent B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B in 30 minutes). For this, aliquots are acidified with an aqueous solution containing 10% TFA, extracted with Et₂O in order to remove the excess MPA before analysis.

After completion of the reaction, the reaction medium is diluted with water (10 mL) and an aqueous solution containing 10% TFA (5 mL) is added. After 3 extractions with Et₂O

(3 × 15 mL) and bubbling argon through for 15 minutes, RP-HPLC purification takes place on a Nucleosil C18 column 120 Å, 5 µm (215 nm, 6 mL/minute, ambient temperature, eluent A water containing 0.05% TFA, eluent B CH₃CN/water 4/1 containing 0.05% TFA, 0-10% B in 5 minutes, then 10-100% B in 150 minutes) provides 9.7 mg of pure K1(fragment 1)-MPA (41%).

2-Synthesis of StBu-K1(fragment 2)-SEAoff



The resin SEA (0.5 mmol, 0.175 mmol/g) is conditioned in DCM. Fmoc-Tyr-OH (2.298 g, 5 mmol) is dissolved in DCM (<5 mL) and a few drops of DMF to aid solubilization, then added to the resin. PyBrop (2.331 g, 5 mmol) is dissolved in a minimum amount of DCM, then added to the resin. DIEA (2.614 mL, 15 mmol) is then added to the resin and the coupling takes 2 hours. The resin is then washed for 4 x 2 minutes with DCM. The resin is then treated with 10% Ac₂O/5% DIEA/DCM (10 mL, 2 minutes) then (10 mL, 20 minutes). The resin is then washed for 5 x 2 minutes with DCM.

Fragment 2 is assembled on the preceding resin (0.5 mmol, 0.175 mmol/g) with a peptide synthesizer (CEM µWaves, Saclay, France) without using microwaves, using the Fmoc/*tert*-butyl strategy. The coupling is carried out with the amino acids (0.2 M, 4 eq.), the activator HBTU (0.5 M, 3.6 eq.) and the base DIEA (2 M, 8 eq.). The washing solvent (DMF) as well as the Fmoc-Met-OH solvent contain 1% thioanisole for maximum prevention of the oxidation of the methionine of the sequence.

The resin is separated into 2 after the glutamine in position 2 (0.25 mmol) in order to couple the Fmoc-Cys(StBu)-OH manually. To do this, the resin is washed with DMF for 4 x 2 minutes, weighed in DMF and divided in 2. HBTU (379.3 mg, 1 mmol) is dissolved in DMF (1100 µL). HOBt (135 mg, 1 mmol) is dissolved in DMF (500 µL) and added to HBTU. Fmoc-Cys(StBu)-OH (431.6 mg, 1 mmol) is dissolved in DMF (500 µL) and added to the HBTU/HOBt mixture. DIEA (522.7 µL, 3 mmol) is then added to the mixture. After stirring for 1 minute the mixture is added to the resin and coupling takes 45 minutes. The resin is then washed with DMF for 4 x 2 minutes. Deprotection of the Fmoc N-terminal is carried out by treatment with 20% piperidine in DMF (15 minutes then 5 minutes). The resin is then washed for with DCM for 4 x 2 minutes then with Et₂O for 3 x 2 minutes and dried.

The final deprotection and cleavage of the peptide from the resin are carried out with TFA/TIS/DMS/Thioanisole/H₂O (90/2.5/2.5/2.5/2.5 by volume, 25 mL) for 2 hours 30 minutes. The peptide is precipitated from a cold diethyl ether/heptane mixture (1/1 by volume), dissolved in a minimum amount of water and lyophilized. 366.14 mg of crude peptide are obtained (yield = 35.6%). C₁₅₆H₂₃₁N₄₁O₄₄S₅ MALDI-TOF [M+H]⁺ calculated (monoisotopic resolution) 3543.6; observed 3542.2.

