

This invention relates to novel cytotoxic T lymphocyte-inducing lipopeptides.

Said invention relates also to the use of such lipopeptides as vaccines.

5           Most vaccines used induce a response through antibodies. However, it has been shown that cytotoxic lymphocytes can effectively protect mice against various pathogenic micro-organisms (Skehel et al., Proc. Natl. Acad. Sci. USA, 1982, 79: 968; Lukacher et al., Exp. Med. 10           1984, 160:814). This has also been shown for human cytotoxic T lymphocytes raised against cytomegaloviruses (Quinnan et al., N. Engl. J. Med. 1982, 307: 7; Rock et al., Am. J. Med. 1984, 76:385). However, little is known about inducing immunity due to lymphocytes.

15           Some authors have tried to induce cytotoxic T lymphocytes (CTL) in vivo using peptides derived from ovalbumin (Carbone et al. J. Exp. Med. 169:603,

DA.

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Ishioka et al. 1989, J. Immunol. 143:1094). These authors obtained immunizations, but these results are specific for peptides derived from ovalbumin.

5 AICHELE et al. ((1990) J. Exp. Med. 171:1815) succeeded, for their part, in inducing a cytolytic T response by repeated injections in vivo of a synthetic peptide in emulsion in incomplete Freund's adjuvant. These authors do not indicate the importance of the adjuvant under their immunization conditions. However, 10 they suggest that an adjuvant is necessary for obtaining such a response.

The application EP-203,676 relates to a vaccine intended to induce a T cell mediated response , comprising some peptide-fatty acid conjugates . The 15 fatty acid which is used is the palmitic acid . However, this vaccine comprises also Freund's adjuvant.

To the knowledge of the applicant, only DERES et al. (Nature, Volume 342, 30 November 1989) have described the use of a synthetic lipopeptide to induce 20 cytotoxic T lymphocytes (CTL) in vivo. In this article,

the NP 147-158 fragment of a nucleoprotein of the influenza virus is coupled to tripalmitoyl-S-glyceryl-cysteinylserylserine (P3CSS). It is shown that the NP 147-158 peptide-P3CSS lipid conjugate induces a CTL response against target cells infected by the influenza virus, whereas mice immunised with the NP 147-158 peptide alone or with the Ser Ser-NP 147-158 peptide do not generate cytotoxic T lymphocytes against this virus.

It should also be noted that lipopeptides have already been used to induce immunological reactions to specific antigens, but the responses generated involve the synthesis of antibodies and not T lymphocyte responses.

HOPP (Molecular Immunology, 21, 13-16, 1984) has shown that antibodies raised against a determinant of the hepatitis B virus could be obtained by immunising rabbits using a molecule consisting of a peptide of 15 amino acids corresponding to the antigenic determinant of the hepatitis B virus and of a pseudolipide residue, dipalmitoyl lysine.



The application EP-93 851 describes some lipopeptides comprising a peptidic sequence of 6 to 50 amino-acids bound to a lipophylic part. This lipophylic part can be a fatty acid such as palmitic, stearic, behenic, oleic or mycolic acids. It is mentioned that these lipopeptides induce the antibodies synthesis.

The publication of Wiesmüller et al (Vaccine, volume 7, n°1, 29-33, 1989) describes the use of a lipopeptide comprising a part of the sequence of the FMDV (VP1) virus and the P3CSS lipid to induce the synthesis of antibodies.

The abstract of the publication of Jacob et al; (Chemical Abstracts, vol. 104, n° 21, 472, abstract 184.455 x , 1986) relates to the induction of the antibodies synthesis by a lipopeptide comprising the tetanus toxine bound to a dipalmitoyl rest.

The abstract of the publication of Watari et al. (Chemical Abstracts, vol. 106, p 516, abstract n° 154 381 u, 1987) relates to the use of some peptides corresponding to the N-terminal region of the D glycoprotein of the HSV virus coupled to the palmitic acid. It is clearly indicated in said publication that there is induction of a T cells mediated response but that this response is not due to



cytotoxic T lymphocytes.

Two other references relate to the synthesis and the structural study of lipopeptides.

The International Application WO 89 10 348 relates to some fatty acids derivated lipopeptides, such as the aminoicosanic, aminodecanoic, aminotetradecanoic, bromodecanoic and bromododecanoic acids.

It is mentioned that these compounds can be used as adjuvants and as carriers for vaccines, but without providing any mean to use these compounds.

The abstract of the publication of Mercy et al. (Chemical Abstracts, vol. 106, n°25, 264, abstract n° 209.643 p, 1987) relates to the structural analysis of a lipopeptide composed of a fragment of a G virus protein and of a lysine-palmitoyl rest.

This analysis of the state of the art therefore shows that no technology applicable to various types of antigenic determinants has been developed which enables the induction of cytotoxic T lymphocytes to be obtained, with a high induced response, and which does not require the administration of an adjuvant.



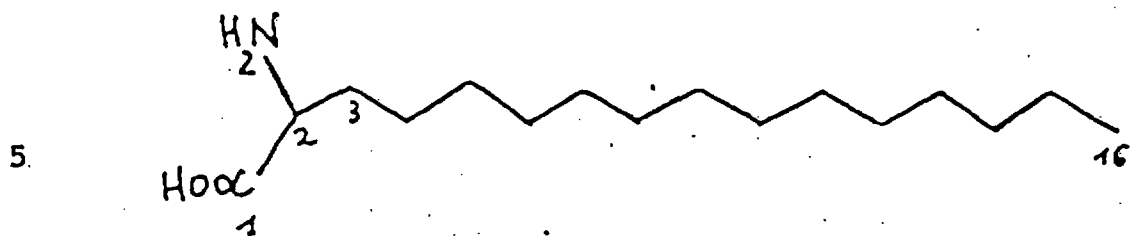
The applicant has found, surprisingly, that a response of cytotoxic T lymphocytes against an antigen could be induced in a host organism by immunising said organism with a lipopeptide complex containing one of the determinants of this antigen.

Even more surprisingly, the applicant has found that this induction could be obtained for a large number of antigenic determinants of various pathogenic agents.

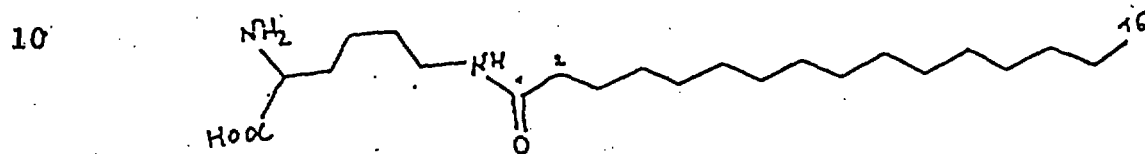
The present invention has thus for object a lipopeptide comprising a peptide part having between 10 and 40 and preferably between 10 and 20 amino acids approximately and comprising at least one antigenic determinant, said lipopeptide also comprising one or more chains derived from fatty acids comprising between 10 and 20 carbon atoms and/or one or more steroid groups modified and coupled on the  $\alpha\text{NH}_2$  or  $\epsilon\text{NH}_2$  functional groups of said amino acids.

Said fatty acid derivatives may be in particular 2-aminohexadecanoic acid (D, L) of the following formula (I):



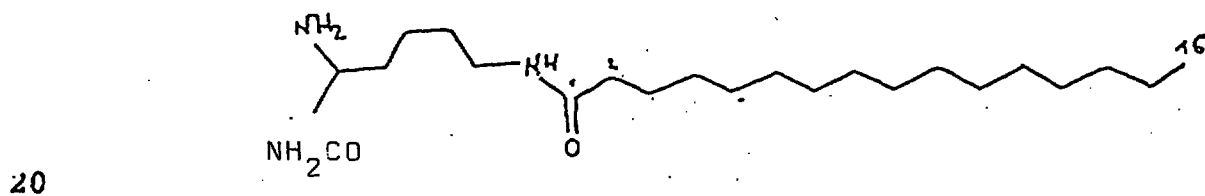


N- $\epsilon$ -palmitoyllysine (L) of the following formula (II):

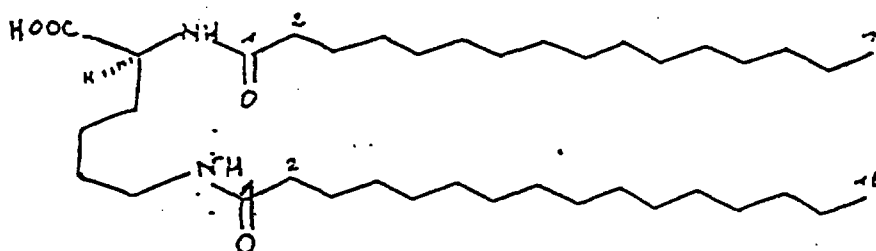


or its derivate having the following formula :

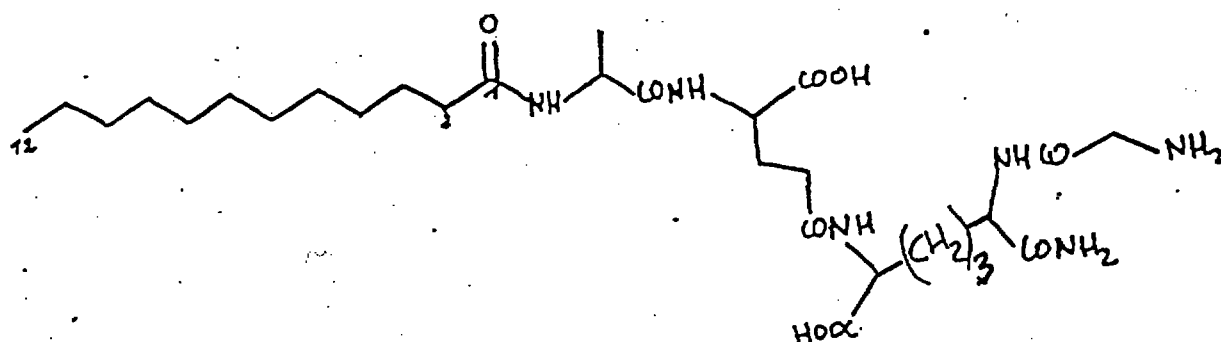
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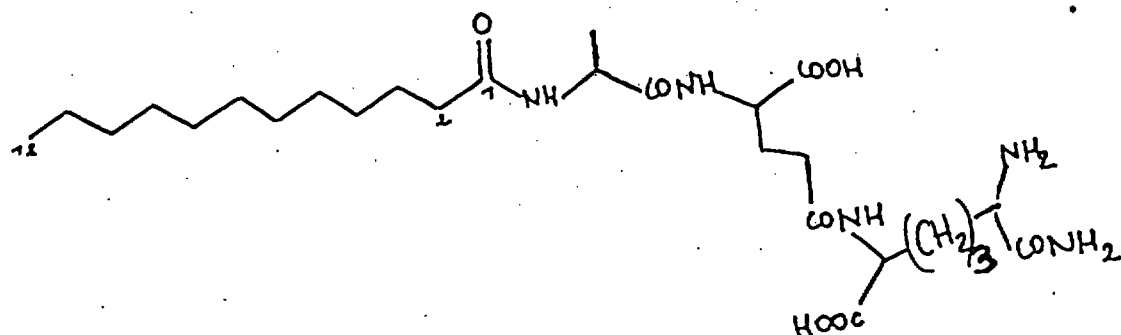
N,N'-dipalmitoyllysine (L) of the following formula  
(III):



