SYNTHESIS AND EVALUATION OF ANTIGENIC PROPERTIES OF PEPTIDES OF THE HIV-1 PROTEIN GP 41

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Scheme is described for the synthesis of 19- and 21-member peptides which constitute a part of the domain (sequence 584–609) from the conservative region of the transmembrane protein gp 41 HIV-1. The synthesis was carried out using standard methods of peptide chemistry in solution. The peptides were built from fragments which were synthesized by means of stepwise joining of amino acid residues starting from the C-terminus, using activated esters. The "complex F" or DCC + HONB methods were employed for condensation of the fragments. Alpha amino groups were protected by means of Z- and Boc- protecting groups. The tert. butyl group was used for the protection of functional side chains. The sulfhydryl group of cysteine was blocked with S-acetamidomethyl group and salt formation or benzyl esters were used for protection of the carboxyl group. During the synthesis, it was necessary to solve problems connected with racemization of Asp and Glu residues in the course of condensation of fragments, as well as formation of a beta-peptide at the position of aspartic acid. HPLC and NMR methods were utilized extensively for the separation and characterization of the structure of the synthesized compounds. The final peptides were tested in enzyme immunoassay. ELISA tests proved that the peptides can be employed as antigenic components for the diagnosis of HIV-1.

The acquired immune defficiency syndrome (AIDS) was first described by Gottlieb et al.[1], Mazur[2] and Siegal[3]. It was ascertained that it is caused by a retrovirus named the human immunodeficiency virus (HIV). Several test systems were elaborated which enable to prove the presence of antibodies to HIV. First, partially purified lysate of the virus was employed as the antigen for the determination of antibodies to HIV proteins[4], which resulted often in screening cross-reacting serum antibodies to other virus antigens or host-cells antigens, rather then antibodies to HIV. Another access is represented by the use of recombinant proteins or synthetic peptides as antigens.

Cosand[5] described a peptide of the sequence 584–609 from the conservative region of the trans-membrane protein gp 41, HIV-1 which corresponds to the main immunodominant domain, the structure of which is as follows:

$$\overset{1}{\text{H-Arg}_{584}}\text{-Ile-Leu-Ala-}\overset{5}{\text{Val}}\text{-Glu-Arg-Tyr-Leu-}\overset{10}{\text{Lys}}\text{-Asp-Gln-Gln-Leu-}$$

$$\text{-}\overset{15}{\text{Leu}}\text{-Gly-Ile-Trp-Gly-Cys-}\overset{21}{\text{Ser}_{604}}\text{-OH}$$

Other workers[6–10] obtained good results by means of enzyme immunoanalysis using various peptides corresponding to the mentioned domains as antigens (the sensitivity and specificity exceeded 99%). All the peptide determinants were synthesized by standard solid-phase methods.

The aim of this work was to elaborate a scheme for the synthesis of peptides with sequences 584–604 (ref.[6]) and 584–602 (ref.[10]) of the HIV-1 surface protein using methods of peptide chemistry in solution and studies of the possibility to employ these antigens for the determination of antibodies to HIV.

In our first attempt of synthesis the peptide sequence (*XXIV*) was divided into four approximately equal fragments, i.e. 1–6, 7–11, 12–16 and 17–21. Fragments 1–6 and 17–21 were split further into shorter fragments 1–3, 4–6 and 17 to 19, 20–21, respectively. Side function groups of aspartic acid, glutamic acid, serine and tyrosine were protected with tert. butyl type protecting group. The sulfhydryl group of cysteine was blocked by S-acetamidomethyl (Acm) protection, arginine was converted into the Boc-(ω-nitro) or Z-(ω-nitro) derivative. Z- or Boc- protecting groups were used for the protection of α-amino groups. The synthesis of the fragment 17–19 in the form of the tert. butyl ester preceded the synthesis of the peptide *XXV*. Tert. butyl esters was used also for the protection of the carboxyl group of serine in the position 21. In all other cases for the protection of carboxyl groups benzyl esters or salt formation were used.

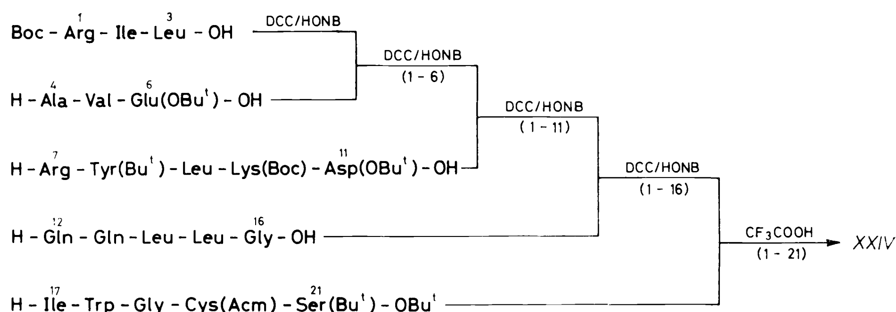
The following fragments were synthesized in this manner:

Boc-Arg-Ile-Leu-OH	(1–3)
Z-Ala-Val-Glu(OBu^t)-OH	(4–6)
Z-Arg(NO_2)-Tyr(Bu^t)-Leu-Lys(Boc)-Asp(OBu^t)-OH	(7–11)
Z-Gln-Gln-Leu-Leu-Gly-OH	(12–16)
Boc-Ile-Trp-Gly-Cys(Acm)-Ser(Bu^t)OBu^t	(17–21)
Z-Ile-Trp-Gly-OBu^t	(17–19)

The condensation of these fragments was carried out in the following sequence (Scheme 1):

{[(1–6) + (7–11)] + (12–16)} + (17–21) in the case of *XXIV*, and

{[(1–6) + (7–11)] + (12–16)} + (17–19) in the case of *XXV*.



SCHEME 1

Fragments 1–3 and 4–6 were synthesized by the method of stepwise growing of the peptide chain. The leucine carboxyl group was protected with benzyl ester, the carboxyl group of glutamic acid was protected by salt formation with Triton B. The synthesized protected peptide with the sequence 1–3 (*II*) was subjected to catalytic hydrogenolysis and activation (DCC + HONB) and then, condensed with the fragment 4–6 after its previous hydrogenolysis. In the case of tripeptide ONB-ester formation an addition of an equivalent amount of pyridine bromohydrate to the reaction mixture caused disruption of the internal salt and rapid quantitative formation of the ONB-ester of the N-terminal fragment[11].

Fragments 7–11 and 12–16 were synthesized by means of stepwise growing of the peptide chain without any difficulties. In the case of the fragment 7–11, the carboxyl group was protected by salt formation. The fragment 14–16 was synthesized in a form of the tert. butyl ester, the C-terminal carboxyl function being protected also by salt formation during further stages of synthesis. Side reactions connected with the formation of pyroglutamic acid were not observed in the course of the synthesis and deprotection of the peptide 12–16.

The fragment 17–21 was synthesized according to the scheme 3 + 2. We obtained Boc-Cys(Acm)-Ser(Bu^t)OBu^t starting from Boc-Cys(Acm)ONp and H-Ser-(Bu^t)OBu^t. Selective removing of Boc-protection from α-amino group of the dipeptide formed was carried out by the action of 90% formic acid. Special conditions were selected (peptide concentration, reaction time) which ensured complete cleavage of the Boc-protection from the α-amino group without damaging the tert. butyl-type protection of the C-terminus serine hydroxy- and carboxyl- functions. Progress of the reaction was checked by means of HPLC method. The condensation of 17–19 and 20–21 fragments was carried out with the aid of "complex F".

