Peptide Synthesis by Means of *tert*-Butyloxycarbonylamino Acid Derivatives of Poly(ethylene-*co*-*N*-hydroxymaleimide)*

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ABSTRACT: Cross-linked poly(ethylene-*co*-*N*-hydroxymaleimide) (PHMI) was prepared by reacting poly(ethylene-maleic anhydride) with hydroxylamine and cross-linking with hydrazine, spermine, or spermidine. PHMI-active esters of the *tert*-butyloxycarbonylamino acid derivatives (Boc-Thr, Boc-γ-benzyl-Glu, Boc-*O*-benzyl-Ser, Boc-*S*-benzyl-Cys, Boc-Pro, and Boc-Ala) were prepared using dicyclohexylcarbodiimide as a coupling agent. The synthesis of the heptapeptide L-Ser-L-Pro-L-Cys-L-Ser-L-Glu-L-Thr-L-Tyr, corresponding to residues 159–165 of bovine carboxypeptidase A, was effected by means of the polymeric active esters synthesized. The synthesis was initiated by reacting *O*-benzyl-L-tyrosine benzyl esters with *t*-Boc-Thr-PHMI in dimethylformamide to yield *N*-*tert*-butyloxycarbonyl-L-threonyl-*O*-benzyl-L-tyrosine benzyl ester. Removal of the *tert*-butyloxycarbonyl group yielded a dipeptide ester which was coupled with the corresponding polymeric active ester to yield an N-blocked tripeptide ester. Repetition of this set of reactions led by stepwise elongation of the peptide chain to the formation of the blocked heptapeptide in 59.5% overall yield. Removal of the blocking groups with liquid HF yielded the desired heptapeptide in 47.6% yield. A quantitative alanylation of poly-ε-benzyloxycarbonyl-L-lysine (mol wt ~10,000) and of insulin was attained by treatment of the synthetic and native macromolecule with *t*-Boc-Ala-PHMI in dimethylformamide at room temperature. The acylation of amines by the polymeric active esters was markedly enhanced at elevated temperatures. The reaction of H_2N-Val-OBzl with Z-Val-PHMI in dimethylformamide at room temperature gives the dipeptide Z-Val-Val-OBzl in quantitative yield within 12–14 hr. When the same reaction was carried out at 70° the coupling was completed within 45–60 min.

The successful use of polymeric reagents in the synthesis of peptides has been described previously (Fridkin *et al.*, 1966, 1968; Sklyarov *et al.*, 1966; Wieland and Birr, 1966, 1967; Laufer *et al.*, 1968). The reagents used were high molecular weight, insoluble, active esters derived from cross-linked polymers and N-blocked amino acids. High yields of N- and C-blocked peptides were obtained on coupling amino acid or peptide esters, possessing free α-amino groups, with excess of the insoluble polyfunctional reagent. The newly formed peptides could be separated readily from the insoluble reagent, and elongated after removal of the N-blocking group and coupling with an insoluble polyfunctional active ester of a desired N-blocked amino acid.

One of the main difficulties encountered in peptide synthesis according to the above procedure using esters of *N*-*tert*-butyloxycarbonyl (*t*-Boc)[1]-amino acids and polyfunctional polymers, such as poly-4-hydroxy-3-nitrostyrene, is their relatively low reactivity (Fridkin *et al.*, 1969). Long reaction times and a large excess of polymeric reagent are required to ascertain completion of the coupling reaction with amino acid or peptide derivatives containing bulky side chains (Fridkin *et al.*, 1969). In an attempt to overcome these difficulties we (Patchornik *et al.*, 1967), and independently Laufer *et al.* (1968), prepared *N*-*t*-Boc-amino acid esters of a poly(ethylene-*co*-*N*-hydroxymaleimide) and investigated their use in peptide synthesis and aminoacyl-transfer reactions. Because of the well-known high activity of the *N*-hydroxysuccinimide esters of *t*-Boc-amino acids (Anderson *et al.*, 1964), high activity of the corresponding polymeric esters might have been predicted.