A portion of fragment 2 is then oxidized before purification. For this, fragment 2 (67 mg) is dissolved in AcOH/water 1/4 (35 mL) then 144 mM I₂ in DMSO (223 µL) is added. After stirring for 30 seconds, 64.8 mM DTT in AcOH/water 1/4 (503 µL) is added to consume the excess I₂. After bleaching the solution, the medium is injected into RP-HPLC (Nucleosil C18 column, 120 Å, 5 µm, 2 x 28 cm, 215 nm, buffer A 100% water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0 to 10% of buffer B in 5 minutes, then 10 to 100% B in 150 minutes, flow rate 6 mL/minute) 11.5 mg of pure peptide is obtained (yield = 17.2 %) C₁₅₆H₂₂₉N₄₁O₄₄S₅ LC-MS [M+H]⁺ calculated (monoisotopic resolution) 3541.6; observed 3541.9.

3-Synthesis of K1(fragment 3)



Fragment 3 is assembled on Novasyn TGR resin (0.5 mmol, 0.25 mmol/g) with a peptide synthesizer (CEM µWaves, Saclay, France) without using microwaves, using the Fmoc/*tert*-butyl strategy. The coupling is carried out with the amino acids (0.2 M, 4 eq.), the activator HBTU (0.5 M, 3.6 eq.) and the base DIEA (2 M, 8 eq.).

The final deprotection and cleavage of the peptide from the resin are carried out with TFA/EDT/H₂O/TIS (94/2.5/2.5/1 by volume, 30 mL) for 2 hours 30 minutes. The peptide is precipitated from a cold diethyl ether/heptane mixture (1/1 by volume), dissolved in a minimum amount of water and lyophilized. After RP-HPLC purification (Vydac C18 column 50 cm x 2 cm, 280 nm, buffer A 100% water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0 to 20% of buffer B in 10 minutes then 20 to 50% of buffer B in 60 minutes, flow rate 30 mL/minute) 272 mg of pure peptide is obtained (yield = 13%) C₁₅₈H₂₃₉N₄₇O₅₂S₄ MALDI-TOF [M+H]⁺ calculated (monoisotopic resolution) 3755.6; observed 3755.7.

4-One-pot ligation protocol:

Synthesis of peptide 4 K1(125-209)

Guanidine HCl (573.24 mg, 6 mmol) is dissolved in 0.1 M phosphate buffer pH = 7.3 (1 mL). 4-mercaptophenylacetic acid (MPAA, 33.68 mg, 0.2 mmol) is dissolved in the latter solution (1 mL). NaOH 5M (150 µL) is added to adjust the pH of the solution to 7.53.

K1(fragment 1)-MPA (9.71mg, 2.8 µmol) and StBu-K1(fragment 2)-SEA(10.65 mg, 2.6 µmol) are dissolved together in the preceding solution (185 µL). The reaction medium is placed in a temperature-controlled bath at 37°C in a glove box.

Ligation is monitored by RP-HPLC on an Xbridge BEH C18 column (4.6 x 250 mm, 300 Å, 5 µm) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B in 30 minutes). For this, an aliquot (1 µL)

of reaction medium is acidified with an aqueous solution containing 10% TFA, extracted with Et₂O to remove the MPAA and MPA before analysis.

After reacting for 20 hours, guanidine HCl (573.15 mg, 6 mmol) is dissolved in 0.1 M phosphate buffer, pH = 7.3 (1 mL). *Tris*(2-carboxyethyl) phosphine hydrochloride (TCEP, HCl, 57.59 mg, 0.2 mmol) is dissolved in the latter solution (1 mL). 5M NaOH is added to adjust the pH of the solution to 7.27.

K1(fragment 3) (16.46 mg, 3.9 μmol) is dissolved in the preceding solution (185 μL) then added to the preceding reaction medium which is again placed under an inert atmosphere at 37°C.

The ligation is monitored by RP-HPLC on an Xbridge BEH C18 column (4.6 × 250 mm, 300 Å, 5 μm) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B in 60 minutes). For this, an aliquot (1 μL) of reaction medium is acidified with an aqueous solution containing 10% TFA, extracted with Et₂O to remove the MPAA and MPA before analysis.

C₄₁₈H₆₄₈N₁₂₂O₁₂₇S₇ MALDI-TOF [M+H]⁺ (average mass) calculated 9640.01, found 9639.