Condensation of the fragments 1–6 and 7–11 was carried out using DCC + HONB. The fragment 1–6 was converted into an activated ester and coupled to

the fragment 7–11. After isolation of protected peptide (1–11) from reaction mixture small sample of the peptide was deblocked for its analytical analysis by HPLC method. It was shown that the sample contains two compounds. We performed a separation of both components on a column (1·6 × 25 cm) with Silasorb C-18 (5 µm, Chemapol, Czechoslovakia) (Fig. 1). Gradient water–methanol (each solvent containing 0·02M triethylamine acetate, pH 4·0) was employed for the separation, the elution time was 50 min at the speed of 10 ml per min. The individual components *A* and *B* were subjected to ^{1}H NMR analysis which reflected all the signals expected in agreement with the amino acid composition in both spectra. At the same time, no resonance lines were ascertained indicating modifications due to the presence of additional chemical groups created by acetylation, alkylation, etc. Spectra of both compounds were similar, however, major differences were found in chemical shifts of amide and α-protons. Differences in chemical shifts of homologic protons reach the minimum values for amino acid residues present at the ends of the peptide chains and the maximum ones for Val and Glu residues. Observations by the Nuclear Overhauser effect (NOE) method showed that the sequences of both peptides are correct and all amino acid residues are bound by α-peptidic bonds.

Analysis of the obtained data and the fact that the condensation of fragments 1–6 and 7–11 was carried out via the glutamic acid residue enabled us to suggest racemization of glutamic acid. Ratio of the compounds *A* and *B* (Fig. 1) enabled us to assign *A* to a peptide with the composition H-Arg-Ile-Leu-Ala-Val-D-Glu-Arg-Tyr--Leu-Lys-Asp-OH and the compound *B* to H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr--Leu-Lys-Asp-OH. Acidic hydrolysis of compounds *A* and *B*, followed by the action of a mixture of L-amino acid oxidase and catalyse[12] on the hydrolyzate supported this hypothesis.

It is necessary to point out that condensation of fragments 1–6 and 7–11 by means of the "complex F" results in an even more dramatic ratio of compounds *A* and *B*, 45 : 55. We succeeded to purify partially the protected undecapeptide 1–11 after three recrystallizations from DMF (content of the major substance determined by HPLC was at least 95% after standard deprotection).

The fragment 1–11 was activated by DCC and HONB. Before the activation, we added once more an equivalent amount of pyridine bromohydrate to the reaction mixture for the purpose of protonization of the second arginine residue. Then we realized the condensation with the fragment 12–16. A small sample of the product was deprotected under conditions mentioned above. The analytical HPLC check showed that we obtained, unfortunately, two compounds at a ratio 1 : 2. We did not carry out their separation and analysis in this case, presuming that, again, racemization took place at the aspartic acid residue. Separation and analysis of peptides of the sequence 1–21 supported this presumption.

The fragment 1–16 obtained in this manner was condensed with the fragment 17–21. After the deprotection (CF_3COOH-H_2O, 9 : 1), we separated two compounds, *D* and *XXIV*, using the method of preparative HPLC under conditions depicted in Fig. 2. It is necessary to stress that in the case that the mixture of diastereoisomers of the N-terminal fragment of the protected peptide 1–11 was employed for the preparation of the peptides 1–16 and then 1–21, the reaction mixture contained several compounds (Fig. 2). We submitted the reaction mixture to preparative HPLC on a column with Silasorb C-18, 7·5 μm (16 × 250 mm) using a gradient

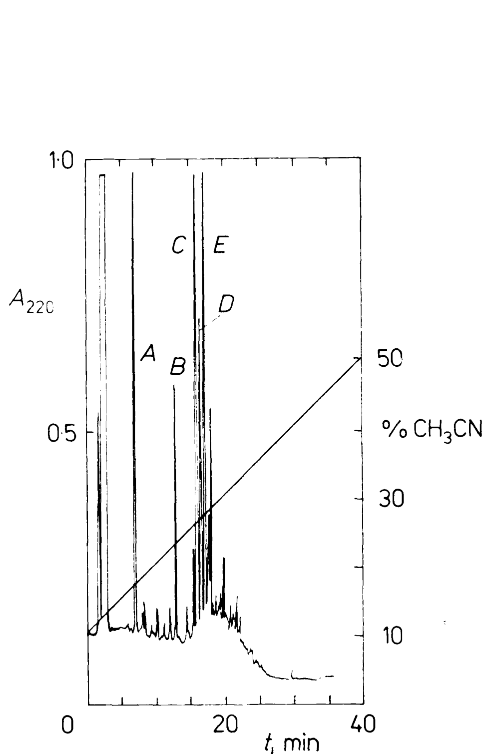


FIG. 1

HPLC of the fragment 1–11: *A* [D-Glu[6]] 1–11; *B* [L-Glu[6]] 1–11; Lichrosorb RP-18 (250 × 4·0 mm); gradient: 0·05M KH_2PO_4 (pH 3·0)–acetonitrile; 20 min

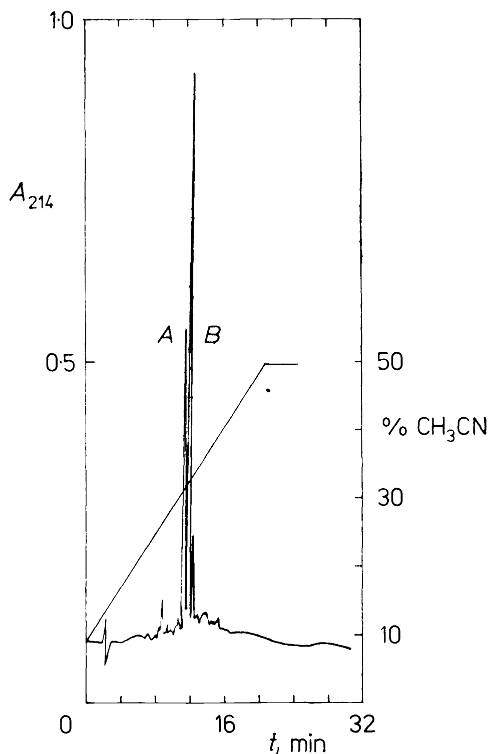
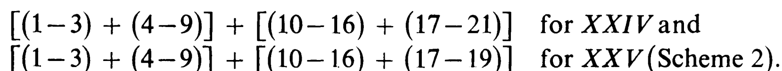


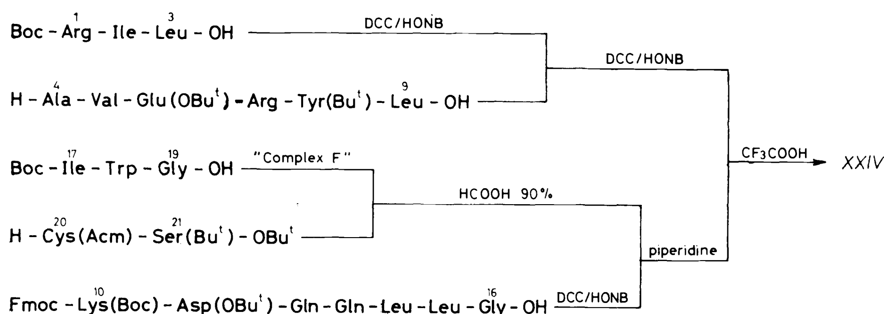
FIG. 2

HPLC of the reaction mixture during the synthesis of the peptide 1–21 (see text): *A* fragment 17–21; *B* N-acetyl derivative of *A*, *C* mixture of [D-Glu[6], D-Asp[11]] 1–21 and [D-Glu[6]] 1–21; *D* [D-Asp[11]] 1–21; *E* 1–21 peptide *XXIV*; TSK ODS-120T (250 × 4·6 mm); gradient: 0·05% TFA–acetonitrile (0·05%) TFA; 40 min

water–acetonitrile (0 – 100%, each solvent containing 0·05% trifluoroacetic acid) and obtained five fractions (*A*, *B*, *C*, *D*, *E*) homogenous according to HPLC. The analysis of the fractions by ^{1}H NMR showed that the fraction *A* is of the sequence 17 – 21 and the fraction *B* is an acetyl derivative of the fragment 17 – 21. In spectra of fractions *C*, *D*, *E* we ascertained all the signals expected for the amino acid composition of the end product – peptide 1 – 21 and no other signals were registered. Differences in chemical shifts due to homologic protons were most significant in the case of glutamic acid and aspartic acid residues. The analysis of ^{1}H NMR spectra* enabled us to ascribe the following configurations to these residues: *C* mixture of [D-Glu^6, D-Asp^{11}]-1-21 and [D-Glu^6]-1-21; *D* [D-Asp^{11}]-1-21; *E* 1 – 21 peptide without racemization. These configurations were confirmed by the analysis of hydrolyzate of fractions *C*, *D* and *E* by gas chromatography on optically active phase[13]. The peptide *XXIV* obtained according to the scheme presented below was proved to be the compound *E* by means of HPLC and ^{1}H NMR.