The synthesis of linear poly(ethylene-*co*-*N*-hydroxymaleimide) (PHMI) by condensing poly(ethylene-*co*-maleic anhydride) with hydroxylamine was described by Laufer *et al.* (1968). The polymer could be cross-linked by exposure to high-energy electron irradiation. The *t*-Boc-amino acid ester derivatives of the linear and the cross-linked PHMI were employed in the synthesis of seventeen different tri- to octapeptides (Laufer *et al.*, 1968).

In the following we describe in some detail our synthesis of cross-linked PHMI from poly(ethylene-*co*-maleic anhydride) using hexamethylenediamine, hydrazine, spermidine, or spermine as cross-linking agents. Several *t*-Boc amino acid PHMI esters were prepared and utilized in the stepwise synthesis of the heptapeptide L-Ser-L-Pro-L-Cys-LSer-L-Glu-L-Thr-L-Tyr. The synthesis of this peptide, corresponding to residues 159–165 of bovine carboxypeptidase A, was undertaken as it consists of amino acid residues of different functional side chains, and thus might serve as a suitable model compound for testing the usefulness of the polymeric reagents discussed, *i.e.*, *t*-Boc-amino acid PHMI esters, in the synthesis of complex oligopeptides. The catalytic activity of the heptapeptide on the hydrolysis of *p*-nitrophenyl acetate is under investigation (Goren and Fridkin, 1971).

In order to determine whether the *t*-Boc-amino acid derivatives of PHMI prepared might be used in the synthesis of high molecular weight polypeptides by the stepwise procedure outlined above, the possible acylation of high molecular weight synthetic and native compound with PHMI-amino acid esters was investigated. Poly-ε-benzyloxycarbonyl-L-lysine (av mol wt ~10,000) and insulin were chosen as the high molecular weight compounds, and *t*-Boc-alanine-PHMI as the acylating agent. The successful results obtained suggest that acyl-PHMI derivatives might be of use in the modifica-

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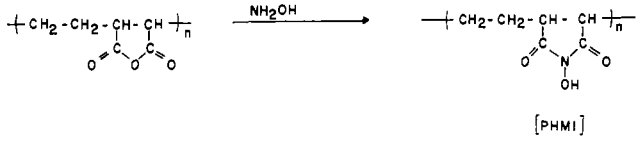
[1] Abbreviations used are: *t*-BOC, *tert*-butyloxycarbonyl; Bzl, benzyl; Z, benzyloxycarbonyl; DNP, 2,4-dinitrophenyl; PHMI, poly(ethylene-*co*-*N*-hydroxymaleimide); DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide.

SCHEME I



tion of synthetic and native macromolecules soluble in the medium in which the acylation reaction is carried out. The enhancement at elevated temperatures of the acylation reaction by means of the polymeric reagents described was also investigated. From the preliminary results obtained it is concluded that the coupling time is markedly shortened at elevated temperatures.

Poly(ethylene-*co*-*N*-hydroxymaleimide) was prepared by reacting poly(ethylene-*co*-maleic anhydride) with hydroxylamine hydrochloride in a mixture of water and pyridine at room temperature (see Scheme I). Cross-linking was effected by means of hexamethylenediamine, hydrazine, spermidine, or spermine. The structure of PHMI was ascertained by infrared analysis. The absorption bands at 1780–1790 and 1710 cm^{-1} (Nujol) revealed the presence of the two carbonyls of the cyclic imide. These replaced the absorption bands at 1870 and 1775–1785 cm^{-1} (Nujol) of the starting ethylene–maleic anhydride copolymer. The cross-linked PHMI polymers synthesized are insoluble in water, methanol, ethanol, chloroform, acetonitrile, dimethylformamide (DMF), and acetic acid. All of them swell in DMF in accord with the degree of cross-linking and the nature of the cross-linking agent. The most suitable polymers for the synthetic procedures to be described below were those obtained by using spermidine (2–5%), spermine (2–4%), or hydrazine (5–10%) as the cross-linking agent. The polymers thus obtained had an average particle size of 200–300 mesh, and could be filtered readily from suspension in the various organic solvents used. They swell in DMF; filtration from this solvent is rather slow. PHMI esters of different *t*-Boc-amino acids were prepared by coupling the appropriate components in DMF or mixtures of DMF and acetonitrile, using dicyclohexylcarbodiimide (DCC) (Anderson *et al.*, 1964) as the coupling agent. The amount of the amino acid derivatives bound to the polymer (1.0–1.7 mmoles/g) was derived from the increase in weight due to esterification, or from amino acid analysis after acid hydrolysis (Fridkin *et al.*, 1968).