When the reaction is complete, the reaction medium is diluted with water (4 mL) and an aqueous solution containing 10% TFA (1 mL) is added. After 4 extractions with Et₂O (4 × 8 mL), the aqueous phase is degassed for 10 minutes by bubbling argon through, diluted with water (36 mL) and filtered. The frit is rinsed with AcOH (1 mL) and rinsed with water (20 mL). The mixture is purified by RP-HPLC on a Vydac C18 column 300 Å, 5 μm (215 nm, 20 mL/minute, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-20% B in 5 minutes, then 20-60% B in 80 minutes) 11.46 mg of pure K1 (125-209) is obtained (Yield = 39.4%).

5-Folding of K1(125-209)

K1(125-209) (3.99 mg, 0.355 μmol) is dissolved in 10 mM PBS buffer, 138 mM NaCl, 2.7 mM KCl pH = 7.4 containing 10% by volume of glycerol, 1 mM reduced glutathione, 0.2 mM oxidized glutathione (10 mL).

The reaction medium is placed at 4°C.

The folding is monitored by LC-MS on an Xbridge BEH C18 column (4.6 × 250 mm, 300 Å, 5 μm) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.1 % TFA, buffer B CH₃CN/water 4/1 containing 0.1 % TFA, 0-100% B in 60 minutes).

After 65 hours, the reaction medium is ultracentrifuged at 8000 rcf, at 4°C for 15 minutes then at 9000 rcf, at 4°C for 15 minutes in order to remove some aggregates.

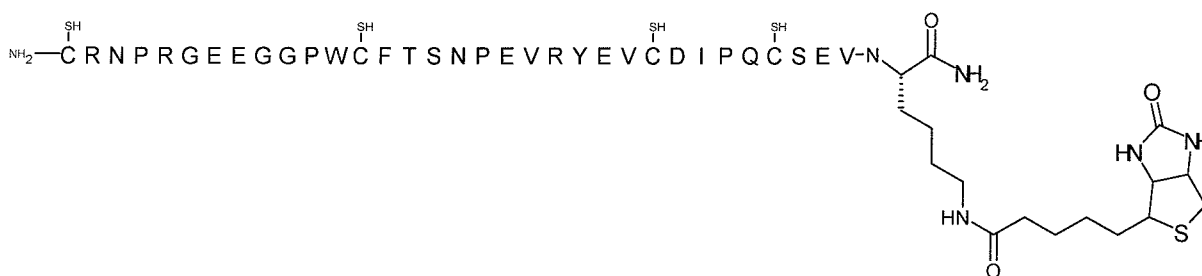
The reaction medium (7.6 mL) is then transferred in several goes into a 2 mL Vivaspin ultrafiltration system with an MWCO of 3000 and ultrafiltered at 12000 rcf, at 12°C for 3 hours 20 minutes. Once concentrated, the reaction medium is approximately 800 μL.

It is then dialyzed at 4°C in a dialysis cassette with an MWCO cut-off of 3500 with a 10 mM PBS buffer, 138 mM NaCl, 2.7 mM KCl pH = 7.4 containing 10% by volume of glycerol (1L) overnight. The reaction medium is approximately 1 mL after dialysis.

5 Folded K1 (125-209) is assayed at 562 nm using the BCA Protein Assay kit (Pierce) producing a BSA standard range. The estimated concentration of K1 (125-209) is 137 μ M.

Example 3

1-Synthesis of K1 (fragment 3)-biotin



10 Fmoc-Lys(Biotin)-OH (2 $\times$ 89 mg, 2 $\times$ 0.15 mmol) is coupled twice on PAL-ChemMatrix resin (0.1 mmol, 0.43 mmol/g) using TBTU (2 $\times$ 30.5 mg, 2 $\times$ 0.15 mmol) and DIEA (2 $\times$ 50 μ L, 2 $\times$ 0.45 mmol) for 2 hours in NMP. After washings with NMP (3 $\times$ 2 minutes) and DMF (3 $\times$ 2 minutes), the 3-biotin fragment is assembled on two 50 μ mol columns with a peptide synthesizer (INTAVIS) using the Fmoc/*tert*-butyl strategy.

