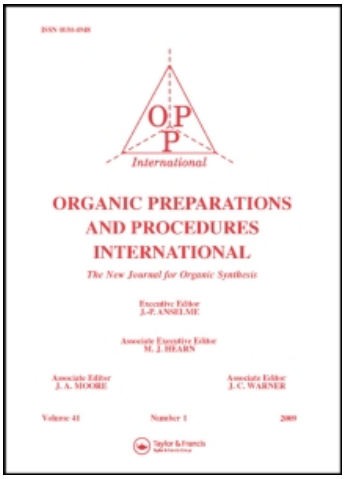

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SOLID-PHASE SYNTHESIS OF A PEPTIDE COMPRISING THE 605-611 DISULFIDE LOOP OF GP41, TRANSMEMBRANE GLYCOPROTEIN OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

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It is known that peptides encompassing the 605-611 disulfide loop of GP41, transmembrane glycoprotein of human immunodeficiency virus type 1 (HIV-1), frequently react with antibodies in the serum from HIV-1 infected individuals,[1] and can be used for the design of simple serological tests for detecting immune responses to HIV-1. We became interested in the synthesis of one of these peptides with the goal of developing an inexpensive test for seroepidemiology of HIV infection in Mexico. The peptide's sequence is H_2N-Ile-Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile-Cys-Thr-Thr-Ala-Val-ProCO_2H. The literature contains no detailed procedure on the synthesis of this peptide, which presents some inherent difficulties. It has a cysteine-cysteine disulfide bond and the cyclization step could induce the oxidation of the indole residue of tryptophan or result in oligomerization. A special design of reactions was necessary in order to prevent these side-reactions and to favor the formation of the monomer. We now describe a detailed procedure for the synthesis of this peptide, which was later shown to be reactive in an enzyme-linked immunosorbent assay (ELISA) with 99% of serum of HIV-1-infected donors from Mexico and scored 100% in specificity and 92% in sensitivity with a control panel from Brazil.

The peptide was prepared by stepwise solid-phase synthesis[2] with the N^{α}-*tert*-butyloxycarbonyl (BOC) derivatives of L-amino acids on Merrifield resin esterified to N^{α}-*tert*-BOC-L-proline. All coupling reactions to form peptide bonds were mediated by N,N'-dicyclohexylcarbodiimide. In general, two coupling steps were used for each amino acid, followed by acetic anhydride capping of any remaining unreacted amine. All coupling steps were monitored by the ninhydrin test of Kaiser *et al.*[3] Removal of the N^{α}-*t*-BOC protected group was carried out with trifluoroacetic acid in dichloromethane. At the completion of the solid phase part of the synthesis, the final N^{α}-*t*-BOC group on the isoleucine residue was deprotected with trifluoroacetic acid in dichloromethane before removal of all side-chain protecting groups (except for the formyl group of tryptophan) and simultaneous cleavage from the resin by liquid hydrogen fluoride in the presence of anisole as scavenger. This sequence of procedure is important to prevent side-reactions caused by long-lived reactive *tert*-butyl cations. The oxidation by potassium ferricyanide was performed in order to generate the disulfide

bond. High dilution was used to suppress dimer formation. Removal of the N^{i}-formyl protecting group of tryptophan was carried out after oxidative formation of the disulfide bond between two cysteines to prevent side-reactions of the indole moiety of tryptophan. The crude peptide was first purified on a column with Sephadex G-15, followed by chromatography on a column of carboxymethyl cellulose. The homogeneity of the peptide was confirmed by the presence of a single sharp peak in analytical reversed-phase HPLC and sequence analysis.

EXPERIMENTAL SECTION

N^{α}-*tert*-Butyloxycarbonyl (BOC) protected amino acids were purchased from Sigma Chemical Co., St. Louis, MO. The following side chain groups were used: O-benzyl for serine and threonine; S-4-methoxybenzyl for cysteine; ε-2- chlorocarbobenzyloxy for lysine; N-formyl for tryptophan. All other reagents were obtained from Aldrich Chemical Co., Milwaukee, WI and were used as received. Chloromethylated copolystyrene-1% divinylbenzene resin (200-400 mesh. 0.7 mmol of Cl/g, Sigma) served as starting material for synthesis. Gel filtration on Sephadex G-15 was performed in 0.1 M ammonium bicarbonate. Carboxymethyl cellulose chromatography was performed with an initial buffer of 0.01 M ammonium acetate of pH 4.5 using a linear 0.01-1 M ammonium acetate gradient at pH 4.5.The peptide was isolated by lyophilization. Repeated lyophilization was required to remove ammonium acetate. Reversed-phase HPLC analyses were performed on a TSK ODS-120 T (5 μm) column (4.6 x 250 mm) with a flow rate of 1 mL/min. Mixture of 0.1% TFA in water (solvent A) and CH_3CN (solvent B) were used as the mobile phase. Sequence determination was performed on a Millipore/Milligen/ Biosearch protein sequencer following Edman's degradation procedure.