The preparation of *t*-Boc-amino acid PHMI esters and their use in peptide synthesis are summarized in Scheme II. The synthesis of the heptapeptide L-Ser-L-Pro-L-Cys-L-Ser-L-Glu-L-Thr-L-Tyr by means of the polymeric active esters listed in Table I is summarized in Scheme III. The *tert*-butyloxycarbonyl group was used to protect the N terminals, whereas the benzyl group was used to protect the thiol of cysteine, the hydroxyl groups of serine and tyrosine, and the γ-carboxyl of glutamic acid. The synthesis was initiated by reacting *O*-benzyl-L-tyrosine benzyl ester with excess (3 equiv) of polymeric insoluble active ester of *t*-Boc-L-threonine in DMF, to yield *N*-*t*-Boc-L-threonyl-*O*-benzyl-L-tyrosine benzyl ester. Removal of the *t*-Boc protecting group with HCl in dioxane and neutralization with triethylamine yielded a dipeptide ester which was coupled with the corresponding polymeric active ester to yield an N-blocked tripeptide ester. Repetition of this set of reactions led, by stepwise elongation of the peptide chain, to the formation of the blocked heptapeptide in 59.5% overall yield. Removal of the blocking groups with liquid HF (Sakakibara *et al.*, 1967) yielded the desired heptapeptide in 47.6% yield.

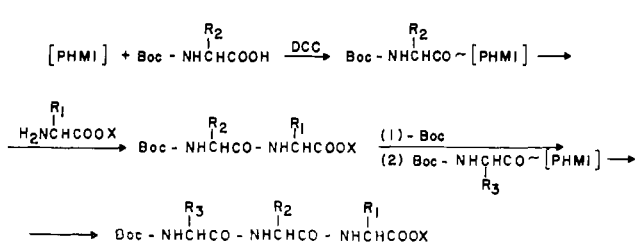
Concomitantly with the synthesis of the heptapeptide according to Scheme III, a classical synthesis of the peptide by the DCC method (Goren and Fridkin, 1971) was performed. A comparison of the properties of the corresponding intermediate peptides obtained by both methods is given in Table

TABLE I: *N*-Butyloxycarbonylamino Acid Esters of Cross-Linked Poly(ethylene-*co*-*N*-hydroxymaleimide).

Compound Bound to Polymer[a]	mmoles of Amino Acid Bound/g of Polyester[d]
N-Boc-L-Thr[b]	1.5
N-Boc-(γ-Bzl)-L-Glu[b]	1.45
N-Boc-(*O*-Bzl)-L-Ser[c]	1.5
N-Boc-(*S*-Bzl)-L-Cys[b]	1.6
N-Boc-L-Pro[b]	1.3
N-Boc-L-Ala[c]	1.4

[a] Binding was effected in DMF by the DCC method. [b] Poly(ethylene-*co*-*N*-hydroxymaleimide) cross-linked with 10% hydrazine was used. [c] Poly(ethylene-*co*-*N*-hydroxymaleimide) cross-linked with 5% spermidine was used. [d] Assayed by increase in weight of starting polymer and by quantitative amino acid analysis after total acid hydrolysis.