15 The couplings are carried out using 4 eq. of each amino acid, 3.6 eq. HBTU, and 8 eq. DIEA. A stage of capping is carried out after each coupling with Ac₂O/DIEA. On completion of the synthesis, the resin is washed with CH₂Cl₂, diethyl ether (2 $\times$ 2 minutes) and dried under vacuum.

20 The final deprotection and cleavage of the peptide from the resin are carried out with TFA/EDT/H₂O/TIS (94/2.5/2.5/1 by volume, 2 $\times$ 5mL) for 2 hours. The peptide is precipitated from a cold diethyl ether/heptane mixture (1/1 by volume), dissolved in a minimum amount of water and lyophilized. After RP-HPLC purification (Nucleosil C18 column 50 cm $\times$ 2 cm, 215 nm, buffer A 100% water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, gradient: 0 to 15% of buffer B in 5 minutes then 15 to 35% of buffer B in 60 minutes, flow rate 6 mL/minute) 36.75 mg of pure peptide is obtained (yield = 8 %)

25 C₁₇₄H₂₆₅N₅₁O₅₅S₅ MALDI-TOF [M+H]⁺ calculated (average mass) 4112.68; observed 4114.3.

2-One-pot ligation protocol:

Synthesis of a 4 K1(125-209) K-biotin peptide

30 Guanidine HCl (573.28 mg, 6 mmol) is dissolved in 0.1 M phosphate buffer pH = 7.3 (1 mL). 4-mercaptophenylacetic acid (MPAA, 33.59 mg, 0.2 mmol) is dissolved in the latter solution (1 mL). NaOH 5M (80 μ L) is added to adjust the pH of the solution to 7.40.

The K1(fragment 1)-MPA (2.76 mg, 0.8 μ mol) and the StBu-K1(fragment 2)-SEA (3.30 mg, 0.8 μ mol) are dissolved together in the preceding solution (57 μ L). The reaction medium is placed in a temperature-controlled bath at 37°C in a glove box.

5 The ligation is monitored by RP-HPLC on an Xbridge BEH C18 column (4.6 x 250 mm, 300 Å, 5 μ m) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B in 30 minutes). For this, an aliquot (1 μ L) of reaction medium is acidified with an aqueous solution containing 10% TFA, extracted with Et₂O to remove MPAA and MPA before analysis.

10 After reacting for 24 hours 40 minutes, guanidine.HCl (573.18 mg, 6 mmol) is dissolved in 0.1 M phosphate buffer, pH = 7.3 (1 mL). *Tris*(2-carboxyethyl)phosphine hydrochloride (TCEP.HCl, 57.80 mg, 0.2 mmol) is dissolved in the latter solution (1 mL). 5M NaOH is added to adjust the pH of the solution to 7.40.

15 The K1(fragment 3) (5.50 mg, 1.2 μ mol) is dissolved in the preceding solution (57 μ L) then added to the preceding reaction medium which is again placed under an inert atmosphere at 37°C.

20 The ligation is monitored by RP-HPLC on an Xbridge BEH C18 column (4.6 x 250 mm, 300 Å, 5 μ m) (215 nm, 1 mL/minute, 30°C, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA, 0-100% B in 60 minutes). For this, an aliquot (1 μ L) of reaction medium is acidified with an aqueous solution containing 10% TFA, extracted with Et₂O to remove MPAA and MPA before analysis.

$C_{434}H_{674}N_{126}O_{130}S_8$ [M+H]⁺ expected (average mass) 9994.49, observed 9994.41.

25 On completion of the reaction, the reaction medium was diluted with water and an aqueous solution containing 10% TFA. After extractions with Et₂O, the aqueous phase was degassed by bubbling argon through, diluted with water, filtered and purified by RP-HPLC on a Vydac C18 column 300 Å, 5 μ m (215 nm, 20 mL/minute, buffer A water containing 0.05% TFA, buffer B CH₃CN/water 4/1 containing 0.05% TFA).

Fragment 1 and fragment 2: K1(fragment 1)-MPA and StBu-K1(fragment 2)-SEA are prepared as described previously in Example 2.

