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TOWARD MECHANIZED PEPTIDE SYNTHESIS VIA POLYMERIC REAGENTS APPROACH

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Simultaneous application of high-molecular-weight active esters and polyvinyl *N,N*-diethylbenzylamine was used as a basis for a continuous peptide synthesis via the polymeric reagents approach. Using the synthetic procedure developed, the hexapeptide Boc-L-Pro-L-Val-L-Lys[Z(p-NO_2)]-L-Val-L-Tyr(Dnp)-L-Pro-OBzl[1] and the tetrapeptide Boc-L-Lys[Z(p-NO_2)]-L-Lys[Z(p-NO_2)]-L-Arg(NO_2)-L-Arg(NO_2)-OBzl, corresponding to residues 19–24 and 15–18 of human ACTH, were synthesized in 63 and 70% overall yields, respectively.

INTRODUCTION

Peptide synthesis via the polymeric reagents approach involves the stepwise elongation of a soluble peptide at its α-amino terminal, using insoluble polymeric active esters (1–4). Such reagents, which can be readily separated from the reaction mixture by filtration, are generally employed in excess and provide rapid reaction rates, high coupling yields, and high product purity. Although many peptides have been successfully synthesized using this technology (e.g., 5–9) one of the method's major advantages, the possibility of its being adopted for automated peptide synthesis, has not yet been realized.

In the present study, we investigate one possible approach to mechanized peptide synthesis with polymeric reagents. It encompasses three operative steps which are performed consecutively without isolation of intermediate compounds: (I) coupling of the free α-amino group of an amino acid ester with a polymeric active ester to yield *N*- and *C*-blocked dipeptide; (II) selective removal by acidolysis of the *N*-protecting group to afford the corresponding ammonium salt of the dipeptide ester; and (III)

[1]Abbreviations for amino acid derivatives and peptides follow the IUPAC-IUB Commission on Biochemical Nomenclature Recommendations. Symbols: see Eur. J. Biochem. 27 : 201–207 (1972).

neutralization of ammonium salt with a weakly basic polymer, concomitantly with coupling of the newly exposed α-amino function with a desired polymeric reagent to produce *N*- and *C*-blocked tripeptide. Further repetitions of these reactions will finally lead to the designed peptide. The principal features of this method and its application to the synthesis of Boc-Pro-Val-Lys[Z(p-NO_2)]-Val-Tyr(Dnp)-Pro-OBzl, a peptide corresponding to residues 19–24 of human ACTH, and of Boc-Lys[Z(p-NO_2)]-Lys[Z(p-NO_2)]-Arg(NO_2)-Arg(NO_2)-OBzl, a peptide corresponding to sequence 15–18 of human ACTH, are described.

MATERIALS AND METHODS

Macroporous polyvinyl *N,N*-diethylbenzylamine (20–50 mesh; bead form; type A-21) was obtained from Rohm and Haas. Thin-layer chromatography was performed on fluorescent silica gel plates (Riedel-De Haen, Hanover) using the solvent systems: chloroform–methanol (9:1 and 3:1, v/v) and acetonitrile–water (9:1, v/v). Peptides were detected by iodine vapors, by charring the plates over a flame, or by fluorescence under an ultraviolet lamp. Peptide derivatives with free α-amino groups were also detected by ninhydrin. Amino acid analyses were performed on a Spinco-Beckman Model 120C amino acid analyzer. The assayed blocked peptide derivatives were hydrolyzed with 6 N hydrochloric acid for 48 h at 110°C in evacuated, sealed tubes. Melting points were determined on a capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter. Insoluble polymeric active esters derived from polystyrene-bound 1-hydroxybezotriazole (PHBT esters) (8) and from (4-hydroxy-3-nitro)-benzylated polystyrene (PHNB esters) (10) were prepared as previously described.

Regeneration of Polyvinyl N,N-Diethylbenzylamine

Polymer A-21 (100 g) was suspended in 1 N NaOH (600 ml), and the mixture was mildly agitated for 15 min at room temperature. The polymer was then filtered, washed with distilled water to neutrality, washed with methanol, ether, and finally dried under high vacuum. To evaluate the content of tertiary amine groups in the polymer, a sample (~25 mg) was suspended in a solution of 0.2 M trifluoroacetic acid (TFA) in dichloromethane (1–2 ml) and stirred for 15 min at room temperature. Excess trifluoroacetic acid in the solution was then backtitrated with sodium methoxide using thymol blue as an indicator (11). The polymer was found to contain about 4 mmol amino groups/g.