SCHEME II



SCHEME III

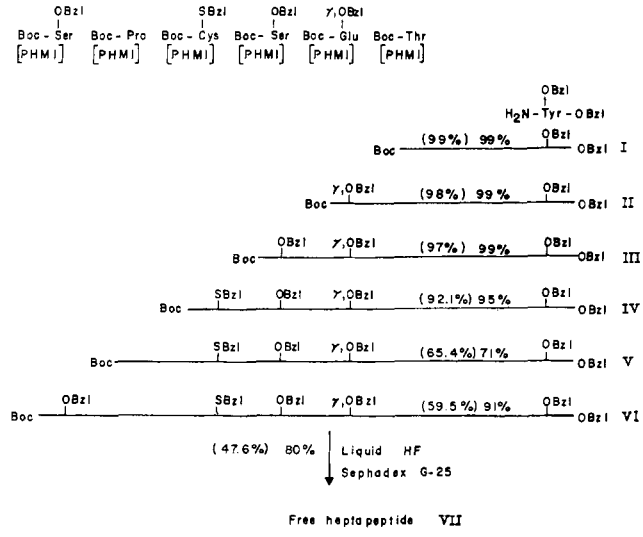


TABLE II

Peptide	Peptides Obtained by Using the PHMI-Amino			
	Yield (%)	Crystal. Solvent	Mp (°C)	$[\alpha]^{23}D$ (deg)
I. Boc-Thr-Tyr-*O*-Bzl (*O*-Bzl; γ-*O*-Bzl, *O*-Bzl)	99 (99)	E–PE[a]	116	−21.4 (*c* 1%, Ch)
II. Boc-Glu-Thr-Tyr-*O*-Bzl (*O*-Bzl, γ-*O*-Bzl, *O*-Bzl)	99 (98)	E–PE	90–92	−23.0 (*c* 1.2%, Ch)
III. Boc-Ser—Glu-Thr-Tyr-*O*-Bzl (*S*-Bzl, *O*-Bzl, γ-*O*-Bzl, *O*-Bzl)	99 (97)	EA–PE	114–118	−12.2 (*c* 1%, Ch)
IV. Boc-Cys—Ser—Glu-Thr-Tyr-*O*-Bzl (*S*-Bzl, *O*-Bzl, γ-*O*-Bzl)	95 (92.1)	EA–PE	142–145	−29.0 (*c* 0.5%, Ch)
V. Boc-Pro-Cys—Ser—Glu-Thr-Tyr-*O*-Bzl (*O*-Bzl) (*O*-Bzl, *S*-Bzl, *O*-Bzl, γ-*O*-Bzl, *O*-Bzl)	71 (65.4)	Et–E[b]	165	−29.0 (*c* 0.9%, DMF)
VI. Boc-Ser-Pro-Cys—Ser—Glu-Thr-Tyr-*O*-Bzl	91 (59.5)	Ch–E	130	−27.1 (*c* 0.5%, Ch)

[a] E = ether, PE = petroleum ether (bp 30–60°), EA = ethyl acetate, Et = ethanol, Ch = chloroform. [b] The peptide was obtained as a solid and triturated with ether (peptides I and V) or with ethanol–ether (peptide V). [c] Thin-layer chromatography

II. The data presented show that identical intermediates were obtained and that in most cases higher yields of intermediates and of the final product were obtained when PHMI-active amino acid esters were employed.

The alanylation of poly-ε-benzyloxycarbonyl-L-lysine by *t*-Boc-alanine-PHMI was carried out as follows: the poly-ε-benzyloxycarbonyl-L-lysine was reacted with the polymeric active ester at room temperature in DMF, and the N-terminal amino acid of the product was assayed by the DNP method. The assay was performed after removal of the terminal *t*-Boc group with anhydrous trifluoroacetic acid. The acid hydrolysate of the dinitrophenylated product was found to contain DNP-alanine but no α-DNP-lysine as determined by thin-layer chromatography (tlc) (Randerath, 1963). The interaction of the polymeric active ester with insulin was similarly carried out at room temperature in DMF. The product was treated with anhydrous trifluoroacetic acid to remove the *t*-Boc blocking groups. Amino acid analysis of the unmasked product revealed the insertion of three additional alanine residues per protein molecule. Dinitrophenylation proved the presence of DNP-Ala instead of DNP-Gly, DNP-Phe, and ε-DNP-Lys to be expected from intact insulin.

