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Synthesis and study of a fragment (584–618) of the transmembrane protein gp41 HIV-I

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A comparison of the immunological activity of the newly synthesized peptide 584–618 fragment of gp41 HIV-I protein with 584–602 and 601–617 fragments highlights the essential role of peptide conformation in immunodiagnostics.

Some well-known advantages of peptide diagnosticums are: their high specificity, the possibility of working with individual substances, and the use of substances which can be standardized easily. Synthetic peptide antigens, representing parts of the conservative region in transmembrane protein gp41, have been shown to be the most promising for HIV-I diagnostics.

Various parts of the immunodominant area of gp41 were studied and offered for HIV-I diagnostics by earlier investigators: 584–604, Wang, 1986;[1] 584–609, Cosand, 1986;[2] 600–618, Smith, 1987;[3] 584–602, R. V. Petrov, 1988.[4]

The main purpose of our work was to develop methods of synthesis of the peptide 584–618 gp41 HIV-I, the sequence of which includes a complete sequence of all the above-mentioned peptides:

$^{(584)}$H–Arg–Ile–Leu–Ala–Val–Glu–Arg–Tyr–Leu–Lys–Asp–Gln–Gln–Leu–Leu–Gly–Ile–Trp–Gly–Cys–Ser–Gly–Lys–Leu–

Ile–Cys–Thr–Thr–Ala–Val–Pro–Trp–Asn–Ala–Ser–OH$^{(618)}$

A comparison of the immunological activity of this peptide (both linear and cyclic forms) with the two peptides 584–602 and 601–617, which have been used to date in medicine, has been also carried out.

The synthetic fragment 584–618 of gp41 protein was obtained by solid phase step-by-step synthesis[5] using Fmoc-techniques on Applied Biosystems Model 431 A and Biosearch Model 9600 peptide synthesizers. For the protection of amino acid side functions, *tert*-butyl protecting groups were used, and cysteine was used in the synthesis as a Fmoc-Cys(Acm) derivative. Carbodiimide (DCC or DIPCDI) in the presence of 1-hydroxybenzotriazole (HOBt) was chosen as a coupling agent. Fmoc-Asn and Fmoc-Gln were coupled as a *p*-nitrophenyl active ether. Fmoc-Ser(OBu^t)-Wang resin, (degree of substitution 0.67) from Bachem, was used in this synthesis. A double coupling procedure was used in the attachment of amino acid residues 584, 585, 604–609.

Having previously used 4-methoxy-2,3,6-trimethylbenzene sulfonyl (Mtr) protecting groups successfully in fragment 584–602 synthesis, we used the same group to protect the side function of arginine in this synthesis as well. Cleavage of the peptide from the resin was carried out using a scavenger mixture 82.5% TFA : 5% phenol : 5% H_2O : 5% thioanisole : 2.5% EDT.[6] Unfortunately, the experiment showed that the time necessary for complete deprotection of peptide 584–618 was too long at 14–16 h. Reducing the exposure time (1.5, 3 and 8 h) does not effect complete cleavage of the Mtr groups. The content of the desired product in the crude material was around 30%, calculated from peak areas at 226 nm on HPLC [Figure 1(*a*), peak I].

This result did not satisfy us, so we decided to use 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) protecting groups, which can be removed under milder conditions. As a result, cleavage time was reduced to 1.5 h and the amount of desired product increased, as expected, to 65–70% [Figure 1(*c*)]. The yield, calculated according to the first amino acid, was 40%.

Purification of the peptide was performed in a two-step procedure: gel-filtration on Sephadex G-25 column in 5% formic acid with subsequent separation on a Toypearl HW-50 column in 5% formic acid. By-products were separated with