Preparation of N-tert-Butyloxycarbonyl-L-prolyl-L-valyl-ε-p-nitrobenzyloxycarbonyl-L-lysyl-L-valyl-O-dinitrophenyl-L-tyrosyl-L-proline Benzyl Ester

A solution of proline benzyl ester hydrochloride (0.27 g, 1.12 mmol) in distilled dichloromethane (20 ml) was placed in a binecked flask (I, Fig. 1), after which polymer A-21 (0.8 g, containing 3.2 mmol tertiary amine) was added, followed by Boc-Tyr(Dnp)-PHBT (3.78 g, containing 1.83 mmol tyrosine). The flask was connected to a Buchi Rotavapor-R (IV, Fig. 1) and the suspension was slowly swung at room temperature. To follow the coupling process, aliquots were taken from the reaction mixture at intervals and analyzed by thin-layer chromatography. Reaction was found to be completed within 60 min. The reaction mixture was then filtered through a sintered-glass funnel (II, Fig. 1) and the solution collected in flask III (Fig. 1). The polymer was washed with CH_2Cl_2 (5×30 ml) and the filtrate and washings were combined and evaporated to dryness. The oily dipeptide residue, Boc-Tyr(Dnp)-Pro-OBzl, contained in flask III was dissolved in anhydrous TFA (7 ml) and, after 7 min at room temperature, the acid was removed *in vacuo*. The dipeptide trifluoroacetate thus obtained was dissolved in CH_2Cl_2 (10 ml) which was evaporated *in vacuo*. Addition of CH_2Cl_2 and evaporation was repeated three more times. The oily residue

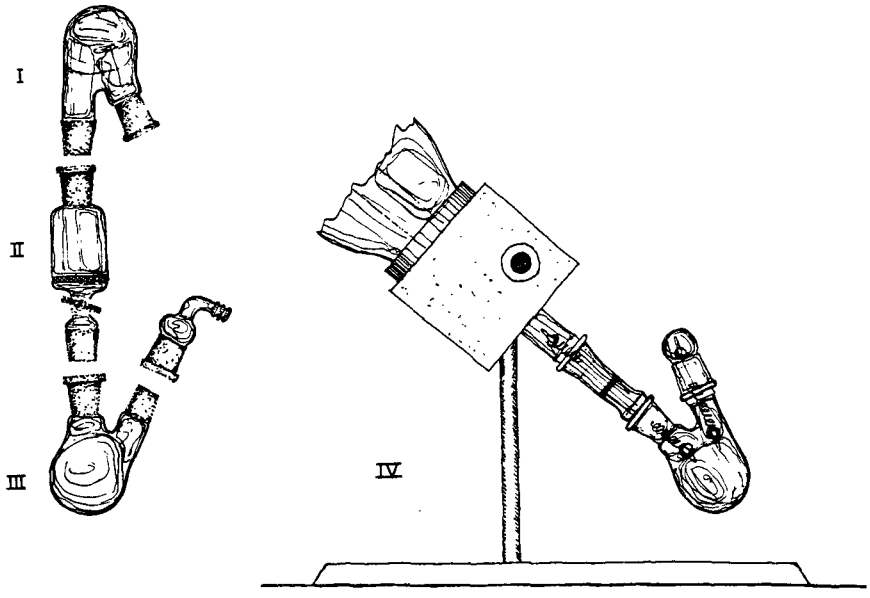

FIG. 1. Equipment used for continuous peptide synthesis.