The reaction of L-H_2N-Val-OBzl with Z-L-Val-PHMI in DMF at room temperature (1:2 molar ratio) was found to yield the dipeptide Z-L-Val-L-Val-OBzl in quantitative yield within 12–14 hr. When the same reaction was carried out at 70°, however, the coupling was completed within 45–60 min. The blocked dipeptides obtained at the two different temperatures were identical in their melting points, optical rotations, and chromatographic properties, with an authentic dipeptide derivative. It could thus be concluded that no significant racemization had occurred during the coupling step at 70°. A similar shortening of the time of the coupling reaction was observed when HNProOBzL was reacted in DMF with Z-Phe-PHMI, and H_2NLeuOMe reacted with Z-Phe-PHMI at 70°.

The experimental findings summarized above show that the polymeric amino acid active esters described, obtained on coupling *tert*-butyloxycarbonylamino acids with cross-linked poly(ethylene-*co*-*N*-hydroxymaleimide), might serve as useful polymeric reagents in the synthesis of low and high molecular weight peptides. The coupling time in DMF is markedly shortened at elevated temperatures. Finally, it is worth mentioning that PHMI-active esters might be useful in the acylation of proteins and other biopolymers.

Experimental Section

All melting points were taken on a capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Infrared spectra were taken in Nujol in a Perkin-Elmer infrared spectrophotometer. Commercially available silica gel and cellulose plates, obtained from Riedel-De Häen AG, Hanover, were used for tlc. The following solvent system was used: system I, chloroform–methanol (3:1, v/v); system II, chloroform–methanol (9:1, v/v); system III, methanol; system IV, 1-butanol–acetic acid–water (4:1:1, v/v). R_F values are uncorrected. The peptides were detected by exposure of the plates to iodine vapors (Klausner and Wolman, 1968) or by charring over a flame (R. D. Hill, 1969, personal communication). Amino acid analyses were performed on a Spinco–Beckman Model 120C. The peptide derivatives assayed were hydrolyzed with 6 N hydrochloric acid for 20 hr in evacuated, sealed tubes at 110°. *N*,*N*-Dimethylformamide was dried

Acid Esters				Peptides Obtained by the DCC Method							
R_{FI}	R_{FII}	R_{FIII}	R_{FIV}	Yield (%)	Crystal. Solvent	Mp (°C)	$[\alpha]^{23}D$ (deg)	R_{FI}	R_{FII}	R_{FIII}	R_{FVI}
0.92	0.84	0.75	0.94	66 (66)	E[b]	112	−21.5 (*c* 1.1%, Ch)	0.92	0.84	0.75	0.94
0.94	0.82	0.72	0.94	93 (61.4)	E–PE	86	−23.5 (*c* 1.1%, Ch)	0.93	0.81	0.71	0.92
0.93	0.78	0.64	0.87[c]	91 (55.9)	EA–PE	114–126	−12.6 (*c* 1%, Ch)	0.93	0.76	0.62	0.87[c]
0.93	0.82	0.66	0.90[c]	80 (44.7)	Et–PE	139–142	−28.2 (*c* 0.6%, Ch)	0.93	0.82	0.66	0.88[c]
0.91	0.82	0.62	0.93[c]	92 (41.1)	E[b]	163					
					Et	168	−27.0 (*c* 1.3%, DMF)	0.91	0.82	0.62	0.93[c]
0.91	0.90	0.60[c]	0.91	62 (25.5)	EA–E	133	−28.0 (*c* 1%, Ch)	0.91	0.90	0.60[c]	0.91

was performed on a cellulose plate.

over molecular sieves (type 5A) obtained from Fischer Scientific Co. and fractionally distilled *in vacuo*. Poly(ethylene-*co*-maleic anhydride) (EMA 21, mol wt 20,000–30,000) was from Monsanto. Insulin was purchased from Sigma Chemical Co. Poly-ε-benzyloxycarbonyl-L-lysine (mol wt ~10,000) was a gift from Miles-Yeda Ltd.