15 pimelauteide of the following formula (IV):

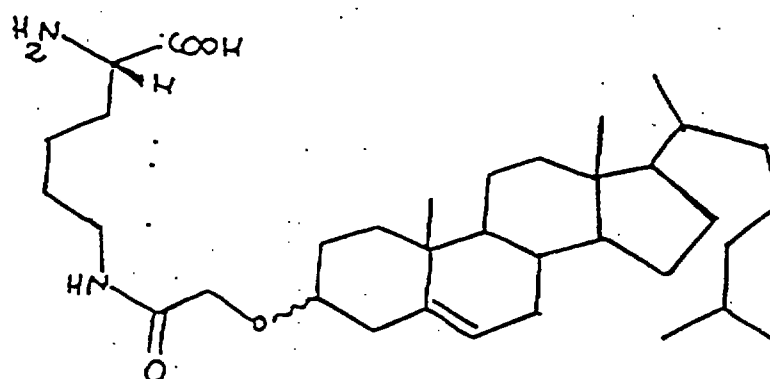


trimexautide of the following formula (V):



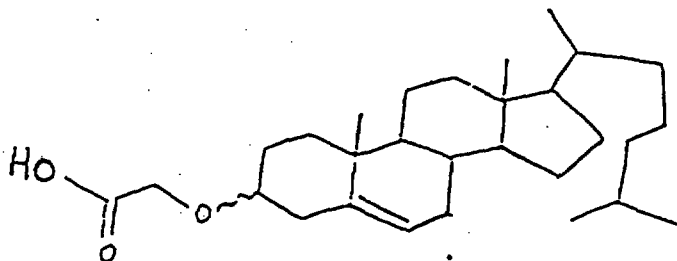
or one of their derivatives.

Said steroid groups may be N<sup>ε</sup>-[(cholest-5-enyl-3-oxy)acetyl]lysine (L) of the following formula (VI):



5 (cholest-5-enyl-3-oxy)acetic acid of the following  
formula (VII)

10



or one of their derivatives.

15 The peptide part may be a fragment of any protein derived from a pathogenic agent having an antigenic determinant.

Such proteins may be especially the proteins of the HIV1 or HIV2 virus, in particular the protein  
20 encoded by the ENV gene. In this case, the fragments

312-327 or 302-336 may be advantageously used depending on the HIV<sub>1</sub>-BRU sequence (Hyers, C.A.B. Rabson, S.F. Josephs, T.F. Smith and E. Wong-Staal (Eds.), 1989, Human retrovirus and AIDS, Los Alamos Laboratory II:59) of this protein, to  
5 form the conjugated lipopeptide molecule.

The present invention has also for object vaccines against viruses or parasites comprising one of the above described lipopeptides and particularly vaccines against diseases linked to HIV viruses, said vaccines comprising  
10 advantageously a fragment of the protein produced from ENV gene.

The present invention relates moreover to pharmaceutical compositions containing an efficient quantity of at least one of the compounds above described  
15 in association with one or several diluents or adjuvants compatible and pharmaceutically acceptable. These compositions are in particular intended for treating the diseases linked with the HIV viruses by induction of cytotoxic T lymphocytes.



The present invention relates also to the use of the above described lipopeptides for immunising the human or animal body against pathogenic agents by inducing cytotoxic T lymphocytes. Such pathogenic agents may be viruses which have substantial cytotoxic activity, in particular the HIV1 and HIV2 viruses, and certain parasites.

Said lipopeptides may also be used against certain cancers in order to induce a CTL response specific for certain tumor cells.

The lipopeptides which are the object of the present invention may be obtained from protein and pseudolipid constituents by methods known to a person skilled in the art, in particular, either by coupling the amino acids comprising the peptide part to the pseudolipid immobilised on a resin, that is to say by solid phase synthesis, or by coupling the pseudolipid to a peptide immobilised in a solid phase.

It should be noted that the lipopeptides



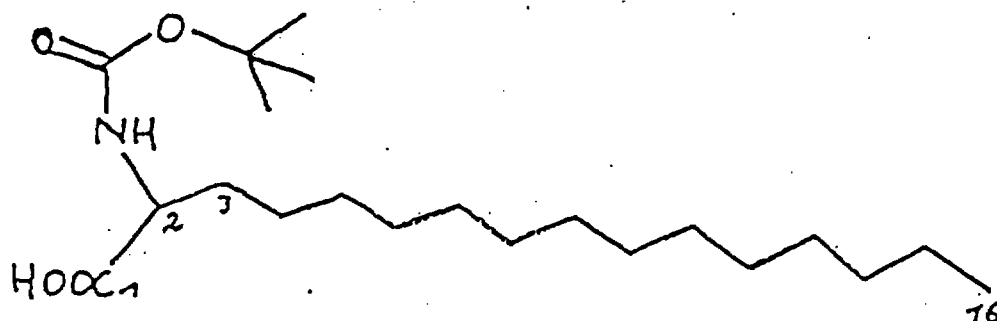
according to the invention have the notable advantage of being capable of being adapted for inducing cytotoxic T lymphocytes against any type of antigenic determinant of any pathogenic agent.

5            Additionally, the present invention also relates to the following synthetic intermediates:

2-tert-butyloxycarbonylaminohexadecanoic acid (D,L) of the following formula (VIII):

10

15

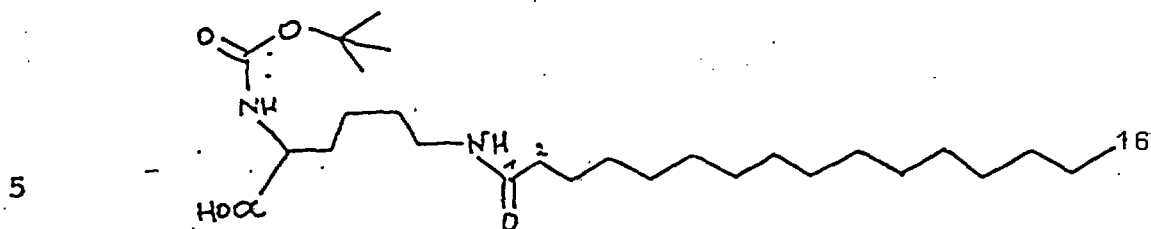


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N- $\alpha$ -terbutyloxycarbonyl-L-palmitoyllysine (L) of the

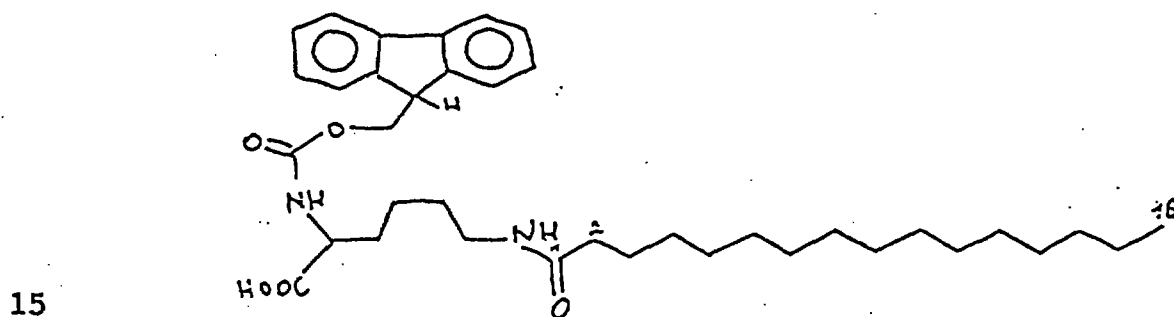


following formula (IX):



N- $\alpha$ -fluorenylmethyloxycarbonyl-L-palmitoyllysine (L) of  
the following formula (X):

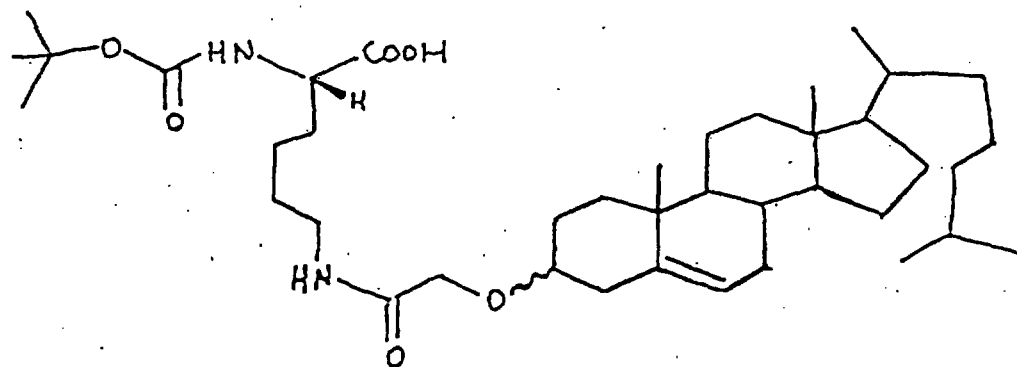
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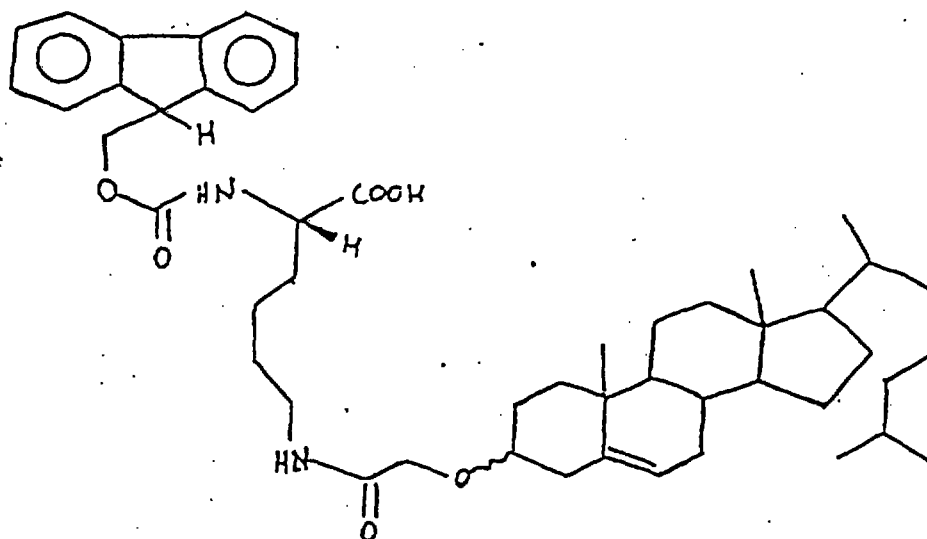
20 N- $\alpha$ -tert-butyloxycarbonyl-L[(cholest-5-enyl-3-



oxy)acetyl]lysine (L) of the following formula (XI):



or N-~~fluorenylmethyloxycarbonyl~~[(cholest-5-enyl-3-oxy)acetyl]lysine (L) of the following formula (XII):



or one of their derivatives.