was dissolved in dichloromethane (20 ml), polymer A-21 (1.25 g, containing 5 mmol tertiary amine) was added, and the suspension was mildly swung for 20 min at room temperature to neutralize any free TFA left after evaporation. Boc-Val-PHBT (2.34 g, containing 2.05 mmol valine) was then added and the coupling reaction proceeded (4 h) to yield the blocked tripeptide Boc-Val-Tyr(Dnp)-Pro-OBzl, isolated similarly to the precedent dipeptide. Removal of the *N*-*t*-butyloxycarbonyl group with TFA, neutralization of TFA-salt, and coupling (2 h) with Boc-Lys[Z(*p*-NO_2)]-PHBT (4.8 g containing 1.93 mmol lysine) yielded the tetrapeptide Boc-Lys[Z(*p*-NO_2)]-Val-Tyr(Dnp)-Pro-OBzl. Similarly, using Boc-Val-PHBT (2.35 g containing 2.05 mmol valine), the next coupling step (2 h) yielded the pentapeptide Boc-Val-Lys[Z(*p*-NO_2)]-Val-Tyr(Dnp)-Pro-OBzl. Finally, deblockage of the *N*-*t*-butyloxycarbonyl group with TFA, neutralization of TFA salt, and coupling (3 h) with Boc-Pro-PHBT (2.5 g containing 2.5 mmol proline) led to the formation of the desired hexapeptide, Boc-Pro-Val-Lys[Z(*p*-NO_2)]-Val-Tyr(Dnp)-Pro-OBzl. Each stage of amino acid addition was performed exactly as described in detail for the preparation of the blocked dipeptide Boc-Tyr(Dnp)-Pro-OBzl.

The chromatographically pure hexapeptide was solidified by treatment with CH_2Cl_2–ether. The yield was 0.88 g (63% based on the amount of proline benzyl ester hydrochloride used in the first synthetic step); m.p. 170–172°C (uncorrected); $[\alpha]_D^{20}$ −35.3 (C, 1.0 *N,N'*-dimethylformamide). Amino acid analysis showed: Val 2.00; Pro 2.16; Tyr 0.84; Lys 0.92. Calculated for $C_{61}H_{76}O_{18}N_{10} \cdot H_2O$ (1255.31): C, 58.36; H, 6.26; N, 11.15. Found: C, 58.27; H. 6.45; N, 11.32.

Preparation of N-tert-Butyloxycarbonyl-ε-p-nitrobenzyloxycarbonyl-L-lysyl-ε-p-nitrobenzyloxycarbonyl-L-lysyl-ω-nitro-L-arginyl-ω-nitro-L-arginine Benzyl Ester

Essentially, this tetrapeptide was synthesized by the procedure described earlier for the preparation of Boc-Tyr(Dnp)-Pro-OBzl, using the same apparatus with several minor alterations.

To a solution of ω-nitroarginine benzyl ester *p*-toluenesulfonate salt (0.39 g, 0.81 mmol) in *N,N'*-dimethylformamide (DMF) (9 ml) was added polymer A-21 (0.5 g containing 2 mmol tertiary amine), followed by Boc-Arg(NO_2)-PHNB (1.84 g containing 1.5 mmol arginine). The heterogeneous mixture was slowly swung at room temperature and coupling was complete within 24 h, as revealed by thin-layer chromatography. This latter analysis also revealed the formation of a by-product, the cyclic *t*-butyloxycarbonyl nitro-L-arginine lactam (12), which, owing to its solu-

bility in ether, could be completely removed from the main product, Boc-Arg(NO_2)-Arg(NO_2)-OBzl, by two precipitations of the latter from ethanol–ether. After removal of the Boc group with TFA and neutralization of the dipeptide's TFA salt with polymer A-21, as described earlier, the synthesis was continued by dissolving the residue in DMF (9 ml) and the addition of Boc-Lys[Z(*p*-NO_2)]-PHBT (2 g, containing 1.38 mmol lysine). Filtration of polymers and evaporation of DMF (after 12 h) yielded the tripeptide Boc-Lys[Z(*p*-NO_2)]-Arg(NO_2)-Arg(NO_2)-OBzl. Repetition of this scheme with Boc-Lys[Z(*p*-NO_2)]-PHBT (2.25 g, containing 1.55 mmol lysine) led to the desired tetrapeptide Boc-Lys[Z(*p*-NO_2)]-Lys[Z(*p*-NO_2)]-Arg(NO_2)-Arg(NO_2)-OBzl. The chromatographically pure product was solidified on trituration with ether. Yield: 0.69 g (70% based on the amount of ω-nitroarginine benzyl ester *p*-toluenesulfonate used in the first synthetic step), m.p. 82°C (uncorrected); $[\alpha]_D^{20} -13.9$ (C, 1.0 *N,N'*-dimethylformamide). Amino acid analysis showed: Lys 1.00; Arg 0.90. Calculated for $C_{52}H_{72}O_{19}N_{16}$ (1225.22): C, 50.97; H, 5.92; N, 18.29. Found: C, 51.08; H, 5.92; N, 17.41.