Solid-Phase Synthesis.- The solid-phase synthesis of the peptide was carried out manually starting with N^{α}-t-BOC-L-proline resin prepared from cesium salt of N^{α}-*t*-BOC-L-proline.[5] A sample of the resin, deprotected and neutralized[6] gave an amine content of 0.44 mmol/g. The following protocol was used for the introduction of each residue into a peptide chain (20 mL of solvent/g of resin for coupling): (*a*) CH_2Cl_2, 3 x 1 min; (*b*) 50% TFA in CH_2Cl_2, 1 x 1 min; (*c*) 50% TFA in CH_2Cl_2, 1 x 30 min; (*d*) CH_2Cl_2, 5 x 1 min; (*e*)isopropanol, 2 x 1 min; (*f*) CH_2Cl_2, 5 x 1 min; (*g*) 10% N, N'-diisopropylethylamine (DIEA)in CH_2Cl_2, 2 x 1 min; (*h*) 10% DIEA in CH_2Cl_2, 1 x 3 min; (*i*) CH_2Cl_2, 5 x 1 min; (*j*) isopropanol, 2 x 1 min; (*k*) CH_2Cl_2, 3 x 1 rain; (*l*) N^{α}-*t*-BOC amino acid (2 equiv) in 5 mL CH_2Cl_2 was added to the vessel followed by dicyclohexylcarbodiimide (2 equiv) in 5 mL CH_2Cl_3; coupling time was 90 min; (*m*) CH_2Cl_2, 3 x 1 min; (*n*) isopropanol, 2 x 1 min; (*o*) CH_2Cl_2, 5 x 1 min; (*p*) second coupling step was performed by repeating steps (*l*) - (*o*); (*q*) acetylation by addition of 1 mL of acetic anhydride in 9 mL of dichloromethane and shaking for 30 min; (*r*) CH_2Cl_2, 5 x 1 min; (*s*) isopropanol, 2 x 1 min; (*t*) CH_2Cl_2, 5 x 1 min; (*u*) 10% DIEA in CH_2Cl_2, 2 x 1 min; (*v*) CH_2Cl_2, 5 x 1 min.

Cleavage.- After the peptide chain assembly was completed, the amine terminal BOC group on isoleucine was deprotected with 50% TFA in CH_2Cl_2; the resin was washed with CH_2Cl_2, isopropanol, neutralized with 10% DIEA in CH_2Cl_2 and dried overnight. The resin (830 mg) was mixed with 1 mL of anisole in a kel-F HF reaction vessel, and HF was condensed at -78° under reduced pressure to a total volume 10 mL. The cleavage reaction proceeded at 0-3° for 2 hrs. After evaporation of HF, the

resin was washed with cold diethyl ether and then with several portions of degassed (100 mL) 10% (v/v) aqueous acetic acid followed by 300 mL of degassed distilled water. The solution was then adjusted to pH 8 with 3 M ammonium hydroxide, and 40 mL of 0.01 M potassium ferricyanide solution was added in order to generate the disulfide bond. The yellow solution was stirred for about 30 min at room temperature and its pH was then adjusted to pH 5 with 50% acetic acid; Dowex 2 (Chloride form, 200-400 mesh, Sigma) was added and the mixture was stirred for about 30 min followed by filtration to yield a clear colorless solution. The solution was then lyophilized and the powder obtained (220 mg) was treated with 1M ammonium bicarbonate (pH 9) at 25° for 24 hrs for the deformylation of tryptophan. The solution was lyophilized and the peptide (166 mg, 48%) was purified by gel filtration on Sephadex G-15 and chromatography on column of carboxymethyl cellulose. The sequence, except for cysteine derivatives, was confirmed by automated sequencing.

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Abbreviations: TFA, trifluoroacetic acid; CH_2Cl_2, dichloromethane; CH_3CN acetonitrile; HF, hydrogen fluoride.

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