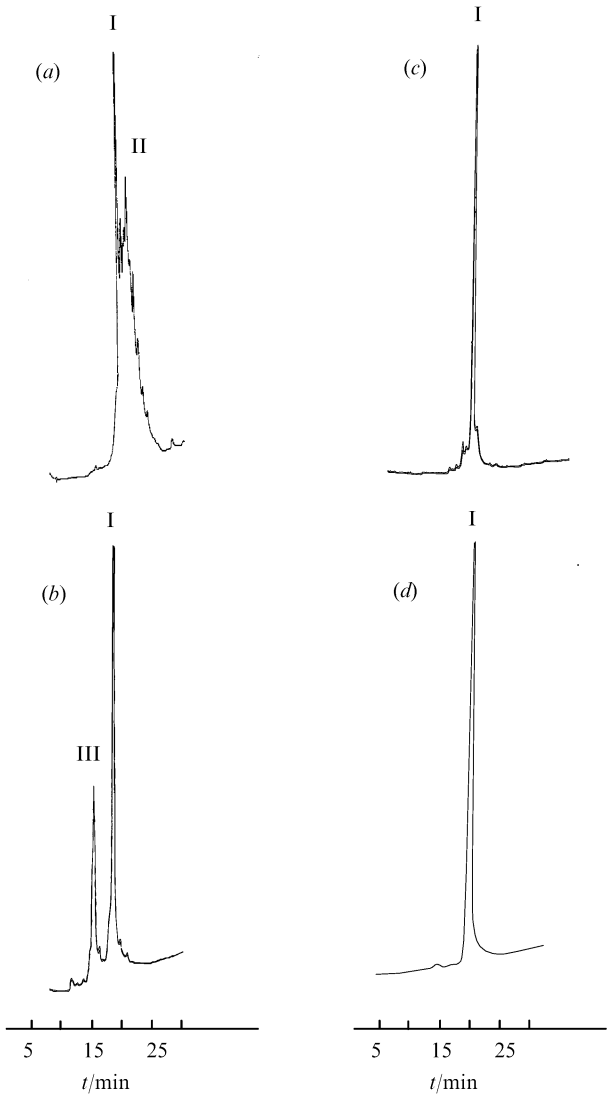
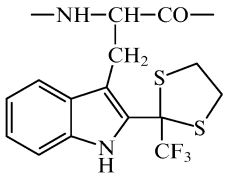


Figure 1 Analytical HPLC elution profile after cleavage of the peptide 584–618 from the resin: (*a*) Mtr-protecting groups in the synthesis on Applied Biosystems Model 431 A; (*b*) Pmc-protecting groups in the synthesis on Biosearch Model 9600; (*c*) Pmc-protecting groups in the synthesis on Applied Biosystems Model 431 A. (*d*) The result of the purification of 584–618 peptide in Acm-form. Column Vydak C-18, 4.6×250 mm, 5 μ, flow rate 1 ml min^{-1}, buffer A: 0.05 M KH_2PO_4 (pH 3.0); B: 70% acetontrile in A; gradient B: 20→80%, in 30 min.

the aid of preparative reversed-phase HPLC on a Vydak column C-18, eluant A: 0.1% aqueous TFA, B: acetonitrile.

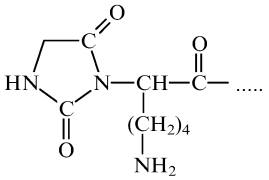
Besides the target product, several by-products were identified. The main component in the crude product [20% HPLC, Figure 1(*a*), peak II] had the correct amino acid content, but its molecular weight according to ESI-mass spectroscopy (MS) was 172 Da higher (4217 Da, the value calculated for 584–618). ^1H and ^{19}F NMR spectra (500 MHz) allowed us to identify this product as the product of a reaction between the indole ring of tryptophan-101, trifluoroacetic acid



(TFA) and 1,2-ethanedithiol (EDT).

The possibilities of such product formation have already been studied.[7]

Another way of synthesising the peptide 584–618 in Biosearch Model 9600 solvent composition, the formation of another by-product had been observed [Figure 1(*b*), peak III]. Amino acid analysis has shown that this is a C-terminal fragment 605–618. Nevertheless, ESI-MS gave a molecular weight of 1559 Da, 27 Da higher than the theoretical value at this fragment. We suppose that this substance arises as a result of peptide chain termination and formation of cyclic product (hydantoin derivative) with participation of Fmoc-Gly-605



and neighbouring Lys-606.