Examples of preparation of the starting peptide fragments

30 A) The synthesis of the SEAon peptide fragments is carried out according to Ollivier N., Dheur J., Mhidia R., Blanpain A., and Melnyk O., Bis(2-sulphanylethyl)amino native peptide ligation., Org. Lett. 12(22), 5238-41 (2010).

By way of illustration the preparation of the peptide fragments

(1a): H-ILKEPVHGG-N(CH₂CH₂SH)₂,
 35 (1b): H-ILKEPVHGA-N(CH₂CH₂SH)₂,
 (1c): H-ILKEPVHGV-N(CH₂CH₂SH)₂ and
 (1d): H-ILKEPVHGY-N(CH₂CH₂SH)₂

is carried out in solid phase

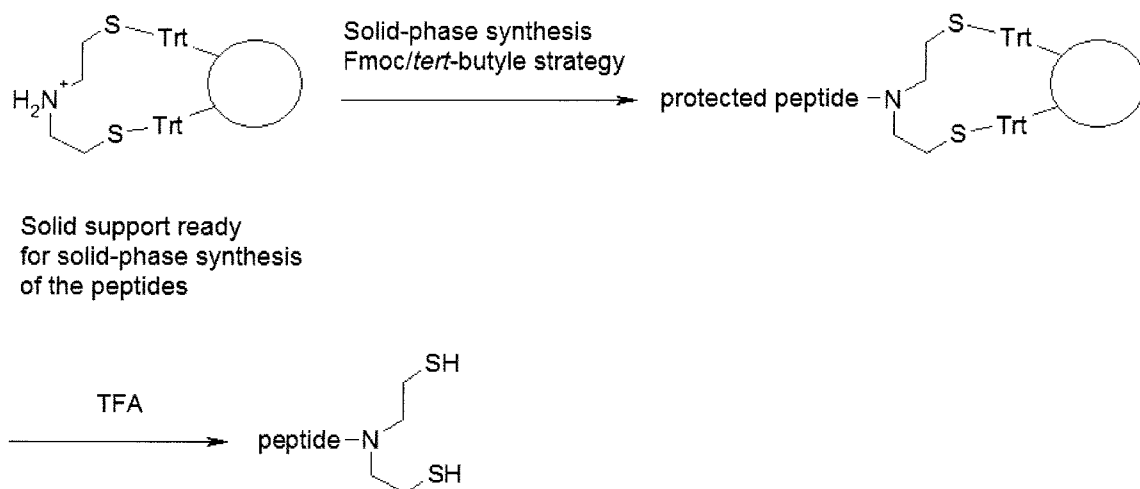
Peptide fragment (1a) is obtained from the primer support prepared with glycine.

Peptide fragment (1b) is obtained from the primer support prepared with alanine.

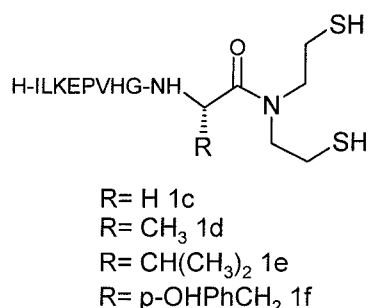
Peptide fragment (1c) is obtained from the primer support prepared with valine.

- 5 Peptide fragment (1d) is obtained from the primer support prepared with tyrosine.

The synthesis of the polypeptides can be summarized as follows:



The peptide fragments thus obtained have the following general formula:



10

Solid phase synthesis of the different polypeptides is carried out using the Fmoc/*tert*-butyl strategy on resins (scale of 0.1 mmol) on a microwave peptide synthesizer (CEM μ WAVES, Saclay, France). The coupling is carried out in using a 5-times molar excess of each amino acid, the activator HBTU is used with a 4.5-times molar excess and the base DiEA is used with a 10-times molar excess.

15

The final deprotection and cleavage of the polypeptide from the resin are carried out with 10 mL of a TFA/TIS/DMS/H₂O mixture (92.5/2.5/2.5/2.5 by volume) for 1 hour. The polypeptide is then obtained by precipitation from 100 mL of a diethyl ether/heptane mixture (1/1 by volume), dissolved in H₂O then lyophilized.