Cross-Linked Poly(ethylene-co-N-hydroxymaleimide). Poly(ethylene-*co*-maleic anhydride) (av mol wt 20,000–30,000) (10 g, corresponding to 80-mmole residues of maleic anhydride) was added with stirring to a solution of pyridine (30 ml) in water (250 ml). Spermidine trihydrochloride (920 mg, 4 mmoles) in 1 N NaOH (12 ml), the trifunctional cross-linking agent, was rapidly added to the mixture at room temperature. It was followed by a solution of hydroxylamine hydrochloride (5.5 g, 80 mmoles) in water (50 ml). Stirring of the heterogeneous reaction mixture was continued for 36 hr at room temperature. The cross-linked copolymer obtained was filtered and washed with ethanol, and ethanol acidified with hydrochloric acid, to remove traces of pyridine. The insoluble fraction left was washed with DMF, filtered, treated with ether, and dried *in vacuo* over P_2O_5 and NaOH pellets. The yield was 10 g of a white powder.

Anal. Calcd for a 5% cross-linked poly(ethylene-*co*-*N*-hydroxymaleimide): N, 9.7. Found: N, 9.5.

The spermidine cross-linked poly(ethylene-*co*-*N*-hydroxymaleimide) obtained had an average particle size of 200–300 mesh. It is insoluble in water, methanol, ethanol, chloroform, acetonitrile, DMF, and acetic acid. It swells in DMF. It could be readily removed by filtration from suspension in any of the above solvents. It should be noted, however, that filtration from DMF was rather slow. The particulate structure of the polymer is retained under normal stirring conditions. Slight particle disintegration has been noticed on prolonged, vigorous stirring at elevated temperatures.

The poly(ethylene-*co*-*N*-hydroxymaleimide), cross-linked with hydrazine or spermine, were obtained by a procedure similar to the one described above using the appropriate cross-linking agent.

N-tert-Butyloxycarbonyl-L-threonyl-PHMI. N-t-Boc-L-threonine (0.88 g, 4 mmoles) in DMF (3 ml) was added to a suspension of poly(ethylene-*co*-*N*-hydroxymaleimide) cross-linked with 10% hydrazine (1 g, containing ~7 mmoles of OH groups) in DMF (10 ml). The mixture was stirred for 10 min at 0°, whereupon DCC (1 g, 5 mmoles) in DMF (5 ml) was added. Stirring was continued for 1 hr at 0° followed by 7 hr at room temperature. The Boc-Thr-PHMI was filtered, washed with three portions of boiling isopropyl alcohol (30 ml each) and with ether, and dried *in vacuo*. The yield was 1.45 g. The Boc-Thr-PHMI synthesized contained 1.5 mmoles of *tert*-butyloxycarbonyl-L-threonine/g as determined by the increase in weight of the starting cross-linked polymer, or by quantitative amino acid analysis.

Polymer-Active Esters of Other N-Boc-amino Acids. High molecular weight insoluble active esters of the *N-tert*-butyloxycarbonylamino acids which have been used in the synthesis of the heptapeptide (see Table I), as well as the polymer ester of *tert*-butyloxycarbonyl-L-alanine, were obtained by coupling the *t*-Boc-amino acids with the cross-linked PHMI polymers according to the procedure given for the synthesis of Boc-Thr-PHMI.