The present invention is illustrated, without being limited in any manner, by the following examples of application in which Figures 1 and 3 represent the reverse-phase HPLC spectra of the lipopeptide V3GP120, 312-327 succinyl during preparative and analytical electrophoreses respectively.

Figure 2 represents, for its part, its mass spectrum.

10 EXAMPLE 1:

Syntheses of pseudolipid residues (or hydrophobic molecules).

1. Synthesis of tert-butyloxycarbonyl-2-aminohexadecanoic acid.

15 1.1. Synthesis of 2-aminohexadecanoic acid.

Empirical formula:  $C_{16}H_{33}NO_2$ .



Molecular weight 271.44

Procedure:

0.0298 mole of 2-bromohexadecanoic acid (10 g) and 100 ml of a 28% solution of  $\text{NH}_4\text{OH}$  are introduced into an autoclave previously cleaned with 50% nitric acid and 10% phosphoric acid. After stirring, the autoclave is heated at  $60^\circ\text{C}$  for 15 hours. The nascent amino derivative precipitates in the reaction medium. After cooling, the autoclave is washed, the water dispersed and the ethanol removed. The reaction mixture is filtered on sintered glass with a porosity of 4. The difference in solubility of the various products in ethanol medium enables the removal of traces of 2-bromohexadecanoic acid by rinsing.

Amount of precipitate obtained: 4.37 g; yield of 54%.

Remark: The insolubility of the amino derivative was checked in numerous solvents (water, ethanol, acetic acid at low temperature, 70% formic acid, ethyl acetate, toluene, toluene/acetonitrile, ethyl acetate/



acetonitrile). Among all the solubilization assays tested, only the action of a specific detergent (trimethylbenzylammonium hydroxide) or boiling acetic acid dissolved the amino derivative.

5           The dried precipitate is taken up in 150 ml of acetic acid which is refluxed until a yellowish clear solution is obtained. The colored pigments are absorbed on vegetable charcoal. After filtering on folded filter paper, the purified amino derivative is obtained by  
10 crystallization from the eluate. The white crystals obtained are recovered on sintered glass with a porosity of 4, washed with cold acetic acid before being dried in a desiccator over  $P_2O_5$ .

Yield: 46% (the product being in acetate form,  
5 a MW of 330.48 is used).

The purification yield is equal to 85%.

1.2. Synthesis of tert-butyloxycarbonyl-2-aminohexadecanoic acid.

- Empirical formula:  $C_{21}H_{41}NO_4$
- 20 - Molecular weight: 371.557.



- Procedure:

Dissolution of 2-aminohexadecanoic acid.

1.1 equivalents of "Triton" (benzyltrimethyl-  
ammonium hydroxide) in solution at 40% in methanol  
5 (2.9 ml) as well as 100 ml of DMF are added to 5 mmol  
of amino acid in acetate form (1.655 g). The reaction  
mixture is left stirred at room temperature until com-  
plete dissolution. The DMF is then evaporated using a  
vane pump. The residue is dried in a desiccator over  
10  $P_2O_5$ .

Protection of the amine functional group.

The dry residue is dissolved in a mixture con-  
sisting of 36 ml of water, 8 ml of 1N  $KHCO_3$  and 30 ml  
of tert-butyl alcohol. 2.5 equivalents of terbutyl-  
15 dicarbonate (MW = 218.25) are added to this solution.  
The pH is adjusted to between 8 and 9 using a 1N  
solution of  $Na_2CO_3$  and maintained constant during the  
initial hours of the reaction. The kinetics of coupling  
are monitored by thin-layer chromatography on silica.

20 After evaporating the tert-butyl alcohol under



vacuum, the product is taken up in 100 ml of water. The aqueous phase is acidified to pH 3 with a 1N solution of HCl. The Boc-amino acid is extracted with ethyl acetate (2 X 100 ml). The organic phase is washed with  
5 distilled water, dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered and then concentrated using a rotating evaporator. 4 ml of hexane are added to the oily residue. Crystallization of the Boc-amino acid is enhanced by cooling in a cold chamber. The white crystals are recovered on  
10 sintered glass with a porosity of 4, washed with hexane and dried in a desiccator over  $\text{P}_2\text{O}_5$ .

Yield: 16 to 18%.

### 1.3. Purification and characterization.

#### 1.3.1. Purification.

15 Successive recrystallizations made it possible to increase the purity (increase in the melting point). The purification led to a reduction in yield of not more than 2% for aminohexadecanoic acid and of not more than 1% for the protected amino acid.

#### 20 1.3.2. Characterization.



A. Melting point:

|   | Product                                        | Melting point obtained |
|---|------------------------------------------------|------------------------|
|   | 2-Bromohexadecanoic acid                       | 56°C                   |
|   | 2-Aminohexadecanoic acid                       | 144°C                  |
| 5 | tert-Butyloxycarbonyl-2-aminohexadecanoic acid | 85°C                   |

B. Thin-layer chromatography on silica.

The solutions (10 to 20  $\mu$ l of a solution at 1 mg/ml) are deposited on thin-layer silica (Merck silica gel 60 without fluorescence indicator).

2-Bromohexadecanoic acid is dissolved in ethanol, 2-aminohexadecanoic acid in boiling acetic acid, and tert-butyloxycarbonyl-2-aminohexadecanoic acid in dichloromethane.

Choice of the migration solvent.

The various systems chosen are:

System A: butanol/ethyl acetate/acetic acid/water in the volume/volume proportions: 1/1/1/1.

System B: ethyl acetate/pyridine/acetic acid/water in the v/v proportions: 5/5/1/3.



System C: chloroform/methanol/acetic acid in the v/v proportions: 10/1/0.1.

Developing.

5 After migration in the system of suitable solvents, the thin layers are dried for 15 minutes at 120°C before being developed after spraying with a developing reagent.

10 - The ninhydrin reagent which is specific for primary amino functional groups enables the detection of the unprotected aminohexadecanoic acid but also of the Boc-amino acid, the spraying of 20% acetic acid followed by drying at 120°C enabling the displacement of the Boc group.

15 The spraying using a reagent comprising 20 g of  $(\text{NH}_4)_2\text{SO}_4$ , 3 ml of  $\text{H}_2\text{SO}_4$  and 100 ml of  $\text{H}_2\text{O}$  enable the simultaneous developing of the three products. In this technique, the drying, after spraying, in the thin-layer chromatography is carried out using an epiradiator (porcelain resistance with infrared radiation).

20



Result:

|    | Product                                         | Solvents | Rf   |
|----|-------------------------------------------------|----------|------|
| 5  | 2-Bromohexadecanoic acid                        | System A | 0.5  |
|    |                                                 | System B | 1    |
|    |                                                 | System C | 1    |
| 10 | 2-Aminohexadecanoic acid                        | System A | 0.82 |
|    |                                                 | System B | 0.94 |
|    |                                                 | System C | 0    |
| 10 | tert-Butyloxycarbonyl-2-amino-hexadecanoic acid | System A | 1    |
|    |                                                 | System B | 1    |
|    |                                                 | System C | 0.67 |

C. Mass spectrometry (PDMS BIO-ION 20).

tert-Butyloxycarbonyl-2-aminohexadecanoic acid spectrum.

| MW (g)            | (M-H) <sup>-</sup> | Fragments: |
|-------------------|--------------------|------------|
| Theoretical MW    | 370.557            |            |
| 5 Experimental MW | 370.8              | 270        |

10 The experimental molecular ion and the theoretical molecular ion have an identical weight. The molecular ion is fragmented; the Boc group (peak at 270) is released. The 296.6 peak represents the ion with a weight of 270 containing the CN group (nitrocellulose).

2. Synthesis of 3-β-(2'-carboxymethoxy)-cholest-5-ene.

2.1. Synthesis of cholesteryl tosylate.

- 15
- Empirical formula :C<sub>34</sub>H<sub>53</sub>SO<sub>3</sub>,
  - Molecular weight: 540.83,
  - Procedure:

After dissolving 25.86 mmol of cholesterol (10 g) in a minimum of pyridine (5 to 10 ml), an excess of tosyl



chloride (10 g, 52.6 mmol) is added. After stirring for 12 hours, the pyridine is removed by evaporating to dryness. The residue is solubilized in 20 ml of acetone at high temperature (the temperature is maintained below 55°C to avoid the formation of oil). The mixture is filtered. The cholesterol tosylate is obtained by crystallisation from the eluate. The white crystals obtained are washed with cold acetone and dried in a desiccator over P<sub>2</sub>O<sub>5</sub>.

Yield : 82 to 85%.

## 2.2 Synthesis of 3 $\beta$ -(2'-hydroxyethoxy)-cholest-5-ene.

- Empirical formula : C<sub>29</sub>H<sub>50</sub>O<sub>2</sub>.

- Molecular weight: 430.71.