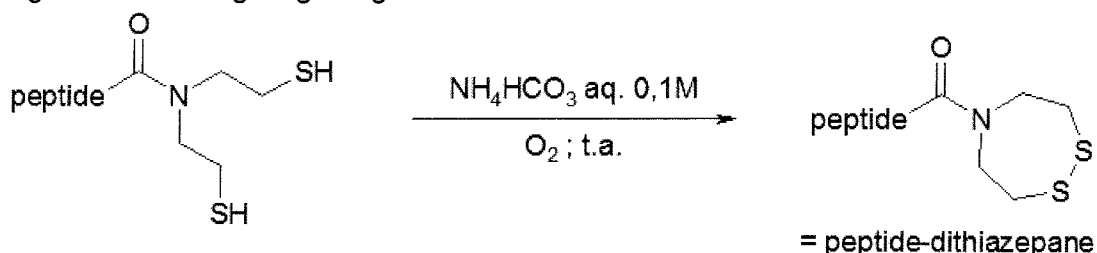
The purity of each peptide fragment is determined by HPLC (91% for peptide fragment (1a) with a glycine, 83% for peptide fragment (1b), 80% for (1c), and 88% for (1d)). MALDI-ToF analysis of the peptide fragments is consistent with the structure of the expected peptide fragment (Peptide fragment (1a) $C_{47}H_{81}N_{13}O_{11}S_2$ $[M+H]^+$ calculated 1068.6 Da, observed 1068.5. Peptide fragment (1b) $C_{48}H_{83}N_{13}O_{11}S_2$ $[M+H]^+$ calculated 1082.6 Da, observed 1082.4. Peptide fragment (1c) $C_{50}H_{87}N_{13}O_{11}S_2$ $[M+H]^+$ calculated 1110.6 Da, observed 1110.5). Peptide fragment 1(d) $C_{54}H_{87}N_{13}O_{12}S_2$ $[M+H]^+$ calculated 1174.6 Da, observed 1174.6).

Purification of the polypeptides is carried out on a Nucleosil C18 column in acetonitrile- H_2O (80-20) in TFA, with a gradient of 0 to 30 % in 30 minutes for peptide fragment (1a), and a gradient of 0 to 10 % in 5 minutes then 10 to 25% in 25 minutes for peptide fragments (1b) and (1c).

The purity determined by HPLC is 96% for peptide fragment (1a) with an overall yield of 35%, 97% for peptide fragment (1b) with a yield of 31%, and of 99% for peptide fragment (1c) with a yield of 27%.

B) oxidation of peptide fragments (1a), (1b), (1c) and (1d) to dithiazepanes

Peptide fragments (1a), (1b), (1c) and (1d) as obtained previously are oxidized according to the following diagram general:



Each peptide fragment is cleaved from the solid support by the action of a TFA/DMS/TiS/ H_2O solution (92.5/2.5/2.5/2.5; v/v). The peptide fragment is then precipitated from a large volume of a diethyl ether/heptane mixture (1/1; v/v) and washed twice using this solution. The crude peptide fragment lyophilized after the cleavage stage is then dissolved in a 0.1M ammonium bicarbonate solution previously degassed for 10 minutes by bubbling nitrogen through (1 mg/mL).

The mixture is then left at ambient temperature under vigorous stirring. The development of the reaction is followed by MALDI-TOF mass spectrometry until the complete disappearance of the reduced form of the polypeptide considered. The polypeptide is finally purified by RP-HPLC (gradient eluent A (H_2O /0.05% TFA)/eluent B (80% Acetonitrile/20% H_2O /0.05% TFA): 0 to 10% in 10 minutes then 10% to 25 % in 25 minutes) then frozen and lyophilized.

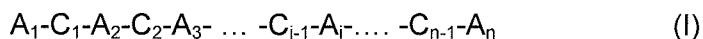
The table below summarizes the results obtained (MALDI-TOF analysis).