N-tert-Butyloxycarbonyl-L-threonyl-O-benyzl-L-tyrosine Benzyl Ester (I). *O*-Benzyl-L-tyrosine benzyl ester (217 mg, 0.6 mmole) in DMF (3 ml) was added to a suspension of Boc-Thr-PHMI (1.1 g containing 1.65 mmoles of threonine) in

DMF (7 ml) and the reaction mixture was stirred for 5 hr at room temperature. Ethyl acetate (75 ml) was added and the polymer was filtered and washed with ethyl acetate (50 ml). The combined filtrate and washing were washed with cold 10% aqueous citric acid, aqueous 5% $NaHCO_3$, and water. Drying was effected with anhydrous sodium sulfate. On evaporation of the ethyl acetate an oily product was obtained which solidified on treatment with ether–petroleum ether (bp 30–60°); yield 334 mg (99%).

N-tert-Butyloxycarbonyl-γ-benzyl-L-glutamyl-L-threonyl-O-benzyl-L-tyrosine Benzyl Ester (II). L-Threonyl-*O*-benzyl-L-tyrosine benzyl ester was obtained on treatment of I with 2 N HCl in anhydrous dioxane for 1 hr at room temperature. The solution was evaporated at room temperature under high vacuum, and the residue was dried over P_2O_5 and NaOH. The hydrochloride was dissolved in DMF and neutralized with triethylamine, and the dipeptide derivative possessing a free α-NH_2 group reacted with Boc-(γ-Bzl)-Glu-PHMI suspended in DMF. The mixture was stirred for 8 hr at room temperature and treated as above. The tripeptide (II) was obtained in 99% yield.

General Procedure for the Preparation of Blocked Peptides. The requisite peptide ester (~0.5 mmole) in DMF (5 ml) was added to the appropriate acyl polymer listed in Table I (~1 g, containing ~1.5 mmoles of activated amino acid) suspended in DMF (5 ml), and the reaction mixture was stirred for 5–8 hr at room temperature. The polymer was filtered and washed twice with DMF (5-ml portions), and with ethyl acetate (100 ml). The filtrate and washings were combined and washed with cold, aqueous 10% citric acid, aqueous 5% $NaHCO_3$, and water. Drying was effected with anhydrous Na_2SO_4. The required product was obtained after evaporation of solvent *in vacuo*. The properties of the blocked peptides obtained are summarized in Table II. Removal of the N-terminal protecting *t*-Boc groups from the blocked peptide derivatives was effected with 2 N HCl in dioxane (peptides I–IV), or with anhydrous trifluoroacetic acid (peptides V and VI).

N-tert-Butyloxycarbonyl-O-benzyl-L-seryl-L-prolyl-S-benzyl-L-cysteinyl-O-benzyl-L-seryl-γ-benzyl-L-glutamyl-L-threonyl-O-benzyl-L-tyrosine Benzyl Ester (VI). The blocked heptapeptide (VI) was obtained in 91% yield on reacting Boc-(*O*-benzyl)-L-Ser-PHMI with the hexapeptide derivative obtained from V on deblocking the *N-t*-Boc protecting group with trifluoroacetic acid: mp 130°, $[\alpha]^{23}D$ −27.1° (*c* 0.5%, chloroform). Amino Acid analysis showed: Thr, 1.00; Ser, 1.73; Glu, 1.10; Tyr, 0.85; *S*-benzylcysteine, 1.00; Pro, 1.01.

Anal. Calcd for $C_{79}H_{91}N_7O_{16}S$: N, 6.87; S, 2.25. Found: N, 6.92; S, 2.12.

A small sample of the heptapeptide (VI) was deblocked by passing HBr gas through a solution of the blocked peptide in anhydrous trifluoroacetic acid, for 90 min. A white powder (90% yield) separated out on addition of absolute ether. Amino acid analysis showed: Thr, 1.00; Ser, 1.85; Glu, 1.07; Tyr, 0.92; *S*-benzylcysteine, 0.94; Pro, 1.00.