The antigenic properties of fractions *C*, *D* and *E* (*XXIV*) were examined by means of the enzyme immunoanalysis of antibodies to HIV and the results were found to be equal (Table I). However, the proposed scheme for the synthesis did not satisfy us. Therefore, we decided to design new ones:


[(1 – 3) + (4 – 9)] + [(10 – 16) + (17 – 21)] for *XXIV* and
[(1 – 3) + (4 – 9)] + [(10 – 16) + (17 – 19)] for *XXV* (Scheme 2).



SCHEME 2

It was necessary to synthesize newly the fragment 4 – 9 (*VIII*) for this purpose and for the preparation of the fragment 10 – 16 (*XV*), the existing fragment 12 – 16 (*XIII*) was used. The synthesis of the fragment 4 – 9 (*VIII*) was carried out without difficulties using the method of stepwise synthesis of the peptide chain. In the stage

* The method for the analysis of optical purity of peptides by means of ^{1}H NMR will be described in other publication.

of di- or tripeptide, we protected the carboxyl group by salt formation. Arginine was introduced in the form of Z-Arg(NO_2)OPfp, then the protected tripeptide was hydrogenated. The guanidine group was not protected during the next steps. Condensation of fragments 1 – 3 (*III*) and 4 – 9 (*VIIIa*) was carried out under conditions identical with those described for the condensation of fragments 1 – 3 and 4 – 6.

I,	Boc-Ile-Leu-OBzl
II,	Boc-Arg(NO_2)-Ile-Leu-OBzl
III,	Boc-Arg-Ile-Leu-OH
IV,	Z-Tyr(Bu^t)-Leu-OH . DCHA
V,	Z-Arg(NO_2)-Tyr(Bu^t)-Leu-OH
VI,	Z-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH
VII,	Z-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH
VIII,	Z-Ala-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH
VIIIa,	H-Ala-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH
IX,	Boc-Arg-Ile-Leu-Ala-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH
X,	Z-Leu-Gly-OBu^t
XI,	Z-Leu-Leu-Gly-OBu^t
XII,	Z-Gln-Leu-Leu-Gly-OBu^t
XIII,	Z-Gln-Gln-Leu-Leu-Gly-OBu^t
XIV,	Z-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-OH
XV,	Fmoc-Lys(Boc)-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-OH
XVI,	Z-Trp-Gly-OH
XVII,	Boc-Ile-Trp-Gly-OH
XVIII,	Boc-Cys(Acm)-Ser(Bu^t)-OBu^t
XIX,	Boc-Ile-Trp-Gly-Cys(Acm)-Ser(Bu^t)-OBu^t
XX,	Z-Trp-Gly-OBu^t
XXI,	Z-Ile-Trp-Gly-OBu^t
XXII,	Fmoc-Lys(Boc)-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys(Acm)-Ser(Bu^t)--OBu^t
XXIII,	Fmoc-Lys(Boc)-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-OBu^t

TABLE I

Results of testing of the peptides

Compound	Mean significance for negative and false-positive sera	Mean significance for positive sera	Sensitivity %	Specificity %
XXV	0·10 ± 0·08	1·18 ± 0·80	78·8	96·1
C	0·09 ± 0·10	1·15 ± 0·82	76·6	92·2
D	0·09 ± 0·09	1·14 ± 0·79	80·3	96·1
XXIV	0·03 ± 0·07	1·23 ± 0·80	86·9	96·1
XXVI	0·20 ± 0·21	1·40 ± 0·67	92·7	86·3

XXIV, H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp- -Gly-Cys(Acm)-Ser-OH

XXV, H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp- -Gly-OH

XXVI, H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp- -Gly-Cys-Ser-OH

It is necessary to point out some peculiarities occurring during the synthesis of the fragment 10–16 (*XV*). The fragment 12–16 (*XIII*) was submitted to catalytic hydrogenolysis (the carboxyl group being protected during the condensation) and Z-Asp(OBu^t)-ONSu was used for the attaching of the aspartic acid residue. The reaction proceeded quantitatively in the course of 5 min. The carbobenzoxy protection group was released from the α-amino group of the hexapeptide 11–16 (*XIV*) by catalytic hydrogenolysis and the resulted peptide H-Asp(OBu^t)-Gln-Gln-Leu- -Leu-Gly-OH was converted into the Triton salt by addition of one equivalent of Triton B in methanol. Simultaneously, a side product was formed with a substantially lower R_F. Addition of two equivalents of Triton B resulted in a quantitative course of this reaction. The compounds formed were separated and analyzed by ^{1}H NMR. Studies using the Nuclear Overhauser effect showed that we came across a wide-branched side reaction yielding mixtures of α- and β-peptides with regard to the aspartic acid residue, with simultaneous cleavage of the tert. butyloxycarbonyl group[13–15]. We overcame these difficulties as follows: The suspension of the hexapeptide H-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-OH in dry DMF was treated with bis(O,N-trimethylsilyl)acetamide. The solubility of the hexapeptide increases rapidly during this treatment. The TMS-group does not prevent the acylation agent from attacking the amino group[16], the reaction with Fmoc-Lys(Boc)-ONSu proceeded rapidly and with high yield (90%) and the cleavage of the silyl group was realized at the stage of isolation of the protected heptapeptide. This heptapeptide of the sequence 10–16 (*XV*) was condensed with the pentapeptide of the sequence 17–21 or with the tripeptide 17–19. The Fmoc cleavage of peptides 10–21 (*XXII*) and 10–19 (*XXIII*) was carried out by means of piperidine and the condensation of the large fragments was performed by the DCC + HONB method. The protected peptides were treated with 90% trifluoroacetic acid for 1 h and purified on a column (2·5 × 100 cm) of Toyo-pearl HW-40 in 0·1% trifluoroacetic acid or by HPLC under conditions showed in Fig. 2. It is necessary to point out that HPLC purification under the mentioned conditions was not favorable due to high adsorption of the peptide to the reverse phase leading to significantly reduced yield (twice) of the final product.

Small sample of the compound *XXIV* was treated with mercury acetate, mercaptoethanol and was purified on a column (2·5 × 100 cm) with Toyo-pearl HW-40 in order to obtain the peptide 1–21 (*XXVI*) with free sulfhydryl groups. The amino

acid sequence of the synthesized peptides was correct and their homogeneity was proved by thin-layer and HPLC chromatography (Fig. 3). The peptides were characterized by ^{1}H NMR (500 MHz).

The synthesized peptides corresponding to the sequences 584–604 and 584–602 of the transmembrane protein gp 41 HIV were tested as antigens for determination of antibodies to HIV.

Testing of the peptides was using the indirect solid-phase immunological method ELISA (ref.[6]) on blood serum plates, preliminary observation by western blotting, immunofluorescence, agglutination "Serodia" test (Fudji Rebio) and solid-phase immunofluorescence method Vinotype (G.D.R.). The serum plate was composed of 36 serum samples negative in all the employed tests, 16 samples negative in western blotting but positive in any other tests (false-positive) and 137 samples positive in western blotting. Results of the testing are summarized in Table I. It is necessary to stress the low level of reaction observed with the peptide *XXIV* in contrast to the high level of the peptide *XXVI* reaction with negative and false-positive sera which can explain the low specificity of the peptide *XXVI*.

The approximately equal level of measurable antibodies to all the peptides enables to use either peptide for the determination of antibodies to HIV. High correlation coefficients (not less than 0·90) confirm almost full identity of the compared peptides in the employed tests.