^1H NMR spectroscopy confirms this hypothesis. A similar side cyclization reaction was described by M. Bodanszky[8] for glycine derivatives possessing urethane protecting groups. A comparison of solvent composition employed in the standard Biosearch Model 9600 protocol and Applied Biosystems Model 431A protocol shows that the main difference between them lies in the usage of DCM (Biosearch) instead of NMP (Applied) while coupling. Carrying out the synthesis on the Biosearch equipment and changing DCM to NMP minimizes by-product formation [Figure 1(*c*)]. So, we assumed a dependence of this reaction on the presence of DCM in the

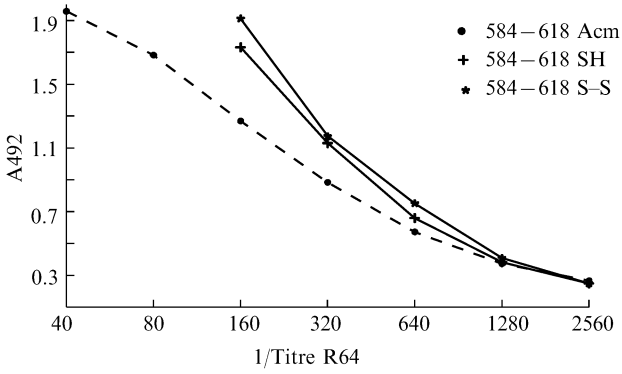


Figure 2 A comparison of the antigenic activities of all three forms of the 584–618 peptide. The peptides were diluted in carbonate buffer pH 99.5 for coating of the plate.

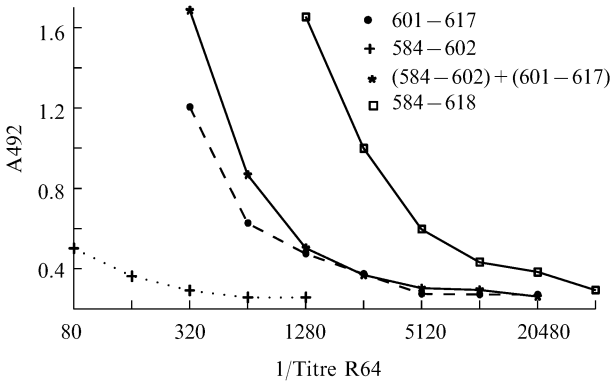


Figure 3 A comparison of the antigenic activity of SH-line peptide 584–618 with the activities of 584–602 and 601–617 fragments and their mixtures in equal concentration.

reaction mixture during condensation.

The next step in our work was to obtain cyclic peptide. Removal of Acm-protection was carried out in 50% acetic acid using mercury(II) acetate with subsequent addition of 2-mercaptoethanol to induce precipitation of excess mercury ions. Isolation and purification of SH-line peptide from some low mass impurities was performed on a Sephadex G-15 column by gel filtration in 5% formic acid. Disulfide bridge formation was carried out in 0.1% water–peptide solution at pH 6.5–7.0 using 3% H_2O_2 water solution. To monitor this step, Elman colour reaction and HPLC were used. This method gave good results: an individual cyclic peptide was obtained in high yield (>90% after deprotection and purification). Its retention time (HPLC) was quite different from the retention times of Acm-protected and SH-line peptide. The purity of all peptides synthesized was >95% [*e.g.*, Figure 1(*d*)] and their structures were correct according to the results of ESI–MS and amino acid analysis. [ESI-MS: 3902 Da for cyclic peptide, 4047 Da for Acm-peptide; A.A.A.: Asx 2.15(2), Thr 1.89(2), Ser 1.69(2), Glx 3.11(3), Gly 2.97(3), Ala 3.0(3), Val 1.98(2), Ile 2.67(3), Leu 4.68(5), Tyr 0.98(1), Lys 2.09(2), Arg 2.03(2)].

Comparison of the antigenic activities of the peptides by enzyme-linked immunosorbent assay (ELISA[1,9]) with R64 (BBI) positive serum is shown in Figure 2. It is clear that the peptide 584–618 with Acm protected SH-groups has the lowest activity, and that the activity of cyclic peptide and SH-line peptide are quite similar.

The most important result for HIV-I diagnostics is that the activity of SH-line peptide 584–618 under final dilution of serum is eight times higher than that of a corresponding mixture of 584–602 and 601–617 fragments, which completely overlap the sequence of 584–618. The integrated results are given in Figure 3.

The results obtained show that the main role in immunodiagnostics using peptides containing the same amino acid sequences is played by the conformation of the peptides.

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