- Procedure:

30 ml of ethylene glycol (480 mmol) are added to 17.5 mmol of cholesteryl tosylate (10 g) dissolved in 120 ml of dioxane. The reaction mixture is refluxed for 4 hours at 120°C. After cooling, it is taken up in 150 ml of distilled water. The alcohol derivative



formed is extracted with diethyl ether (3 X 200 ml). The ethereal phase is successively washed with 5% NaHCO<sub>3</sub> (2 X 200 ml) and distilled water (2 X 200 ml). After drying over anhydrous Na<sub>2</sub>SO<sub>4</sub>, the ethereal solution is concentrated until an oil is obtained. After adding 4 ml of hexane, the crystallization is started by rubbing and cooling in a cold chamber (4°C). The white crystals are recovered on sintered glass with a porosity of 4 and washed with hexane before being dried in a desiccator over P<sub>2</sub>O<sub>5</sub>.

Yield: 32 to 34%.

2.3. Synthesis of 3 $\beta$ -(2'-carboxymethoxy)-cholest-5-ene.

- Empirical formula: C<sub>29</sub>H<sub>48</sub>O<sub>3</sub>,

- Molecular weight: 444.69.

- Procedure:

The oxidizing solution is prepared beforehand: it comprises 2.67 g of chromic anhydride, 2.3 ml of concentrated H<sub>2</sub>SO<sub>4</sub>, the volume being brought to 10 ml with distilled water. The oxidizing medium is added



dropwise to 4.66 mmol ( 2g) of 3- $\beta$ -(2'-hydroxyethoxy)-cholest-5-ene dissolved in 50 ml of acetone. The progress of the reaction is monitored by thin-layer chromatography. Once the reaction is completed, the reaction medium is taken up in 250 ml of distilled water. The acid derivative is extracted with ethyl acetate (3 x 200 ml). The organic phase is washed with distilled water (2 x 200 ml), dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated until an oil is obtained. 4 ml of petroleum ether are added to the oil. The crystallisation of the acid derivative is enhanced by cooling in a cold chamber (4°C). The white crystals are recovered on sintered glass with a porosity of 4, washed with petroleum ether and dried in a desiccator over P<sub>2</sub>O<sub>5</sub>.

Yield : 29 to 31%.

#### 2.4. Purification and characterization.

##### 2.4.1. Purification.

Cholesteryl p-toluenesulfonate is purified by successive recrystallizations in acetone.  $\beta$ -(2'-hydroxyethoxy)-cholest-5-ene and the acid derivative



have both been purified by thick-layer chromatography on silica.

A. Thick-layer chromatography on silica.

5 The depositions are carried out on a thick layer of silica (Merck silica gel 60 PF<sub>254</sub> with a fluorescence indicator), the spots being detected by ultraviolet radiation.

10 A solution containing 0.250 mg of product is deposited on the silica plate, the products have both been dissolved in dichloromethane.

| Product                                         | Solvent                                                                     | R <sub>f</sub> |
|-------------------------------------------------|-----------------------------------------------------------------------------|----------------|
| 3 $\beta$ -(2'-Hydroxyethoxy)-cholest-5-ene     | Petroleum ether/<br>ethyl ether<br>Volume/volume<br>proportions: 1/1        | 0.48           |
| 15 3 $\beta$ -(2'-Carboxymethoxy)-cholest-5-ene | Petroleum ether/<br>ethyl ether/<br>methanol<br>v/v proportions:<br>10/10/3 | 0.52           |
| 20                                              |                                                                             |                |

The two products were extracted from the silica



with methanol. A loss equivalent to about 30% of the amount deposited is observed for each of the products.

#### 2.4.2. Characterization.

##### A. Melting point.

| 5  | Product                                         | Melting point<br>(literature) | Melting<br>point |
|----|-------------------------------------------------|-------------------------------|------------------|
|    | Cholesteryl para-toluene-<br>sulfonate          | 131.5°C-132.5°C               | 128°C            |
| 3  | $\beta$ (2'-Hydroxyethoxy)                      | 102°C-104°C                   | 99°C             |
| 10 | 3 $\beta$ (2'-Carboxymethoxy)-<br>cholest-5-ene | 160°C-161°C                   | 157°C            |

##### B. Thin-layer chromatography

15 The depositions (10 to 20  $\mu$ l) of a 1 mg/ml solution are carried out on a thin layer of silica with a fluorescence indicator (Merck Kieselgel 60F<sub>254</sub>).

The dissolution of the various products is carried out in dichloromethane.

20 After migration in the suitable solvent system, the thin layers are dried in air before being developed either by ultraviolet radiation or after spraying with HClO<sub>4</sub> (20%) and drying in an oven (120°C for 20 minutes).



Result:

Various solvent systems.

System A: Ethyl ether/petroleum ether in the volume/  
volume proportions: 1/1.

5 System B: Ethyl ether/petroleum ether in the v/v pro-  
portions: 1/2.

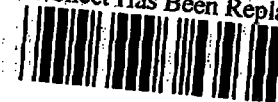
System C: Petroleum ether/ethyl ether/methanol in the  
v/v proportions: 5/5/1.

10 System D: Petroleum ether/ethyl ether/methanol in the  
v/v proportions: 10/10/3.

System E: Petroleum ether/ethyl ether/methanol in the  
v/v proportions: 5/5/2.

System F: Ethyl ether.

|    | Product                                             | Solvent system | R <sub>f</sub> |
|----|-----------------------------------------------------|----------------|----------------|
| 15 | Cholesterol                                         | System A       | 0.54           |
|    |                                                     | System B       | 0.3            |
|    |                                                     | System F       | 0.95           |
|    | Cholesteryl para-<br>toluenesulfonate               | System A       | 0.85           |
| 20 |                                                     | System B       | 0.62           |
|    |                                                     | System F       | 1              |
|    | <sup>3</sup> β-(2'-Hydroxyethoxy)-<br>cholest-5-ene | System A       | 0.41           |
|    |                                                     | System B       | 0.24           |



|    |                                 |                            |      |
|----|---------------------------------|----------------------------|------|
|    |                                 | System F                   | 0.9  |
|    | 3 $\beta$ -(2'-Carboxymethoxy)- |                            |      |
|    | cholest-5-ene                   | System A                   | 0    |
|    |                                 | System B                   | 0    |
| 5  |                                 | System C                   | 0.1  |
|    |                                 | System D                   | 0.42 |
|    |                                 | System E                   | 0.78 |
|    |                                 | System F Streaking effect: |      |
| 10 |                                 | R <sub>f</sub> O           | →0.5 |

C. Mass spectrometry (PDMS)

Analysis of the 3 $\beta$ -(2'-carboxymethoxy)-cholest-5-ene.

|    |                 |                    |
|----|-----------------|--------------------|
|    | MW (g)          | (M-H) <sup>-</sup> |
|    | Theoretical MW  | 443.69             |
| 15 | Experimental MW | 443.1              |

The experimental molecular ion and the theoretical molecular ion have an identical weight.

D. <sup>13</sup>C nuclear magnetic resonance.

Analysis of the 3  $\beta$ -(2'-carboxymethoxy)cholest-5-ene spectrum was carried out by comparison with the <sup>13</sup>C NMR spectrum of cholesterol.

The dissolution of the cholesterol and the 3  $\beta$ -(2'-carboxymethoxy)cholest-5-ene was performed in deuterated chloroform.



Cholesterol spectrum.

|    | Peaks     | d(ppm) obtained | attribution       | theoretical<br>d(ppm)                                      |
|----|-----------|-----------------|-------------------|------------------------------------------------------------|
| 5  | 1         | 140.7606        | C5 or C6          | alkene func-<br>tional group:<br>d(ppm) from<br>100 to 145 |
|    | 2         | 121.7064        | C5 or C6          |                                                            |
|    | 3         | 78.5715         | CDCl <sub>3</sub> |                                                            |
| 10 | 4         | 76.9981         | CDCl <sub>3</sub> |                                                            |
|    | 5         | 75.4010         | CDCl <sub>3</sub> |                                                            |
|    | 1' to 22' | 71 to 11        |                   | alkane func-<br>tional groups.                             |

<sup>3</sup>β-(2'-Carboxymethoxy)cholest-5-ene spectrum.

|     | Peaks | d(ppm) obtained | attribution | theoretical<br>d(ppm)                                      |
|-----|-------|-----------------|-------------|------------------------------------------------------------|
| '15 | 1     | 172.2923        | C2'         | acid func-<br>tional group:<br>d(ppm) from<br>160 to 185   |
| 20  | 2     | 139.8394        | C5 or C6    | alkene func-<br>tional group:<br>d(ppm) from<br>100 to 145 |
| 25  | 3     | 122.5233        | C5 or C6    | alkene func-<br>tional group                               |



|    |         |          |                   |                |
|----|---------|----------|-------------------|----------------|
|    |         |          |                   | ether func-    |
|    |         |          |                   | tional group:  |
|    |         |          |                   | d(ppm) from 45 |
|    |         |          |                   | to 80          |
| 5  | 5       | 79.2943  | Cl'               | + slight dis-  |
|    |         |          |                   | placement      |
|    | 6       | 78.5903  | CDCl <sub>3</sub> |                |
|    | 7       | 77.0089  | CDCl <sub>3</sub> |                |
|    | 8       | 75.4146  | CDCl <sub>3</sub> |                |
| 10 | 9 to 31 | 65 to 11 |                   | alkane func-   |
|    |         |          |                   | tional groups. |

E. Elemental analysis.

Elemental analysis of 3 $\beta$ -(2'-carboxymethoxy)cholest-5-ene gave the following results:

|    |            |             |          |
|----|------------|-------------|----------|
| 15 |            | Theoretical | Obtained |
|    | % carbon   | 78.3        | 76.25    |
|    | % hydrogen | 10.9        | 10.9     |
|    | % oxygen   | 10.8        | 12.5     |

EXAMPLE 2: - Synthesis of the lipopeptides.

20 I. Method for coupling 2-aminohexadecanoic acid

The 312-327 region of gp120 of the HIV-1 LAV-BRU virus was chosen for sequences constructed in lipopeptide form in order to study the cytotoxic T response. 3 preparations were carried out using this

sequence.

The synthesis was carried out in solid phase (MERRIFIELD R.B., 1963, J. Am. Chem. Soc. 85, 2149-2210).

5 All the lipopeptides were synthesized on a benzhydrylamine resin (charged at 0.72 millimole/gram). In all cases, the first amino acid grafted was 2-Boc-aminohexadecanoic acid (2 equivalents). This made it possible to obtain constructs where the C-terminal  
10 amino acid was 2-aminohexadecanoic acid in amide form in order to avoid the presence of a charge near the hydrophobic aliphatic chain. After acetylation with acetic anhydride in basic medium, in order to block the free reactive sites, the cleavage of the N-terminal Boc  
15 and then the coupling of the first amino acid of the sequence were carried out.