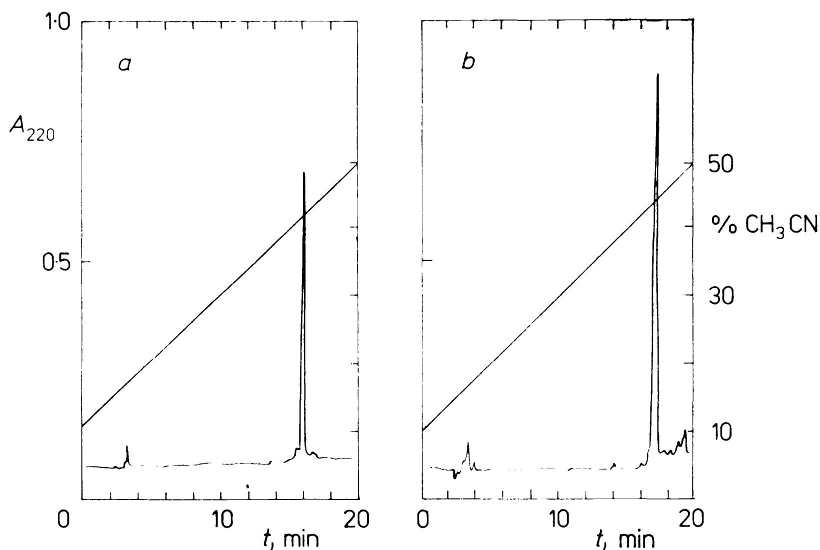


FIG. 3

HPLC of the peptides: *a* 1–19 (*XXV*) and *b* 1–21 (*XXIV*). TSK ODS-120T (250 × 4·6 mm); gradient: 0·05% TFA–acetonitrile (0·05%) TFA; 20 min

EXPERIMENTAL

Amino acids and their derivatives used throughout the work were purchased from Reanal (Hungary) and Bachem (Switzerland). The purity of the compounds was checked by TLC on plates Kieselgel 60, Merck (F.R.G.) in the following solvent systems: chloroform–methanol–32% acetic acid, 60 : 45 : 20 (A); 1-butanol–acetic acid–water, 3 : 1 : 1 (B); 1-butanol–acetic acid–pyridine–water, 10 : 5 : 6 : 1 : 7·5 (C); ethyl acetate–pyridine–acetic acid–water, 45 : 20 : 6 : 11 (D); chloroform–ethyl acetate–methanol–acetic acid, 6 : 3 : 1 : 0·5 (E); chloroform–methanol, 4·5 : 1 (F); chloroform–methanol–acetic acid, 32 : 2 : 1 (G); chloroform–methanol–acetic acid, 9 : 1 : 0·5 (H); chloroform–ethyl acetate–methanol, 6 : 3 : 1 (I); chloroform–methanol–acetic acid–water, 7 : 4 : 1 : 1 (K); ethyl acetate–hexane, 1 : 1 (L); chloroform–methanol, 9 : 1 (M); trichloromethane–methanol–acetic acid. 9 : 1 : 0·5 (N). The compounds were visualized with ninhydrin or chlorine–benzidine. Hydrogenation of the peptides was carried out in the presence of 10% Pd/C (5—10% w/w) from Fluka or Merck. The uncorrected melting points were measured on Boetius apparatus (G.D.R.).

Optical rotations were determined on a polarimeter Perkin–Elmer (U.S.A.). Amino acid analysis of peptides (hydrolyzed with 6M-HCl with 2% phenol at 110°C, 24 h) was carried out with the aid of the automatic analyzer Labotron Liquimat (F.R.G.), cysteine and tryptophan were not determined. HPLC was performed on Altex 332 apparatus with a Altex/Hitachi 155-40 detector at the wavelength of 220 nm. We employed acetonitrile (Merck Lichrosolv), water and trifluoroacetic acid three times distilled in glass apparatus and KH_2PO_4 (Sigma) for the separation. The identity of the individual components was confirmed by ^1H NMR spectra recorded on Bruker WH-500 spectrometer (F.R.G.) at a frequency of 500 MHz at 27°C. The samples were prepared by dissolving 1 mg of the compound in 0·5 ml hexadeuterodimethylsulfoxide containing 1% TFA.

All organic solvents were dried over Na_2SO_4 after the extraction and washing with water. The solutions were evaporated on rotary evaporators at 40°C. Column chromatography was carried out on Silicagel L 40—100 (Czechoslovakia).

Boc-Ile-Leu-OBzl (*I*)

To the solution of 2·1 g (5·6 mmol) of TosOH.H-Leu-OBzl in 50 ml DMF 0·8 ml (5·75 mmol) of triethylamine and 2·0 g (5·7 mmol) of Boc-Ile-ONp were added. The reaction mixture was left for 18 h at 20°C and then evaporated. The residue was dissolved in 80 ml ethyl acetate, rinsed subsequently with 5% ammonium, water, 2% H_2SO_4 and water, dried and evaporated. Crystallization from ether afforded 2·24 g (92%) of compound *I*; m.p. 107—108°C; $[\alpha]_D$ −21·0° (*c* 0·2, DMF); R_F 0·68 (B), 0·70 (H), 0·94 (I). ^1H NMR: Boc: 1·36; Ile: NH 6·66, α-CH 3·81; Leu: NH 8·19, α-CH 4·36; OBzl: CH_2 5·09.

Boc-Arg(NO_2)-Ile-Leu-OBzl (*II*)

The compound *I* (0·96 g, 2·2 mmol) was treated with 25 ml 1M-HCl in acetic acid for 1 h. The reaction mixture was evaporated. The oily residue was treated with 50 ml hexane, the crystalline matter formed was filtered, dried in a vacuum desiccator over NaOH and dissolved in 10 ml DMF. Then, 0·24 ml (2·2 mmol) of methylmorpholine was added, followed with the solution of 0·97 g (2 mmol) Boc-Arg(NO_2)OPfp in 10 ml DMF. The reaction mixture was left for 3 h at 20°C and evaporated. The residue was dissolved in 80 ml ethyl acetate, rinsed with 2% $NaHCO_3$, water, dried and evaporated. After the treatment with the mixture ethyl acetate–ether, the yield was 0·95 g (74%) of compound *II* in a form of amorphous powder; $[\alpha]_D$ −15·0° (*c* 0·2, DMF);

R_F 0·22 (B), 0·41 (H), 0·58 (I). ^{1}H NMR: Boc: 1·37, Arg: NH 7·01, α-CH 3·91; Ile: NH 7·57, α-CH 4·22; Leu: NH 8·38, α-CH 4·33; OBzl: CH_2 5·09.

Boc-Arg-Ile-Leu-OH (*III*)

Compound *II* (0·9 g, 1·4 mmol) was hydrogenated in glacial acetic acid in the presence of Pd/C. The catalyst was filtered off, the filtrate evaporated. The oily residue was mixed with 25 ml of ether, the precipitate formed was filtered, rinsed with ether and dried over NaOH. Yield: 0·69 g (97%) of compound *III* in a form of amorphous powder; $[\alpha]_D$ +6·0° (*c* 0·2, DMF); R_F 0·55 (A), 0·57 (D), 0·59 (B). ^{1}H NMR: Boc: 1·37; Arg: NH 7·05, α-CH 3·92; Ile: NH 7·53, α-CH 4·23; Leu: NH 8·21, α-CH 4·20.

Z-Tyr(Bu^t)-Leu-OH DCHA (*IV*)

Z-Tyr(Bu^t)-ONSu (4·0 g, 8·54 mmol) was dissolved in 50 ml DMF. Solution of 1·12 g (8·54 mmol) of leucine in 8·5 ml 1M-NaOH was added to this solution. The reaction mixture was stirred for 12 h at room temperature and evaporated. The residue was dissolved in 100 ml ethyl acetate, rinsed with 2% H_2SO_4, water and evaporated. The oily residue was dissolved in 100 ml of ether and 1·7 ml (8·7 mmol) DCHA were added. The crystals formed were filtered, rinsed with ether and dried. Yield: 4·67 g (82%) of compound *IV*; m.p. 154–107°C; $[\alpha]_D$ −4·5° (*c* 0·2, DMF); R_F 0·39, 0·14 (DCHA) (H), 0·95, 0·67 (DCHA) (B), 0·40, 0·09 (DCHA) (N). ^{1}H NMR: Z: CH_2 4·93; Tyr(Bu^t): NH 7·41, α-CH 4·26, Bu^t 1·26; Leu: NH 8·18, α-CH 4·24.