RESULTS

We have described in detail the preparation of two blocked peptides, Boc-Pro-Val-Lys[Z(*p*-NO_2)]-Val-Tyr(Dnp)-Pro-OBzl (**V**; Fig. 2) and Boc-Lys[Z(*p*-NO_2)]-Lys[Z(*p*-NO_2)]-Arg(NO_2)-Arg(NO_2)-OBzl (**VIII**; Fig. 3) corresponding to residues 19–24 and 15–18 of human ACTH, respectively. Hexapeptide **V** was synthesized in 63% overall yield, based on the amount of proline benzyl ester used in the initial step of the coupling scheme shown in Fig. 2. Figure 3 presents the set of reactions which led to the synthesis of tetrapeptide **VIII** in 70% overall yields based on the amount of ω-nitroarginine benzyl ester initially reacted. The two syntheses involved the simultaneous use of a polymeric reagent and a basic neutralizing polymer, all reactions being executed with a simple combination of ordinary laboratory equipment (Fig. 1) and without the necessity for isolating intermediate synthetic compounds.

DISCUSSION

Anhydrous trifluoroacetic acid was used throughout the syntheses as a reagent for the deblockage of *N*-terminal tert-butyloxycarbonyl protecting groups. A key step in the synthesis developed here is the neutralization of the trifluoroacetate ammonium salts produced in this deprotection and of some free trifluoroacetic acid usually left with product in spite of the

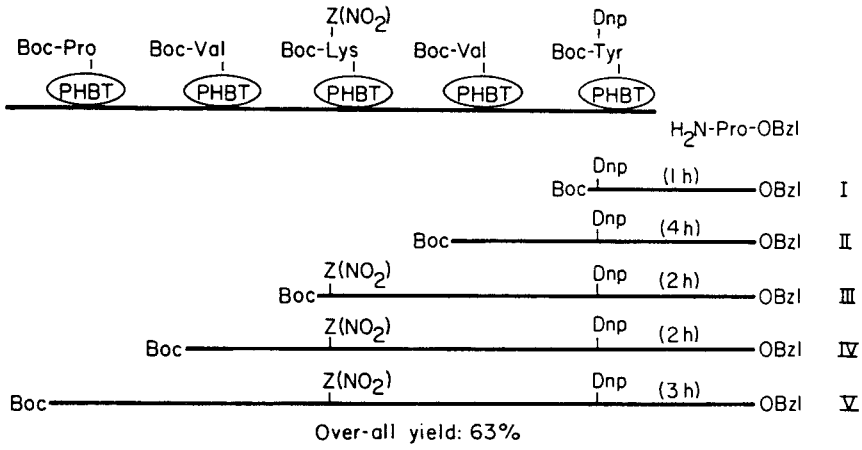

FIG. 2. Synthetic scheme used for the preparation of Boc-L-Pro-L-Val-L-Lys[Z(*p*-NO_2)]-L-Val-L-Tyr(Dnp)-L-Pro-OBzl. Experimental procedure is described in the Materials and Methods section.

repeated evaporations *in vacuo* performed after acidolysis. The commercially macroporous polymer A-21 normally used by us for this purpose, as well as a gel-type polyvinyl *N,N*-diethylbenzylamine (derived from copolystyrene–2% divinylbenzene, 200–400 mesh) synthesized in our laboratory, easily neutralizes in 2:1 molar excess free trifluoroacetic acid in an organic solvent such as CH_2Cl_2, $CHCl_3$, or CH_3OH, and, less efficiently though quantitatively, in DMF as well. However, trifluoroacetate salts of peptides cr of amino acid esters, as well as hydrochloride, hydrobromide, or *p*-toluenesulfonate salts, are only partially