All these stages were carried out manually, which made it possible to carry out very precise controls of the coupling of the pseudolipid amino acid  
20 and the first amino acid on the latter.



The coupling activator is benzotriazolyl-N-oxytriadimethylaminophosphonium hexafluoro-phosphate (BOP) in the presence of hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIEA). By virtue of this very efficient coupling method, the yield of these two coupling reactions was always greater than 99.5% despite the substantial steric hindrance due to 2-Boc-aminohexadecanoic acid.

The rest of the synthesis was carried out in a conventional manner, automatically, up to the last amino acid. At this stage, the peptidyl-resin was divided into 3 batches, treated manually:

- 1 batch was preserved as such;
- 1 batch was grafted using 2-Boc-aminohexadecanoic acid.

The manual coupling (using BOP) of the latter was followed by cleavage of the N-terminal Boc and acetylation of all the amine functional groups thus exposed. This made it possible to avoid the presence of a charge near the hydrophobic aliphatic chain of the pseudolipid amino acid, 1 batch was grafted using diBoc, N $\alpha$ , $\epsilon$ -lysine. Manual

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The rest of the synthesis was carried out in a conventional manner, automatically, up to the last amino acid. At this stage, the peptidyl-resin was divided into 3 batches, treated manually:

- 1 batch was preserved as such;
- 1 batch was grafted using 2-Boc-aminohexadecanoic acid.

The manual coupling (using BOP) of the latter was followed by cleavage of the N-terminal Boc and acetylation of all the amine functional groups thus exposed. This made it possible to avoid the presence of a charge near the hydrophobic aliphatic chain of the pseudolipid amino acid, 1 batch was grafted using diBoc,  $N\alpha,\epsilon$ -lysine. Manual



grafting (using BOP) of the latter was followed by cleavage of the two BOC groups and the manual coupling of palmitic acid (using BOP). We thus obtained peptides having a dipalmitoyl-lysine in the N-terminal position.

5           These couplings, carried out manually, were the subject of a strict control which revealed yields that were always higher than 99.5%. These results confirm the advantage of using BOP as activating agent in peptide synthesis, especially for coupling pseudolipid amino acids  
10       or for coupling to the latter. The synthesized lipopeptides were then cleaved from their support. The lipopeptides derived from the 312-327 sequence were cleaved by anhydrous hydrofluoric acid treatment. The cleavage yields are quite low, between 40 and 70%.

15           There are at least two explanations for these values:

1) the cleavage of a peptide grafted on a benzhydrylamine resin is never total under the usual conditions of cleavage;

20           2) the presence of 2-aminohexa-

decanoic acid, directly in contact with the resin, certainly amplified this phenomenon.

After washing twice (TFA-ether), the identity of the lipopeptides was assessed by amino acid analysis after total acid hydrolysis and, for some, by PDMS mass spectrometry. Their homogeneity was checked by thin layer chromatography on silica and analytical reverse phase HPLC.

## II. Methods for coupling pimelautide and trimexautide

### 1) Method for coupling pimelautide (or trimexautide) to the N-terminal end of a peptide by means of a succinyl link.

This method applies to the fixing of unprotected pimelautide (or trimexautide) on a peptide constructed in a solid phase, still fixed to the resin, and to protected side chains.

Both trimexautide and pimelautide (Rhône Poulenc) have two free carboxylic functional groups and a free primary amine functional group. The creation of



a predetermined amide bond between the peptide and the pimelautide (or trimexautide) can only be achieved by using the pimelautide (or trimexautide) as amino partner.

5           The succinylation of the peptide on the resin makes it possible to make it the carboxylic partner.

#### DEPROTECTION

10           The tertbutoxycarbonyl group, which temporarily protects the N-terminal end of the peptide on the resin, is cleaved by the action of a 40% trifluoroacetic acid solution in dichloromethane for 20 minutes with stirring.

The resin is washed with:

- twice 20 ml of dichloromethane,
- 15           - twice 20 ml of 5% diisopropylethylamine in dichloromethane,
- twice 20 ml of dimethylformamide (for 3 minutes for each wash).

#### SUCCINYLATION.

20           It is achieved by carrying out the coupling



three times by bringing the resin of the succinylation solution into contact with:

- a fivefold excess of succinic anhydride (5% solution in N-methylpyrrolidone)

5           - diisopropylethylamine in a stoichiometric amount relative to the amines of the resin (for 20 minutes with stirring).

ACTIVATION.

10           The activation of the carboxyl now present on the resin is carried out as follows:

the resin is subjected to the action of the activating solution (for 15 minutes at room temperature and with stirring):

15           - BOP (benzotriazolyloxytrisdimethylaminophosphonium hexafluorophosphate): 3 excesses with respect to the carboxyls,

- HOBT (hydroxybenzotriazole): 3 excesses with respect to the carboxyls,

20           - diisopropylethylamine: 7 excesses with respect to the carboxyls in solution in N-



methylpyrrolidone.

WASHING:

The solution is washed with:

- 3 times 30 ml of dimethylformamide,

5                   - 3 times 30 ml of dichloromethane.

COUPLING:

The resin is subjected to the action of the coupling solution:

10                   - pimelautide (or trimexautide): 3 excesses  
with respect to the activated ester of  
hydroxybenzotriazole,

- diisopropylethylamine: 3 excesses with  
respect to the ester,

- 10% dimethyl sulfoxide

15                   - 90% N-methylpyrrolidone: sufficient amount to  
dissolve pimelautide (or trimexautide).

The saturated solution is at about 4% pimelautide or  
trimexautide after sonication and passage for 2 minutes  
at 50°C.

20                   2) Synthesis in solid phase of V3GP120, 312-327



succinyl.

a) N-Protection of the pimelautide (or trimexautide) by the tert-butyloxycarbonyl group.

5 500 micromoles of pimelautide (or trimexautide) are dissolved in 10 ml of a 0.1 molar solution of carbonate buffer at pH 9.5.

10 ml of a solution of di-tert-butyl pyrocarbonate at 100 mmol/l are added.

A pH of between 9 and 10 is maintained for 10 100 hours by adding disodium carbonate.

The reaction mixture is then diluted with 10 ml of water and 10 ml of diethyl ether and the washed aqueous phase is acidified to pH 2.5 with potassium hydrogen-sulfate.

15 An extraction with 100 ml of dichloromethane followed by evaporation of the solvent using a rotating evaporator leads to the crystallization of Boc-pimelautide (or Boc-trimexautide).

20 The incorporation of Boc-pimelautide (or Boc-trimexautide) by peptide synthesis in a solid phase



generates two position isomers.

#### b) CLEAVAGE AND PURIFICATION

The cleavage of the peptide at the end of the synthesis is performed using anhydrous hydrofluoric acid.

The peptide is then purified by gel filtration and type C<sub>4</sub> reverse-phase preparative HPLC.

Figure 1 represents the preparative HPLC spectrum at 235 nm obtained for 20 mg of lipopeptide dissolved in HCOOH.

The lipopeptide obtained is then analyzed by total acid hydrolysis, by analytical C<sub>4</sub> HPLC chromatography and mass spectrography.

Figure 2 represents the mass spectrum. A distinct peak is observed at 2422.2 which corresponds to the weight of the lipopeptide.

Figures 3a and 3b represent the analytical C<sub>4</sub> HPLC chromatography of the lipopeptide and of the control without lipopeptide respectively.

The chromatographic conditions are as follows:

solvent (A) : 0.5 0/00 trifluoroacetate,  
gradient: solvent B : 0.75 % acetonitrile  
0.5% trifluoroacetate,  
gradient from 10% to 80% in 120 min,  
5 measurement at a wavelength of 215 nm.

During the total acid hydrolysis, the diaminopimelic acid (Dap) present in pimelautide and trimexautide constitutes a good coupling marker.

10

Results of the total acid hydrolysis

|    | Amino acids | nanomoles | measured | theoretical | measured/<br>theoretical |
|----|-------------|-----------|----------|-------------|--------------------------|
|    | Thr         | 3.2500    | 0.97     | 1           | 0.97/1                   |
| 15 | Glu         | 7.1000    | 2.11     | 2           | 1.06/1                   |
|    | Pro         | 3.1800    | 0.95     | 1           | 0.95/1                   |
|    | Gly         | 10.3500   | 3.08     | 3           | 1.03/1                   |
|    | Arg         | 6.6900    | 1.99     | 2           | 1.00/1                   |
|    | Val         | 3.3900    | 1.01     | 1           | 1.01/1                   |
| 20 | Ile         | 9.5800    | 2.85     | 3           | 0.95/1                   |



|     |        |      |   |        |
|-----|--------|------|---|--------|
| Phe | 3.3500 | 1.00 | 1 | 1.00/1 |
| Lys | 3.5200 | 1.05 | 1 | 1.05/1 |
| Arg | 9.9200 | 2.95 | 3 | 0.98/1 |
| Dap | 3.500  | 1.05 | 1 | 1.05/1 |

5 EXAMPLE 3 -

Immunization against the peptide NP 147-158.

The immunizations of mice are carried out as follows:

10

Immunizations:

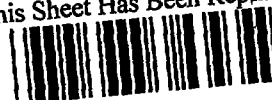
The mice were injected with the lipopeptide preparations intraperitoneally (i.p.) or subcutaneously (S.C.), with or without adjuvant.

15 At least two injections (at intervals of 8 to 30 days) are necessary in order to obtain CTLs.

Each injection contains  $5 \times 10^{-8}$  mole of lipopeptide.

Detection of the CTLs

20 8 to 21 days after the last injection, the



spleen of the immunized mice was removed, the splenocytes of these mice were cultured in vitro in an amount of  $5 \times 10^6$  splenocytes/2 ml of conventional culture medium (DMEM + 10% FeS + pyruvate + no essential amino acids +  $\beta$ -2-mercaptoethanol) containing  $5 \mu\text{M}$  of the peptide corresponding to that involved in the lipopeptide construction.

From the 5th day of the in vitro culture, the activity of the CTLs may be detected by the conventional test of  $^{51}\text{Cr}$  release (Martinon et al., J. Immunol., 142;3489-3494, 1989).