Z-Arg(NO_2)-Tyr(Bu^t)-Leu-OH (*V*)

Compound *IV* (2·26 g, 3·4 mmol) was suspended in 50 ml ethyl acetate, rinsed three times with 2% H_2SO_4, with water up to neutral pH and evaporated. The oily residue was dissolved in 50 ml ethanol and hydrogenated in the presence of Pd/C. The catalyst was filtered off, to the filtrate 1·4 ml (3·4 mmol) of Triton B in methanol were added and the mixture was evaporated. The residue was dissolved in 50 ml DMF, the volume was adjusted to 20 ml and the solution of 1·77 g (3·4 mmol) Z-Arg(NO_2)-OPfp in 15 ml DMF was added. The reaction mixture was left for 4 h at room temperature, evaporated and 30 ml of 2% H_2SO_4 were added to the residue. After extraction with ethyl acetate, the organic phase was washed with water until the pH was neutral, the residue was triturated with hexane and dried. Recrystallization from ether afforded 2·0 g (86%) of compound *V*; m.p. 113–117°C; $[\alpha]_D$ −11·5° (*c* 0·2, DMF); R_F 0·11 (H), 0·85 (D), 0·88 (B). ^{1}H NMR: Z: CH_2 5·01; Arg: NH 7·37, α-CH 3·95; Tyr(Bu^t): NH 7·90, α-CH 4·54, Bu^t 1·24; Leu: NH 8·18, α-CH 4·23.

Z-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH (*VI*)

Compound *V* (1·82 g, 2·67 mmol) was hydrogenated in glacial acetic acid in the presence of Pd/C. After removing the catalyst, the filtrate was evaporated. The oily residue was treated with 30 ml of ether, the precipitate was filtered, rinsed with ether, dried in a vacuum desiccator over NaOH and dissolved in 15 ml DMF. Solution of 1·5 g (3·45 mmol) Z-Glu(OBu^t)-ONSu in 15 ml DMF was added. The reaction mixture was left for 4 h at 20°C, evaporated and the residue was recrystallized twice from ethyl acetate with ether. Yield: 1·59 g (72%) of compound *VI*; m.p. 138–142°C; $[\alpha]_D$ −9·5 (*c* 0·2, DMF); R_F 0·67 (D), 0·74 (B), 0·05 (I). ^{1}H NMR: Z: CH_2 5·02; Glu(OBu^t): NH 7·43, α-CH 4·02, OBu^t 1·37; Arg: NH 7·94, α-CH 4·28; Tyr(Bu^t): NH 7·97, α-CH 4·54, Bu^t 1·25; Leu: NH 8·22, α-CH 4·22.

Z-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH (*VII*)

Compound *VI* (1·33 g, 1·61 mmol) was hydrogenated in 25 ml ethanol in the presence of Pd/C. After removing the catalyst, the filtrate was evaporated and the residue was dissolved in 10 ml DMF. Solution of 0·66 g (1·77 mmol) Z-Val-ONp in 10 ml DMF was added to this solution and the reaction mixture was agitated for 12 h at room temperature. Oily residue obtained after evaporation was dissolved in 40 ml of chloroform and separated on a column (2·5 × 15 cm) of Silicagel using a gradient chloroform–methanol. Fractions corresponding to the major product were pooled, evaporated and precipitated from ethanol with ether. The crystalline product was filtered and dried under vacuum. Yield: 0·91 g (61%) of compound *VII*; m.p. 215–218°C; $[\alpha]_D$ +7·0° (*c* 0·1, DMF); R_F 0·73 (D), 0·74 (B). ^{1}H NMR: Z: CH_2 5·03; Val: NH 7·30, α-CH 3·87; Glu(OBu^t): NH 8·02. α-CH 4·25. OBu^t 1·37; Arg: NH 7·92, α-CH 4·25, Tyr(Bu^t): NH 7·95, α-CH 4·54, Bu^t 1·25; Leu: NH 8·22, α-CH 4·22.

Z-Ala-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH (*VIII*)

Compound *VII* (0·9 g, 0·97 mmol) was hydrogenated in 15 ml ethanol in the presence of Pd/C. After removing the catalyst, the filtrate was evaporated and the residue was dissolved in 5 ml DMF. Solution of 0·41 g (1·19 mmol) Z-Ala-ONp in 5 ml DMF was added to this solution and the reaction mixture was agitated for 10 h at 20°C. After evaporation the product was precipitated from chloroform by ether, and dried in desiccator. Yield: 0·76 g (79%) of compound *VIII*; m.p. 254–256°C; $[\alpha]_D$ −31·5° (*c* 0·2, DMF); R_F 0·73 (D), 0·71 (B). Amino acid analysis: Glu 0·98 (1); Ala 0·95 (1); Val 0·93 (1); Leu 1·00 (1); Tyr 1·08 (1); Arg 1·05 (1). Content of the main product 97%. ^{1}H NMR: Z: CH_2 5·01; Ala: NH 7·48, α-CH 4·11; Val: NH 7·74, α-CH 4·16; Glu(OBu^t): NH 7·99, α-CH 4·27, OBu^t 1·35; Arg: NH 7·92, α-CH 4·29; Tyr(Bu^t): NH 7·97, α-CH 4·59, Bu^t 1·25; Leu: NH 8·26, α-CH 4·18.

H-Ala-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH (*VIIIa*)

Compound *VIII* (0·58 g, 0·58 mmol) was hydrogenated in 20 ml ethanol in the presence of Pd/C. The catalyst was filtered off, the filtrate evaporated and the solid residue was triturated with 2 ml of water and purified on a column Lobar (310–25) Lichroprep RP-8 using a gradient water-methanol (both solvents contained 0·01M ammonium acetate). Fractions corresponding to the main product were pooled, evaporated and crystallized from ethanol with ether. The product was filtered and dried in a desiccator under vacuum. Yield: 0·43 g (85%) of compound *VIIIa*; m.p. 227–230°C; $[\alpha]_D$ −28·1° (*c* 0·2, DMF); R_F 0·40 (D), 0·30 (B).

Boc-Arg-Ile-Leu-Ala-Val-Glu(OBu^t)-Arg-Tyr(Bu^t)-Leu-OH (*IX*)

Compound *III* (0·15 g, 0·30 mmol) was dissolved in 5 ml DMF and 48 mg (0·3 mmol) of pyridine bromohydrate was added together with 86 mg (0·48 mmol) HONB. The reaction mixture was cooled to −15°C and solution of 0·82 g (0·4 mmol) of DCC in 3 ml DMF was added. The reaction mixture was left for 12 h at +4°C and then for 5 h at 20°C. The precipitated N,N'-dicyclohexylurea was filtered off, the filtrate was evaporated and the solid was treated with 20 ml of ether. The oily product was washed three times by decantation with ether and dissolved in 5 ml DMF. Solution of 0·21 g (0·24 mmol) of compound *VIIIa* in 5 ml DMF was added to this solution. The reaction mixture was agitated for 10 h at room temperature, diluted with 100 ml ethyl acetate and the precipitate formed was filtered, rinsed with ethyl acetate and ether, and dried in vacuum desiccator. Yield: 0·31 g (95%) of compound *IX* in a form of amorphous powder; $[\alpha]_D$ −18·5° (*c* 0·2, DMF); R_F 0·54 (D), 0·35 (B), 0·63 (C). Amino acid analysis: Glu 0·97 (1);

Ala 0·96 (1); Val 1·07 (1); Ile 1·00 (1); Leu 2·07 (2); Tyr 1·05 (1); Arg 2·13 (2). Content of the main product 100%. ^{1}H NMR: Boc: 1·37; Arg: NH 7·06, α-CH 3·91; Ile: NH 7·58, α-CH 4·13; Leu: NH 8·07, α-CH 4·30; Ala: NH 7·91, α-CH 4·23; Val: NH 7·74, α-CH 4·13; Glu(O-Bu^t): NH 7·96, α-CH 4·25, OBu^t 1·38; Arg: NH 7·89, α-CH 4·25; Tyr(Bu^t): NH 7·95, α-CH 4·53, Bu^t 1·25; Leu: NH 8·22, α-CH 4·22.