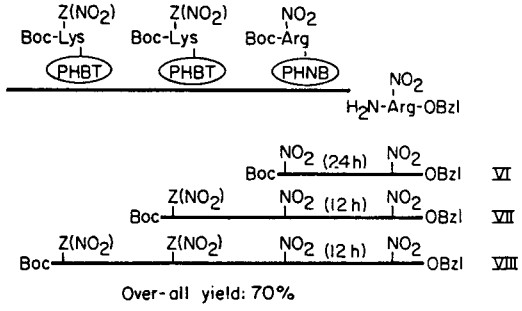

FIG. 3. Synthetic scheme used for the preparation of Boc-L-Lys[Z(*p*-NO_2)]-L-Lys[Z(*p*-NO_2]-L-Arg-(NO_2)-L-Arg(NO_2)-OBzl. Experimental procedure is described in the Materials and Methods section.

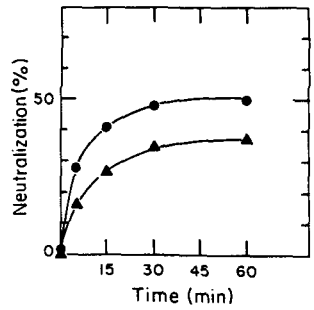

FIG. 4. Neutralization of glycine benzyl ester *p*-toluenesulfonate with polymer A-21. Ammonium salt (152 mg; 0.45 mmol) was dissolved in *N,N'*-dimethylformamide (10 ml) and the solution was mixed with beads of polymer A-21 (0.5 g containing 2 mmol tertiary amine, ●; or 0.25 g containing 1 mmol tertiary amine, ▲). Neutralization was followed by nonaqueous titration of residual soluble *p*-toluenesulfonate salt with sodium methoxide using thymol blue as an indicator (13).

neutralized by an excess (2 equiv) of the neutralizing polymer (A-21), as shown in Fig. 4 for neutralization of glycine benzyl ester *p*-toluenesulfonate. It is reasonable to assume that an equilibrium (Fig. 5) is formed due to competition between the amino groups of the polymer and those of the peptide. As shown in Fig. 5, the equilibrium can be shifted toward complete neutralization of the peptide's salt by addition of a polymeric reagent, i.e., by peptide bond formation.

As can be seen in the experimental section, as well as in Figs 2 and 3, there is a considerable increase in the time needed for completion of the couplings which led to peptide **VIII**, as compared with those of peptide **V**. This decrease in reaction rates may be due to the more polar nature of the tetrapeptide and its components, which can result in the formation of hydrogen or other nonspecific polar bonds between the peptide and the free hydroxyl or free tertiary amino groups of polymer reagents and the neutralizing polymer, respectively. Such interactions can bring about

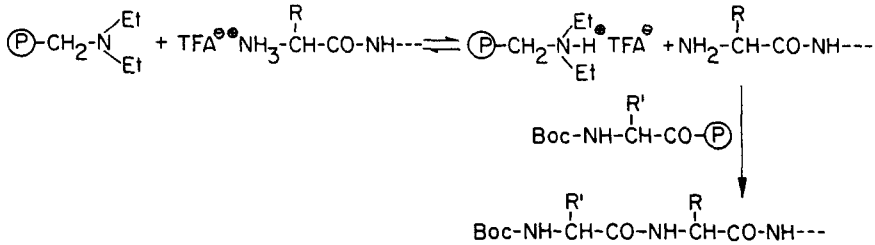

FIG. 5. Equilibrium established during neutralization of peptide's ammonium salt with polymer A-21. Ⓟ = $(-CH(C_6H_4)-CH_2-)_n-$.

lowering of the effective concentrations of reactants, i.e., of the reaction rate. However, more "prosaic" steric considerations should not be exluded.

In connection with the synthesis of tetrapeptide **VIII**, it should be noted that in the first synthetic step, an active ester derivative of (4-hydroxy-3-nitro)-benzylated polystyrene, Boc-Arg(NO_2)-PHNB, was used. This compound was chosen in order to minimize the formation of the cyclic *t*-butyloxycarbonyl nitro-L-arginine lactam by-product. Boc-Arg(NO_2)-PHBT, when used for elongation of peptide chain, led almost exclusively to cyclic lactam formation.

In conclusion, in view of the experimental findings described it seems to us that an automatic peptide synthesis via the polymeric reagents approach is plausible. We are currently pursuing this challenge in our laboratory.

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