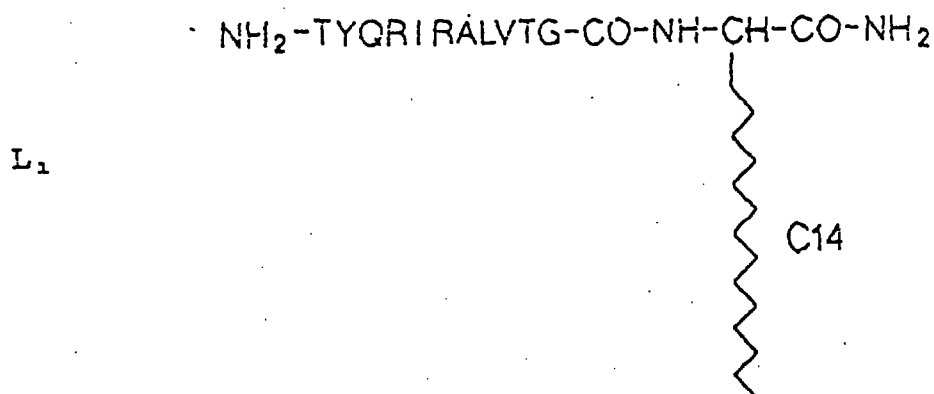
The CTL activity is tested against some syngenic target cells in the presence of the peptide (NP 147-158 R<sup>-</sup>, P<sub>3</sub>CSS, PepNP or L<sub>1</sub>-Pep.NP) or against some syngenic target cells infected by the influenza A virus.

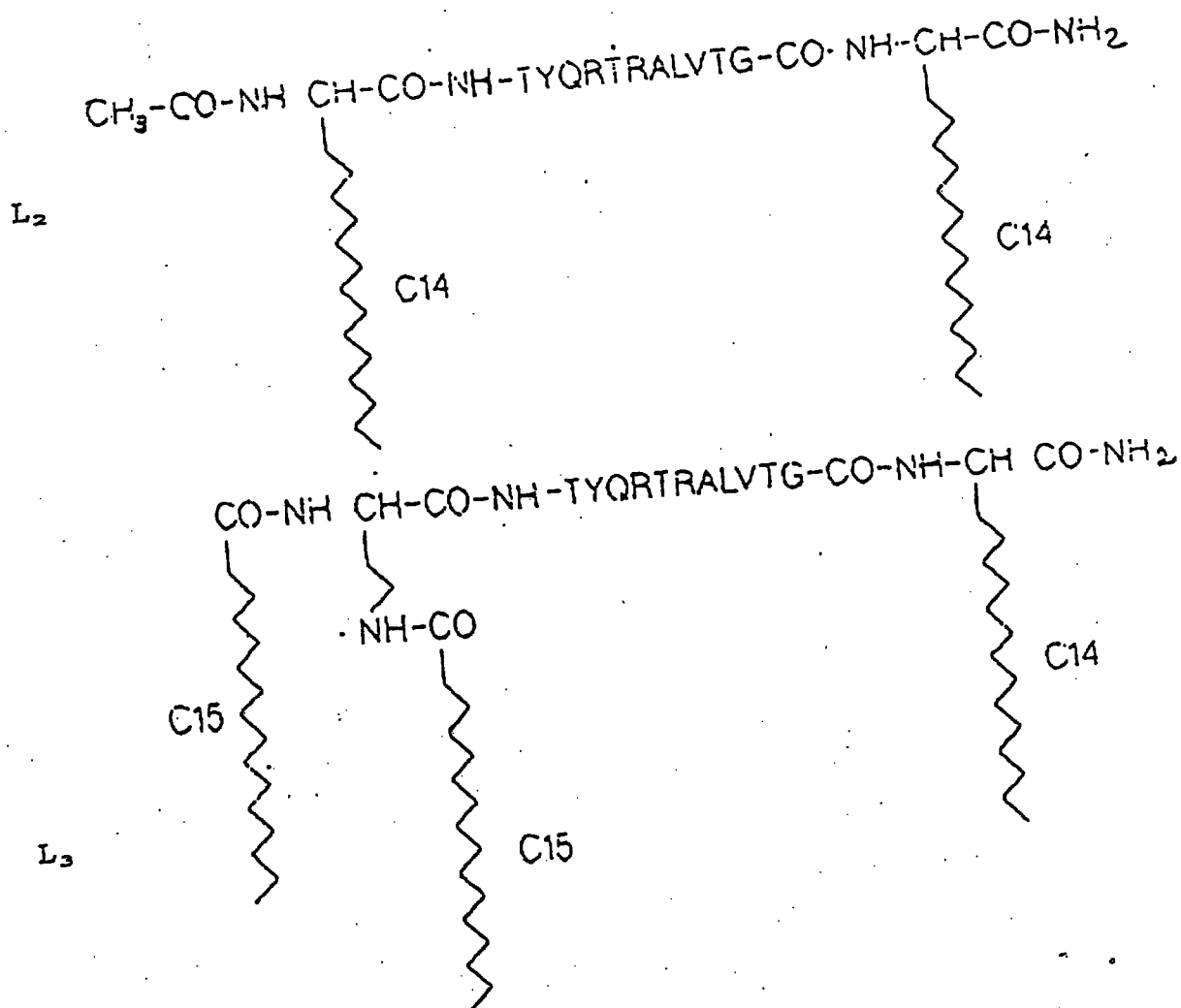
The results obtained are summarized in Table I. The first part of the table relates to the results already obtained with the whole influenza virus, the NP 147-158 R protein of the influenza virus and the



P3CSS-PEPNP lipopeptide, which consists of the NP 147-158 peptide coupled to tripalmitoyl-S-glyceryl-cysteinylserylserine (DERES et al. previously mentioned).

5           The second part of the table relates, on the one hand, to the immunization trials carried out with liposomes containing the NP 147-158 peptide and with the lipopeptides L1, L2 and L3. These lipopeptides are molecules containing a peptide part (NP 147-158) and a  
10   lipid part, respectively. The lipopeptides L1, L2 and L3 are therefore of the following formulae





The cytolytic activities after 5 days, 12 days and more than 21 days show that a very high activity is obtained for the lipopeptide L1 compared to the other trials carried out.

5     EXAMPLE 4:

Immunization by CB1, CB2 and CB3 against the  
ENV 312-327 peptide .

          This peptide is a protein fragment encoded by the ENV gene of the HIV virus.

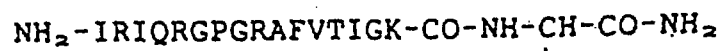
10        The experimental procedures are identical to those described in Example 3.

          Table II summarizes the results obtained.

          In this table, CB1, CB2 and CB3 correspond to lipopeptides formed from the peptide derived from the  
15        ENV protein and a lipid.

          The formulae of CB1, CB2 and CB3 are as follows:





C14

CB<sub>1</sub>



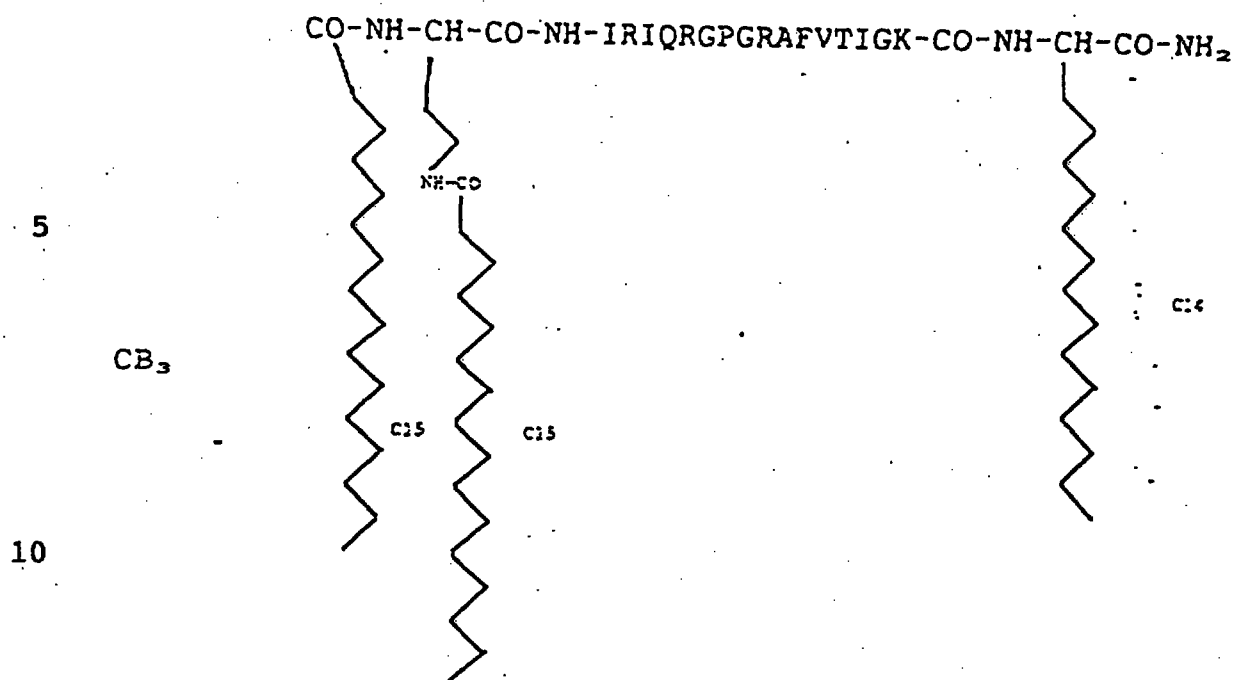
C14

CB<sub>2</sub>



C14



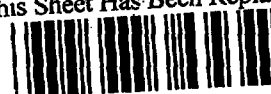


15           The results in Table II show a substantial activity for one of the lipopeptides (CB1).

EXAMPLE 5:

Immunization against the peptide ENV 302-335.

20           This peptide is the 302 to 335 fragment of the ENV protein of the HIV virus.



The experimental procedures are identical to those described in Example 3.

The results are shown in Table III.

5      The formulae of the lipopeptides CB<sub>6</sub>, CB<sub>7</sub> and CB<sub>8</sub> are as follows:

NH<sub>2</sub>-TRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAH-CO-NH-CH-CO-NH<sub>2</sub>

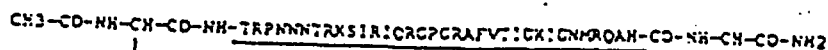
CB<sub>6</sub>



Cl<sub>4</sub>



CB<sub>7</sub>

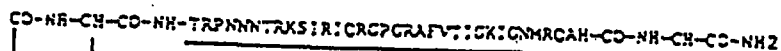


C14

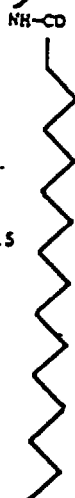


C14

CB<sub>8</sub>



C15



C15



C14



It can be observed in Table III that the two lipopeptides, CB<sub>6</sub> and CB<sub>7</sub>, show cytolytic activities substantially higher than the control.