Z-Leu-Gly-OBu^t (*X*)

To the solution of 5 g (21·7 mmol) of H_3PO_3 H-Gly-OBu^t in 50 ml water were added 2·3 g (21·7 mmol) of Na_2CO_3 and then, 7·8 g (20·2 mmol) Z-Leu-ONp and 2·7 g (20·0 mmol) HOBT in 80 ml tetrahydrofuran were added under continuous stirring. The reaction mixture was left for 20 h at room temperature, evaporated under vacuum and the residue was dissolved in 150 ml ethyl acetate, washed with 2% H_2SO_4, 5% $NaHCO_3$ and water. The ethyl acetate fraction was dried over sodium sulfate and evaporated. The oily product was dried under vacuum. Yield: 7·3 g (96%) of compound *X*; R_F 0·81 (E).

Z-Leu-Leu-Gly-OBu^t (*XI*)

The compound *X* (7·6 g, 20·0 mmol) was hydrogenated in 140 ml methanol in the presence of Pd/C. The catalyst was filtered off, the residue dissolved in 80 ml dioxane and solution of 7·7 g (19·9 mmol) Z-Leu-ONp and 2·8 g (20·0 mmol) HOBT in 80 ml dioxane was added. The reaction mixture was held 2 h at room temperature. Dioxane was evaporated under vacuum, the residue was dissolved in 150 ml ethyl acetate and washed with 2% H_2SO_4, 5% $NaHCO_3$ and water. The ethyl acetate fraction was evaporated under vacuum and the product was crystallized from ethyl acetate with ether. Yield: 8·9 g (91%) of compound *XI*; m.p. 135–137°C; $[\alpha]_D$ −21·4° (*c* 0·6, DMF); R_F 0·70 (E), 0·62 (B), 0·35 (L). ^{1}H NMR: Z: CH 5·02; Leu: NH 7·40, α-CH 4·05; Leu: NH 7·85, α-CH 4·35; Gly: NH 8·23, α_1-CH 3·74, α_2-CH 3·63; OBu^t: 1·39.

Z-Gln-Leu-Leu-Gly-OBu^t (*XII*)

The compound *XI* (5·0 g, 10·2 mmol) was hydrogenated in 100 ml of methanol in the presence of Pd/C. The catalyst was filtered off, the filtrate evaporated and the residue dissolved in 50 ml DMF. Solution of 4·0 g (10·0 mmol) Z-Gln-ONp and 1·35 g (10·0 mmol) HOBT in 80 ml DMF was added under continuous stirring and the reaction mixture was left for 2 h at room temperature. The precipitate formed during this time was filtered, rinsed with ethyl acetate, ether and dried under vacuum. Yield: 6·13 g (99%) of compound *XII*; m.p. 232–234°C; $[\alpha]_D$ −27·3° (*c* 0·2, DMF); R_F 0·51 (E), 0·33 (L), 0·54 (I). ^{1}H NMR: Z: CH 5·02; Gln: NH 7·44, α-CH 3·97; Leu: NH 7·92, α-CH 4·31; Leu: N 7·86, α-CH 4·32; Gly: NH 8·19, α_1-CH 3·74, α_2-CH 3·63; OBu^t: 1·38.

Z-Gln-Gln-Leu-Leu-Gly-OBu^t (*XIII*)

The compound *XII* (6·0 g, 9·6 mmol) was hydrogenated in 80 ml of methanol in the presence of Pd/C. The catalyst was filtered off, washed with methanol, the filtrate was evaporated and the residue dissolved in 50 ml DMF. Solution of 3·9 g (9·7 mmol) Z-Gln-ONp and 1·3 g (9·7 mmol) HOBT in 50 ml DMF was added to the solution and the reaction mixture was agitated for 20 h at room temperature. The gel formed was filtered off, washed with ethyl acetate, ether and dried under vacuum. Yield: 7·3 g (98%) of compound *XIII*; m.p. 235–237°C; $[\alpha]_D$ −27·2° (*c* 0·3, DMF); R_F 0·30 (H), 0·28 (L), 0·55 (I). ^{1}H NMR: Z: CH 5·02; Gln: NH 7·48, α-CH 3·97; Gln: NH 8·07, α-CH 4·23; Leu: NH 7·94, α-CH 4·30; Leu: N 7·84, α-CH 4·32; Gly: NH 8·19, α_1-CH 3·74, α_2-CH 3·63; OBu^t: 1·39.

Z-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-OH (*XIV*)

A) The compound *XIII* (6·7 g, 8·96 mmol) was dissolved in 80 ml of 100% TFA. TFA was evaporated during the course of 1 h, 100 ml ether was added to the residue and the precipitate formed was filtered off, washed with ether, dried, dissolved in 80 ml of the mixture DMF–water (1 : 1) and hydrogenated in the presence of Pd/C. The catalyst was filtered off, washed with water, the filtrate was evaporated with dioxane under vacuum to afford 4·5 g (90%) H-Gln-Gln-Leu--Leu-Gly-OH with R_F 0·32 (A), 0·40 (B).

B) The compound obtained in step *A*) (1·95 g, 2·68 mmol) was dissolved in 50 ml DMF, 1·7 ml (3·5 mmol) of 40% Triton B in methanol was added to the solution. The reaction mixture was evaporated to a volume of 20 ml and 2·2 g (5·2 mmol) Z-Asp(OBu^t)-ONSu in 50 ml DMF were added to it under continuous stirring. The mixture was stirred for further 5 min at room temperature, then DMF was evaporated under vacuum reducing the volume to 1/3. After treatment with 60 ml 2% H_2SO_4, the product was filtered, washed with water and isopropanol and dried under vacuum. Yield: 2·6 g (87%) of compound *XIV*; m.p. 210–213°C; $[\alpha]_D$ –10·5° (*c* 0·2, DMF); R_F 0·55 (A), 0·63 (B), 0·25 (G). ^{1}H NMR: Z: CH 5·04; Asp(OBu^t): NH 7·59, α-CH 4·38, OBu^t 1·36; Gln: NH 8·03. α-CH 4·20; Gln: NH 8·07, α-CH 4·21; Leu: NH 7·91, α-CH 4·30; Leu: N 7·84, α-CH 4·32; Gly: NH 8·11, α_1-CH 3·78, α_2-CH 3·68.

Fmoc-Lys(Boc)-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-OH (*XV*)

The compound *XIV* (0·44 g, 0·5 mmol) was hydrogenated in glacial acetic acid in the presence of Pd/C. The catalyst was filtered off, the filtrate was treated with 15 ml ether and the precipitate formed was filtered off, washed with ether on the filter, dried in a vacuum desiccator over NaOH and suspended in 3 ml DMF. To the suspension obtained in this manner, 0·28 ml (1·25 mmol) bis(O,N-trimethylsilyl)acetamide was added, followed by 0·34 g (0·6 mmol) Fmoc-Lys(Boc)--ONSu in 3 ml DMF. The reaction mixture was held for 12 h at room temperature, evaporated and the residue was treated with 40 ml water. The precipitate formed was filtered, washed with water and dried in a vacuum desiccator over P_2O_5. Crystallization from ethanol afforded 0·48 g (81%) of compound *XV*; m.p. 210–213°C; $[\alpha]_D$ –15·5° *c* 0·2, DMF); R_F 0·75 (A), 0·62 (B), 0·79 (K). Amino acid analysis: Asp 1·00 (1); Glu 1·94 (2); Gly 1·01 (1); Leu 2·14 (2); Lys 1·01 (1). Content of the main compound 100%. ^{1}H NMR: Fmoc-Lys(Boc): NH 7·47, α-CH 3·96, Boc 1·35; Asp(OBu^t): NH 8·24, α-CH 4·55. Bu^t 1·36; Gln: NH 7·84, α-CH 4·20, Gln: NH 8·04, α-CH 4·21; Leu: NH 7·89, α-CH 4·29; Leu: NH 7·82, α-CH 4·32; Gly: NH 8·11, α_1-CH 3·78, α_2-CH 3·68.

Z-Trp-Gly-OH (*XVI*)

Z-Trp-ONSu (10·0 g, 23·0 mmol) were dissolved in 80 ml dioxane and added under stirring to the solution of 2·6 g (34·6 mmol) glycine with 3·66 g (34·5 mmol) sodium carbonate in water. The reaction mixture was agitated for 24 h at room temperature, evaporated under vacuum and the oily product was treated with 70 ml ethyl acetate and rinsed twice with 2% H_2SO_4. The aqueous layer was extracted with ethyl acetate, washed with water to neutral pH, dried over sodium sulfate and evaporated. Yield: 8·9 g (98%) of compound *XVI* in a form of oil; R_F 0·27 (H).