5

peptide fragment	m/z $[M+H]^+$ _{calc.}	M/z $[M+H]^+$ _{obs.}	Final yield(%)
(1a)	1066.6	1066.6	17
(1b)	1080.6	1080.6	13
(1c)	1108.6	1108.6	23
(1d)	1172.6	1172.6	20

CLAIMS

1. A method for preparing a peptide assembly of n fragments and n-1 amino acids bearing a thiol function, represented by the formula:



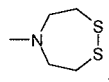
5 in which $A_1, A_2, A_3, \dots, A_i, \dots, A_n$ are peptide fragments,
 $C_1, C_2, C_3, \dots, C_{i-1}, \dots, C_{n-1}$ are amino acid residues bearing a thiol function,
 n is comprised between 3 and 50, preferably between 3 and 20, or between 3 and 10, and
 i is any integer comprised between 2 and n,

characterized by implementation of the preparation of a peptide-thioester of formula:



in which A_1 is a peptide fragment and SR is an alkyl thioester residue, R being able to be an optionally substituted alkyl radical,

starting from a bis(2-sulphanylethyl)amino peptide: $A_1-SEAoff$ in which SEAoff is a cyclic bis(2-sulphanylethyl)amino group



15 by action of a thiol R-SH, in the presence of a cyclic disulphide reducing agent,
 followed by condensation on a peptide fragment of structure:



in which C_1, A_2 and SEAoff are defined as above and (SR') represents a disulphide residue on the thiol of the amino acid C_1 , in the presence of an aromatic thiol ArSH,

followed by conversion of the new peptide fragment obtained, of structure:



in which A_1, C_1, A_2 and SEAoff are as defined previously, to a peptide-thioester of formula:



in which A_1, C_1, A_2 and R are as defined previously, by action of a thiol R-SH, in the presence of a cyclic disulphide reducing agent,

25 and condensation on a peptide fragment of structure:



in which SEAoff and R' and C_2 and A_3 are defined as above, in the presence of an aromatic thiol ArSH, in order to produce a peptide fragment of structure:



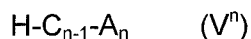
30 in which A_1, C_1, A_2, C_2, A_3 and SEAoff are as defined previously,

and reiteration of these 2 operations up to n-2 times, in order to obtain a peptide fragment of structure:



5 in which $A_1, A_2, A_3, \dots, A_i, \dots, A_{n-1}, C_1, C_2, C_3, \dots, C_{i-1}, \dots, C_{n-2}$ and SEAoff are as defined previously, followed by

implementation of a native ligation reaction of this obtained peptide fragment (IV^m), with a peptide of formula:



10 in which C_{n-1} and A_n are as defined previously, by reduction in the presence of a cyclic disulphide reducing agent, in order to produce the multiple peptide assembly of general formula (I).

2. A method according to claim 1, characterized in that a peptide assembly of 3 or 4 fragments is prepared, of structures $A_1-C_1-A_2-C_2-A_3$ or $A_1-C_1-A_2-C_2-A_3-C_3-A_4$.

3. A method according to claim 2, characterized in that a peptide assembly of 3
15 fragments is prepared in a "one pot" reaction.

4. A method according to one of claims 1 to 3, characterized in that $C_1, C_2, C_3, \dots, C_{i-1}, \dots, C_{n-1}$ represent Cys residues.

5. A method according to one of claims 1 to 4, characterized in that one or more of the peptide fragments $A_2, \dots, A_i, \dots, A_{n-1}$ can bear one or more modified amino acids.

20 6. A method according to one of claims 1 to 5, characterized in that the condensation of the peptide-thioester with a peptide fragment of structure (III) is carried out in the presence of 4-mercaptophenylacetic acid.

7. Method for manufacturing a pharmaceutical composition characterized in that it comprises:

- 25
- the manufacture of a polypeptide or peptide according to the method for manufacturing a polypeptide of formula (I) according to one of claims 1 to 5, and
 - the preparation of pharmaceutical compositions comprising a polypeptide or peptide thus prepared, in the pure state or in combination with one or more compatible and pharmaceutically acceptable adjuvants.

30 8. A method for manufacturing a diagnostic device characterized in that it comprises:

- the manufacture of a polypeptide or peptide according to the method for manufacturing a polypeptide of formula (I) according to one of claims 1 to 5, and
- the formulation or forming of this polypeptide in a form suitable for use for a diagnostic device.