EXAMPLE 6 :

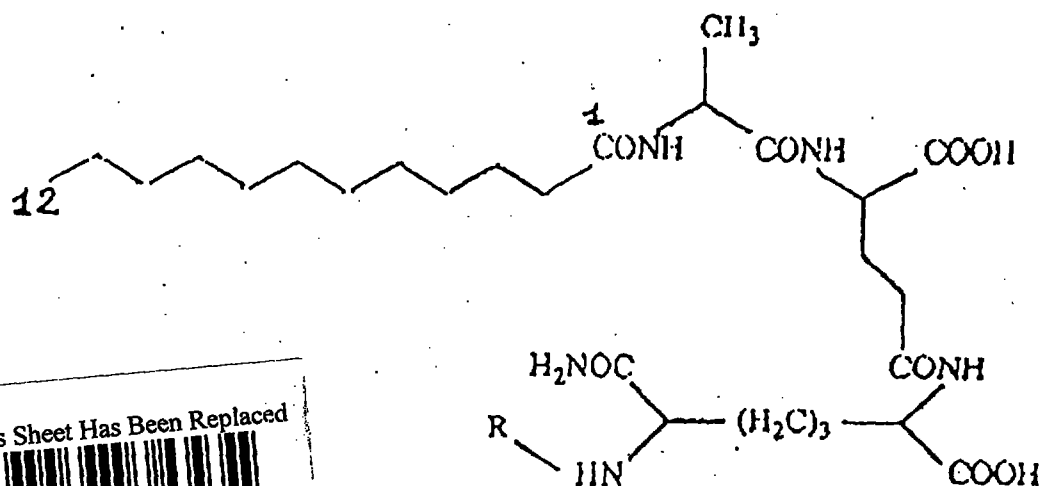
5        Immunization against the peptide ENV 312-327 by  
CB<sub>1</sub>, CB<sub>4</sub>, CB<sub>5</sub>, CB<sub>17</sub>, CB<sub>19</sub>, CB<sub>21</sub> and CB<sub>25</sub>.

The experimental procedures are substantially identical to those described in example 3.

The table IV summarizes the results obtained.

10        The formula of CB<sub>1</sub> is indicated in example 4.

The formulae of CB<sub>4</sub>, CB<sub>5</sub>, CB<sub>17</sub>, CB<sub>19</sub>, CB<sub>21</sub> and CB<sub>25</sub> are as follows :

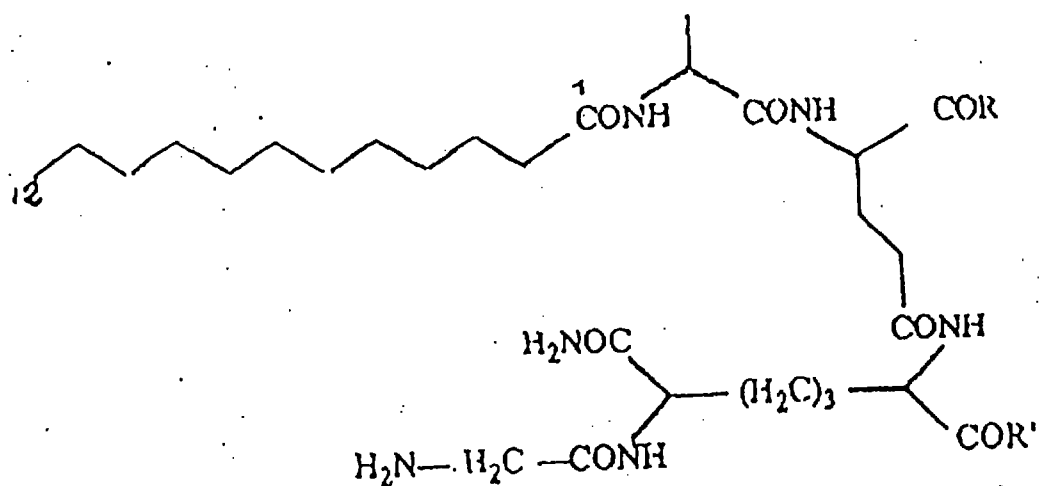


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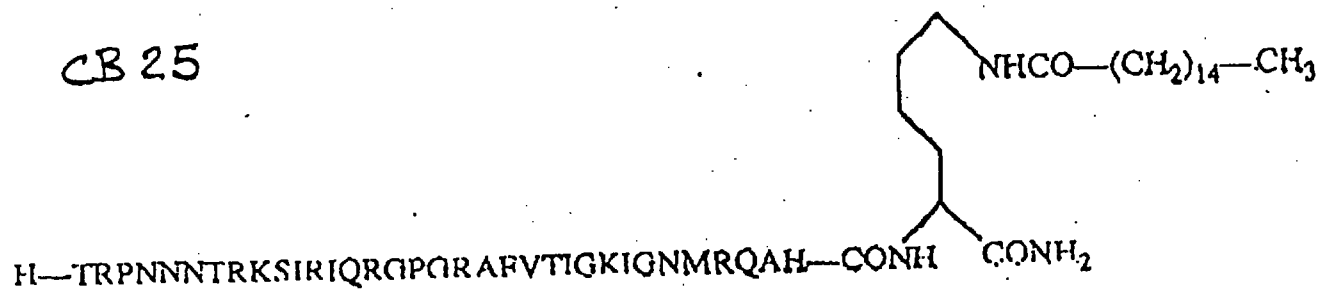
CB 5. R = CO (CH<sub>2</sub>)<sub>2</sub>—CONH—IRIQRGPGRAIVTIGK—OH

CB 19 = R · CO —CH<sub>2</sub>—NH—CO (CH<sub>2</sub>)<sub>2</sub>—CONH—IHQRGPGRAIVTIGK—OH

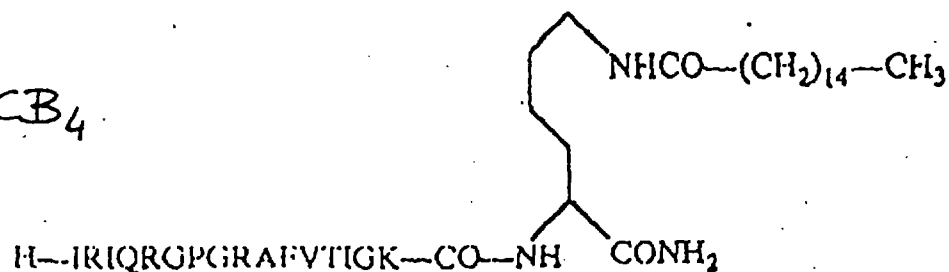


CB 21 Mixture of A: R = OH  
 R' = NH—IRIQRGPGRAFVTIOK—OH  
 B: R = NH—IRIQRGPGRAFVTIGK—OH  
 R' = OH

CB 25

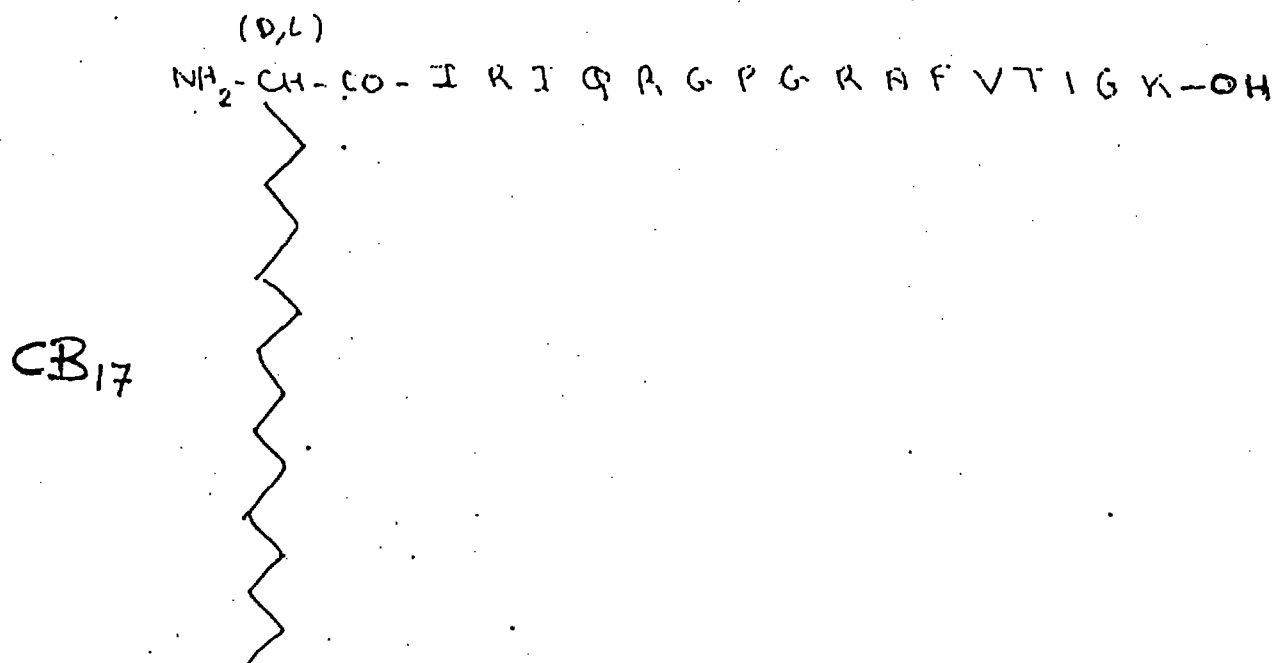


CB<sub>4</sub>



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15            The C-terminal end of the 312-327 sequence of HIV-1 (LAV-BRU) GP120 protein is according to a carboxylic condition. A racemic  $\alpha$ -amino-hexadecanoic acid has been fixed on the N-terminal region. The N-terminal functional group is according to an amine condition.