Boc-Ile-Trp-Gly-OH (*XVII*)

The compound *XVI* (7·4 g, 18·7 mmol) was hydrogenated in 50 ml of methanol in the presence of Pd/C. The catalyst was filtered off, the filtrate was evaporated and the oily residue was dissolved in 15 ml water with pH adjusted to 8 with sodium carbonate and mixed with solution of

7·4 g (22·5 mmol) Boc-Ile-ONSu in 15 ml dioxane. The reaction mixture was stirred for 24 h at room temperature, dioxane was evaporated under vacuum and the reaction mixture was treated further as compound *XVI*. The yield of compound *XVII* was 8·1 g (91%) after recrystallization from ethyl acetate with ether; m.p. 128–132°C; $[\alpha]_D$ −17·3° (*c* 0·2, DMF); R_F 0·78 (B), 0·20 (H), 0·11 (L). ^{1}H NMR: Boc: 1·36; Ile: NH 6·70, α-CH 3·76; Trp: NH 7·81, α-CH 4·64; Gly: NH 8·29, α_1-CH 3·73, α_2-CH 3·73.

Boc-Cys(Acm)-Ser(Bu^t)-OBu^t (*XVIII*)

To the suspension of 1·52 g (6·0 mmol) HCl H-Ser(Bu^t)-OBu^t in 100 ml ethyl acetate saturated solution of sodium bicarbonate was added until the solution became clear. The ethyl acetate layer was separated, washed with water, evaporated and to the residue, 30 ml of isopropanol were added. Oily product formed after evaporation was dissolved in 30 ml DMF and solution of 2·48 g (6·0 mmol) Boc-Cys(Acm)-ONp in 30 ml DMF was added. The reaction mixture was left for 20 h at room temperature, evaporated and the residue was dissolved in 100 ml ethyl acetate, washed with 1% ammonium and water and evaporated again. The yield of compound *XVIII* (in a form of amorphous powder) was 2·3 g (78%) after precipitation from ethyl acetate with ether; $[\alpha]_D$ +0·5° (*c* 0·2, DMF); R_F 0·84 (B), 0·54 (I). ^{1}H NMR: Boc: 1·38; Cys(Acm): NH 6·98, α-CH 4·27, Acm 1·87; Ser(Bu^t): NH 7·74, α-CH 4·31, Bu^t 1·12; OBu^t: 1·39.

Boc-Ile-Trp-Gly-Cys(Acm)-Ser(Bu^t)-OBu^t (*XIX*)

The compound *XVIII* (0·49 g, 1·0 mmol) was treated with 85 ml of 90% formic acid during 2·5 h. The reaction mixture was evaporated, the oily product was decanted twice with ether, dissolved in a mixture methanol–water (7 : 3) and treated with ion-exchange resin Dowex-1 in OH^- cycle, until the pH reached value 8·0. The resin was filtered off and washed on filter with methanol, the residue was dissolved in 50 ml of isopropanol and evaporated. The oily product was dissolved in 10 ml DMF and solution of compound *XVII* (0·47 g, 1·0 mmol) in 5 ml DMF was added. "Complex F" (1·0 g, 2·5 mmol) was added to the mixture cooled to 0°C. The reaction mixture was left for 20 h at 20°C and the precipitated N,N′-dicyclohexylurea was filtered off. The filtrate was evaporated, the residue was treated with 50 ml ethyl acetate and the precipitate formed was filtered off, washed on the filter with ethyl acetate and dried. Yield: 0·56 g (66%) of compound *XIX* in a form of amorphous powder; $[\alpha]_D$ −8·5° (*c* 0·2, DMF); R_F 0·77 (B), 0·39 (I), 0·54 (H). ^{1}H NMR: Boc: 1·36; Ile: NH 6·70, α-CH 3·77; Trp: NH 7·85, α-CH 4·60; Gly: NH 8·22, α_1-CH 3·76, α_2-CH 3·70; Cys(Acm): NH 8·09, α-CH 4·71, Acm 1·87; Ser(Bu^t): NH 7·96, α-CH 4·32, Bu^t 1·12; OBu^t: 1·39.

Z-Trp-Gly-OBu^t (*XX*)

Z-Trp-OH (6·8 g, 20·0 mmol) was dissolved in 100 ml DMF. N-Methylmorpholine (2·2 ml, 20·0 mmol) was added and the solution was cooled to −15°C. Isobutyl chloroformate (2·75 ml, 20·0 mmol) was added under continuous stirring and the reaction mixture was stirred for further 2 min at −15°C. Then, solution of 5·06 g (23·7 mmol) H_3PO_4.H-Gly-OBu^t and 2·63 ml (23·9 mmol) N-methylmorpholine in 50 ml DMF was added and the reaction mixture was stirred for 30 min at −15°C and 1 h at room temperature. The solvent was evaporated under vacuum, the residue was dissolved in 200 ml ethyl acetate and washed with 2% H_2SO_4, saturated sodium bicarbonate and water until reaching neutral pH. The organic layer was dried over sodium sulfate and evaporated under vacuum. The oily product was dried under vacuum. Yield: 9·0 g (99%) of compound *XX*; R_F 0·66 (M), 0·76 (E).

Z-Ile-Trp-Gly-OBu^t (*XXI*)

The compound *XX* (4·5 g, 10·0 mmol) was hydrogenated in 100 ml methanol in the presence of Pd/C. The catalyst was filtered off, washed with methanol, the filtrate was evaporated under vacuum and the oily residue was dissolved in 50 ml DMF. Solution of 3·9 g (10·0 mmol) Z-Ile--ONp and 1·35 g (10·0 mmol) HOBT in 50 ml DMF was added under stirring. The reaction mixture was left for 2 h at room temperature and then, treated in the same manner as compound *XX*. Crystallization from the mixture ethyl acetate–ether afforded 5·5 g (97%) of compound *XXI*; m.p. 158–161°C; $[\alpha]_D$ −14·4° (*c* 0·2, DMF); R_F 0·79 (E), 0·43 (L), 0·55 (H). ^{1}H NMR: Z: CH_2 5·03; Ile: NH 7·58, α-CH 3·87, Trp: NH 7·92, α-CH 4·62; Gly: NH 8·34, α_1-CH 3·73, α_2-CH 3·68; OBu^t: 1·40.

Fmoc-Lys(Boc)-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly--Cys(Acm)-Ser(Bu^t)-OBu^t (*XXII*)

The compound *XIX* (0·46 g, 0·54 mmol) was treated with 45 ml of 90% formic acid in the course of 0·5 h. The reaction mixture was evaporated, the oily residue was decanted twice with ether and dissolved in a mixture methanol–water (7 : 3) and treated with ionexchange resin Dowex-1 in OH^- cycle until the pH reached value 8·0. The resin was filtered off, washed with methanol on the filter, the filtrate was evaporated and the residue dissolved in 20 ml isopropanol and evaporated again. The oily residue was dissolved in 5 ml DMF and solution containing the compound *XV* (0·49 g, 0·43 mmol) in 5 ml DMF was added. Then, after cooling to −20°C, 0·78 g (1·00 mmol) of the "complex F" was added under stirring and the reaction mixture was stirred for additional 5 min at −20°C, 1 h at 0°C and 5 h at room temperature. The precipitated N,N′-dicyclohexylurea was filtered off, the filtrate was evaporated to a total volume 2 ml, diluted with 30 ml ether and dried. Yield: 0·78 g (99%) of compound *XXII* in a form of amorphous powder; $[\alpha]_D$ −16·0° (*c* 0·2, DMF); R_F 0·88 (A), 0·79 (B), 0·64 (F). Amino acid analysis: Asp 1·12 (1); Ser 0·79 (1); Glu 2·21 (2); Gly 2·0 (2); Ile 0·95 (1); Leu 2·17 (2); Lys 1·05 (1). Content of the main compound 100%. ^{1}H NMR: Fmoc-Lys(Boc): NH 7·48, α-CH 3·96, Boc 1·35; Asp(OBu^t): NH 8·24, α-CH 4·55, OBu^t 1·36; Gln: NH 7·85, α-CH 4·19; Gln: NH 8·04, α-CH 4·20; Leu: NH 7·90, α-CH 4·29; Leu: NH 7·87, α-CH 4·27; Gly: NH 8·07, α_1-CH 3·75, α_2-CH 3·64; Ile: NH 7·62, α-CH 4·19; Trp: NH 8·03, α-CH 4·57; Gly: NH 8·21, α_1-CH 3·73, α_2-CH 3·67; OBu^t: 1·40.

Fmoc-Lys(Boc)-Asp(OBu^t)-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-OBu^t (*XXIII*)

The compound *XXI* (85 mg, 0·15 mmol) was hydrogenated in ethanol in the presence of Pd/C. The catalyst was filtered off, the filtrate was evaporated and the residue was dissolved in 2 ml DMF. To this solution 120 mg (0·1 mmol) of compound *XV* and, after cooling to −20°C, 190 mg (0·25 mmol) of the "complex F" were added under stirring. The reaction mixture was stirred for additional 5 min at −20°C, 1 h at 0°C and 5 h at room temperature. The product was treated further in the same manner as compound *XXII*. Yield: 128 mg (80%) of compound *XXIII* in a form of amorphous powder; $[\alpha]_D$ −21·5° (*c* 0·2, DMF); R_F 0·90 (A), 0·87 (B), 0·69 (F). Amino acid analysis: Asp 1·00 (1); Glu 1·96 (2); Gly 1·94 (2); Ile 0·89 (1); Leu 2·05 (2); Lys 1·00 (1). Content of the main compound 100%. ^{1}H NMR; Fmoc-Lys(Boc): NH 7·48, α-CH 3·96, Boc 1·35; Asp(OBu^t): NH 8·24, α-CH 4·55, OBu^t 1·35; Gln: NH 7·85, α-CH 4·20; Gln: NH 8·04; Leu: NH 7·90, α-CH 4·29; Leu: NH 7·86, α-CH 4·28; Gly: NH 8·08, α_1-CH 3·77, α_2-CH 3·63; Ile: NH 7·67, α-CH 4·18; Trp: NH 8·05, α-CH 4·54; Gly: NH 8·08, α_1-CH 3·77, α_2-CH 3·72; Cys-(Acm): NH 8·07. α-CH 4·72, Acm 1·87; Ser(Bu^t): NH 7·97, α-CH 4·33, Bu^t 1·12; OBu^t: 1·40.

H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-
-Leu-Gly-Ile-Trp-Gly-Cys(Acm)-Ser-OH (*XXIV*)

The compound *IX* (620 mg, 0·46 mmol) was suspended in 10 ml DMF, then 74 mg (0·46 mmol) of pyridine bromohydrate were added (the solid dissolved completely) (Solution A). To the solution of 956 mg (0·5 mmol) of compound *XXII* 0·1 ml of piperidine was added. The reaction mixture was left for 1 h at room temperature, then it was diluted with 200 ml of ether and the precipitate was filtered off, dried under vacuum and dissolved in 10 ml DMF. To this solution, solution A was added together with 130 mg (0·72 mmol) HONB, the mixture was cooled to −10°C and 126 mg (0·6 mmol) of DCC were added under continuous stirring. The reaction mixture was left to stand for 48 h at 4°C, the precipitated N,N′-dicyclohexyl urea was filtered off, the filtrate was evaporated to a volume of 10 ml and diluted with 70 ml ethyl acetate. The precipitate was filtered, washed with ethyl acetate, dried and treated with 40 ml of trifluoroacetic acid–water (9 : 1) mixture for 1 h. The residue after evaporation was treated with 80 ml of ether, the precipitate was filtered off, washed with ether, dried in a vacuum desiccator over NaOH, dissolved in 16 ml 0·1% trifluoroacetic acid and purified on a column (2·5 × 100 cm) with Toyo-pearl HW-40 (elution with 0·1% TFA). Fractions corresponding to the main product were pooled and dialyzed. Yield: 800 mg (69%) of compound *XXIV* in a form of amorphous powder; R_F 0·44 (A), 0·12 (B), 0·44 (C). Amino acid analysis: Asp 1·09 (1); Ser 0·91 (1); Glu 3·32 (3); Gly 2·17 (2); Ala 1·00 (1); Val 0·95 (1); Ile 1·74 (2); Leu 3·72 (4); Tyr 0·99 (1); Lys 1·00 (1); Arg 1·86 (2). Content of the main compound 87%. ^{1}H NMR: Arg: NH 8·13, α-CH 3·86, Ile: NH 8·42, α-CH 4·22; Leu: NH 8·19, α-CH 4·33; Ala: NH 7·90, α-CH 4·35; Val: NH 7·79, α-CH 4·14; Glu: NH 8·01, α-CH 4·26; Arg: NH 7·91, α-CH 4·26; Tyr: NH 7·89, α-CH 4·47; Leu: NH 8·04, α-CH 4·30; Lys: NH 7·90, α-CH 4·24; Asp: NH 8·23, α-CH 4·52; Gln: NH 7·90; Leu: α-CH 4·29; Gly: NH 8·08, α_1-CH 3·76, α_2-CH 3·64; Ile: NH 7·64, α-CH 4·19; Trp: NH 8·03, α-CH 4·58; Gly: NH 8·15, α_1-CH 3·77, α_2-CH 3·72.

H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-
-Leu-Gly-Ile-Trp-Gly-OH (*XXV*)

From 82 mg (0·06 mmol) of compound *IX* and 104 mg (0·078 mmol) of compound *XXXIII* were obtained 108 mg (80%) of compound *XXV* in a form of amorphous powder, according to the method described for the preparation *XXIV*; R_F 0·41 (A), 0·15 (B), 0·67 (C). Amino acid analysis: Asp 1·14 (1); Glu 3·24 (3); Gly 1·86 (2); Ala 1·00 (1); Val 1·09 (1); Ile 1·75 (2); Leu 3·85 (4); Tyr 0·99 (1); Lys 0·82 (1); Arg 2·00 (2). Content of the main compound 74%. ^{1}H NMR: Arg: NH 8·13, α-CH 3·86; Ile: NH 8·42, α-CH 4·22; Leu: NH 8·19, α-CH 4·32; Ala: NH 7·90, α-CH 4·35; Val: NH 7·78, α-CH 4·14; Glu: NH 8·01, α-CH 4·26; Arg: NH 7·91, α-CH 4·26; Tyr: NH 7·89, α-CH 4·47; Leu: NH 8·04, α-CH 4·30; Lys: NH 7·90, α-CH 4·24; Asp: NH 8·23, α-CH 4·52; Gln: NH 7·90; Gly: NH 8·09, α_1-CH 3·78, α_2-CH 3·64; Ile: NH 7·68, α-CH 4·19; Trp: NH 8·06, α-CH 4·54; Gly: NH 8·11, α_1-CH 3·78, α_2-CH 3·73; Cys: NH 8·07, α-CH 4·68; Ser: NH 8·8, α-CH 4·28.

H-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-
-Leu-Gly-Ile-Trp-Gly-Cys-Ser-OH (*XXVI*)

The compound *XXIV* (135 mg, 0·05 mmol) was dissolved in 30 ml of 20% acetic acid and to this solution 26 mg (0·87 mg) mercury acetate were added. The reaction mixture was left to stand for 1 h at room temperature, 100 microliters of mercaptoethanol were added and the mixture was left for 12 h at 4°C. Then, after reducing the volume to 8 ml by evaporation, it was purified on a column (2·5 × 100 cm) with Toyo-pearl HW-40 (elution with 0·1% TFA). Fractions cor-

responding to the main product were pooled and lyophilized. Yield: 112 mg (85%) of the compound *XXV*. The product was eluted during the HPLC analysis, carried out under conditions described in Fig. 3, in one major peak with the retention time 18·36 min (retention time for the compound *XXIV* was 22·68 min). According to the HPLC, the content of the main product was at least 88%.

The synthesized major compound was employed for the immunological determination of antibodies against HIV without further purification.

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