This Sheet Has Been Replaced



**TABLE I**  
**ANTI-NP 147-158R PEPTIDE IMMUNIZATIONS (influenza virus)**

| In vivo injection                                    | In vitro stimulation     | Target                      | Cytolytic activity |      |       |
|------------------------------------------------------|--------------------------|-----------------------------|--------------------|------|-------|
|                                                      |                          |                             | d5                 | d12  | ≥ d21 |
| 0                                                    | NP.147-158R              | NP.147-158R (a)             | -                  | -    | ++    |
| Influ. virus                                         | NP.147-158R <sup>-</sup> | Influ. virus (b)            |                    |      | (+)   |
|                                                      |                          | NP.147-158R <sup>-</sup>    | (++)               | (++) | (+++) |
|                                                      |                          | Influ. virus                | (++)               | (++) | (+++) |
| NP.147-158R <sup>-</sup>                             | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | (-)                | -    | +     |
|                                                      |                          | Influ. virus                | (-)                | -    |       |
| P <sub>3</sub> CSS-Pep.NP                            | Influ. virus             | Influ. virus                | (-)                |      |       |
|                                                      | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | (++)               |      |       |
|                                                      |                          | Influ. virus                | (++)               |      |       |
|                                                      |                          | P <sub>3</sub> CSS-Pep. NP. | (++)               |      |       |
| LIPOSOME-Pep.NP. (c)                                 |                          |                             |                    |      |       |
| [1 x s.c. syntex]                                    | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | -                  | -    |       |
| [1 x i.p.]                                           | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | -                  | -    |       |
| [2 x s.c. syntex]                                    | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | -                  | ++   |       |
| [2 x i.p.]                                           | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | -                  | ++   |       |
| LIPOPEPTIDES-Pep.NP.                                 |                          |                             |                    |      |       |
| L1-Pep.NP. [2 x i.p.]                                | NP.147-158R              | NP.147-158R <sup>-</sup>    | -                  | +++  |       |
|                                                      |                          | Influ. virus                | -                  |      |       |
|                                                      |                          | L1-Pep.NP.                  | +++                | +++  |       |
| L2-Pep.NP. [2 x i.p.]                                | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | -                  | -    |       |
|                                                      |                          | Influ. virus                | -                  | -    |       |
|                                                      |                          | L1-Pep.NP.                  | -                  | -    |       |
| L3-Pep.NP. [2 x i.p.]                                | NP.147-158R <sup>-</sup> | NP.147-158R <sup>-</sup>    | -                  | -    |       |
|                                                      |                          | Influ. virus                | -                  | -    |       |
|                                                      |                          | L1-Pep.NP.                  | -                  | -    |       |
| The results in brackets have already been published. |                          |                             |                    |      |       |

(a) syngenic target cells in the presence of 3 μm of the peptide NP.147-158 R<sup>-</sup>

(b) syngenic target cells infected by the virus influenza A

(c) liposomes loaded with peptide NP 147-158 R<sup>-</sup>

TABLE II  
ANTI-ENV.312-327 PEPTIDE IMMUNIZATIONS (HIV-BRU).

| In vivo injection        | In vitro stimulation | Target                                            | Cytolytic activity d5 | d12        | ≥ d21      |
|--------------------------|----------------------|---------------------------------------------------|-----------------------|------------|------------|
| 0                        | ENV.312-327          | ENV.312-327 (a)<br>Vac-env (b)                    | -<br>-                | -<br>-     | ++<br>-    |
| Vac-env                  | CB1<br>ENV.312-327   | ENV.312-327 (a)<br>ENV.312-327 (b)<br>Vac-env (b) | -<br>(++)<br>(++)     |            | - (?)      |
| ENV.312-327              | ENV.312-327          | ENV.312-327 (a)<br>Vac-env (b)                    | -<br>-                | -          | ++         |
| LIPOPEPTIDES-ENV.312-327 |                      |                                                   |                       |            |            |
| CB1 [1 X i.p.]           | ENV.312-327          | ENV.312-327 (a)                                   | -                     |            |            |
| CB1 [2 X i.p.]           | ENV.312-327          | ENV.312-327 (b)<br>Vac-env                        | +++<br>+++            | +++<br>+++ | +++<br>+++ |
| CB1 [1 X s.c.syntax]     | ENV.312-327          | ENV.312-327 (a)                                   | -                     |            |            |
| CB1 [2 X s.c.syntax]     | ENV.312-327          | ENV.312-327 (b)<br>Vac-env (b)                    | ++<br>++              |            |            |
| CB2 [2 X i.p.]           | ENV.312-327          | ENV.312-327 (a)<br>Vac-env (b)                    | -<br>-                |            | ++<br>+++  |
| CB3 [2 i.p.]             | ENV.312-327          | ENV.312-327 (a)<br>Vac-env (b)                    | -<br>-                |            | -          |

(a) syngenic target cells in the presence of 3 μM of peptide Env 312-327

(b) syngenic target cells infected by a vaccine virus allowing the expression of the gene env of HIV



TABLE III  
ANTI-ENV.302-335 PEPTIDE IMMUNIZATIONS (HIV-BRU)

| In vivo injection        | In vitro stimulation             | Target                            | Cytolytic activity<br>d5 | d12       | ≥d21 |
|--------------------------|----------------------------------|-----------------------------------|--------------------------|-----------|------|
| ENV.302-336              | ENV.302-336<br>or<br>ENV.312-327 | ENV.312-327 (a)                   | -                        | -         | -    |
| LIPOPEPTIDES-ENV.302-335 |                                  |                                   |                          |           |      |
| CB6 [2 x i.p.]           | ENV.302-336<br>or<br>ENV.312-327 | ENV.312-327 (a) ++<br>Vac-env (b) |                          | +++       |      |
| CB7 [2 x i.p.]           | ENV.302-336<br>or<br>ENV.312-327 | ENV.312-327 (a)<br>Vac-env (b)    |                          | ++<br>+++ |      |
| CB 5 [2 x i.p.]          | ENV.302-336<br>or<br>ENV.312-327 | ENV.312-327 (a) -<br>Vac-env (b)  |                          | -<br>-    |      |

(a) Syngenic target cells in the presence of 3 $\mu$ M of peptide ENV 312-327

(b) Syngenic target cells infected by a vaccine virus allowing the expression of the gene ENV of HIV.

TABLE IV

Anti- ENV.312-327 peptide immunizations by CB<sub>1</sub>, CB<sub>4</sub>, CB<sub>5</sub>, CB<sub>17</sub>, CB<sub>19</sub>, CB<sub>21</sub> and CB<sub>25</sub>.

| in vivo injection  | in vitro stimulation | target                         | cytotoxic activity |                   |
|--------------------|----------------------|--------------------------------|--------------------|-------------------|
|                    |                      |                                | <14 days           | <21 days          |
| CE1 (2 x s.c.)     | ENV 312-327          | ENV 312-327,<br>Vac-ENV<br>CB1 | +++<br>+++<br>+++  | +++<br>+++<br>+++ |
| CB4 (2 x i.p.)     | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>+++         | +++<br>+++        |
| CB17 (2 x i.p.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | -<br>-             | ++<br>+           |
| CB19 (2 x i.p.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +<br>-             | ++<br>+           |
| CB21 (2 x i.p.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | ++<br>++           | +++<br>+++        |
| CB25 (2 x i.p.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>++          | NT                |
| CB25 (2 x i.p.FIA) | ENV 312-327          | ENV 312-327<br>Vac-ENV         | ++<br>+            | NT                |
| CB25 (3 x i.p.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>++          | NT                |
| CB25 (3 x i.p.FIA) | ENV 312-327          | ENV 312-327<br>Vac-ENV         | ++<br>++           | NT                |
| CB25 (2 x s.c.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>++          | NT                |
| CB25 (2 x s.c.FIA) | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>+++         | NT                |
| CB25 (3 x s.c.)    | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>++          | NT                |
| CB25 (3 x s.c.FIA) | ENV 312-327          | ENV 312-327<br>Vac-ENV         | +++<br>+++         | NT                |
| CB5 (2 x i.p.)     | ENV 312-327          | ENV 312-327<br>Vac ENV         | ++<br>++           | +++<br>+++        |



CLAIMS:

1. A lipopeptide comprising a peptide part having between 10 and 40 amino acids approximately and preferably between 10 and 20 amino acids approximately and comprising at least one antigenic determinant, said lipopeptide also comprising one or more chains derived from fatty acids comprising 10 to 20 carbon atoms and/or one or more steroid groups modified and coupled on the  $\alpha\text{NH}_2$  or  $\epsilon\text{NH}_2$  functional groups of said amino acids.

2. The lipopeptide according to claim 1, characterized in that the fatty acid-derived chain is 2-aminohexadecanoic acid or one of its derivatives.

3. The lipopeptide according to claim 1, characterized in that the fatty acid-derived chain is N- $\epsilon$ -palmitoyllysine or one of its derivatives.

4. The lipopeptide according to claim 3, characterized in that the fatty acid-derived chain is N,N'-dipalmitoyllysine.



5. The lipopeptide according to claim 1, characterized in that the fatty acid-derived chain is pimelautide or trimexautide or one of their derivatives.

6. The lipopeptide according to claim 1, characterized in that the steroid group is N-ε-[(cholest-5-enyl-3-oxy)-acetyl]lysine or one of its derivatives.

7. The lipopeptide according to claim 1, characterized in that the steroid group is (cholest-5-enyl-3-oxy)acetic acid or one of its derivatives.

8. The lipopeptide according to one of claims 1 to 7, characterized in that the peptide part is a fragment of a protein of the HIV1 or HIV2 viruses.

9. The lipopeptide according to claim 8, characterized in that the peptide part is a fragment of the protein encoded by the ENV gene and in particular the 312-327 fragment or 302-336 fragment.

10. A vaccine against a pathogenic agent containing one of the lipopeptides according to one of claims 1 to 9.

11. The vaccine according to claim 10 against diseases linked to HIV viruses, characterized in that it



contains one lipopeptide or lipopeptides according to one of claims 8 and 9.

12. The use of the lipopeptides according to one of claims 1 to 9 for immunizing the human or animal body against pathogenic agents by inducing cytotoxic T lymphocytes.

13. The use according to claim 12, characterized in that the pathogenic agents are viruses, in particular the HIV1 and HIV2 viruses, in which case the immunization is carried out by means of the lipopeptides according to one of claims 8 and 9.

14. The use according to claim 12, characterized in that the pathogenic agents are parasites.

15. Pharmaceutical composition, characterized in that it contains an efficient quantity of at least one compound according to one of claims 1 to 9 in association with one or several diluents or adjuvants compatible and pharmaceutically acceptable.



16. Composition according to claim 15, characterized in that it is intended for treating the diseases related with HIV viruses by induction of the cytotoxic T lymphocytes.



This Sheet Has Been Replaced