The *S*-benzyl heptapeptide gave on paper electrophoresis at pH 1.9, 3.5 and 6.5, R_{Glu} 0.52–0.53 (traces at R_{Glu} 0.70), R_{Glu} −4.5 (traces at R_{Glu} −11) and R_{Glu} 0.21 (traces at the origin), respectively. The peptide could be revealed by ninhydrin or by the Pauly reagent.

L-Seryl-L-prolyl-L-cysteinyl-L-seryl-L-glutamyl-L-threonyl-L-tyrosine (VII). All the protecting groups of the heptapeptide (VI) (*O*-benzyl and *S*-benzyl) were removed with anhydrous hydrofluoric acid according to Sakakibara *et al.* (1967). The blocked peptide (50 mg) was placed in a polyethylene bottle to which anisole (0.1 ml), dimethyl sulfide (0.1 ml), trifluoroacetic acid (0.2 ml), and HF (10 ml) were added. The colorless solution turned brown-red within several minutes. It was kept at room temperature for 115 min whereupon the hydrogen fluoride was evaporated in a stream of dry, and oxygen-free, nitrogen gas. The residue was dried for 30 min *in vacuo* over NaOH and triturated with absolute ethyl acetate, and the solvent was removed by centrifugation. The colorless powder was dried *in vacuo* over P_2O_5 and NaOH, dissolved in 0.5 M acetic acid, and chromatographed on a Sephadex G-25 column which had been equilibrated with 0.5 M acetic acid. The major peak was collected and lyophilized: yield 80%, mp 187–192° dec, $[\alpha]^{23}D$ −58.6° (*c* 0.4%, 0.5 M acetic acid). The heptapeptide VII prepared by the classical stepwise synthesis (Goren and Fridkin, 1971) melted at 190–195° dec and showed an optical rotation of $[\alpha]^{23}D$ −60.0° (*c* 0.5%, 0.5 M acetic acid). Amino acid analysis showed: Thr, 1.00; Ser, 1.87; Glu, 1.12; cysteic acid, 0.82; Tyr, 0.90; Pro, 1.10.

The free SH content of the heptapeptide as determined by the Ellman method (1959) amounted to 70–86% of the theoretical.

Paper electrophoresis of VII at pH 1.9, 3.5, and 6.5 gave R_{Glu} 0.55–0.56 (traces at R_{Glu} 0.9), R_{Glu} −5.3 and R_{Glu} 0.24 (traces at the origin), respectively. The peptide was revealed by ninhydrin or by the Pauly reagent. The mobilities recorded were found identical with those obtained for an authentic sample of VII prepared by a classical stepwise synthesis (Goren and Fridkin, 1971).

On treatment of the heptapeptide for 90 min with HF at 0° only 40–50% of the *S*-benzyl was cleaved.

On treatment of the heptapeptide with Na in liquid NH_3, the peptide-bond Ser–Pro was cleaved to an extent of ~50% (Wilchek *et al.*, 1965). This could be detected by the decrease in the value of Ser and by paper electrophoresis.

tert-Boc-Ala-(ε-Z-Lys)$_n$-COOH. A solution of poly-ε-benzyloxycarbonyl-L-lysine hydrochloride (30 mg) (mol wt ~ 10,000) in DMF (3 ml) was added to a suspension of Boc-Ala-PHMI (200 mg, containing 0.28 mmole of alanine) in 2 ml of DMF. The mixture was stirred overnight at room temperature. The polymer was filtered, and washed twice with 10-ml portions of DMF. The solution (filtrate + washings) was evaporated to yield a gel which solidified on trituration with ether. The colorless solid was washed with methanol and ether and dried *in vacuo*. The *t*-Boc-protecting groups of the polylysine derivatives was removed by dissolution in anhydrous trifluoroacetic acid (2 ml). The solution was kept for 1.5 hr at room temperature, the trifluoroacetic acid was evaporated to dryness, and the residue was triturated with ether; yield, 20 mg.

DNP-analysis (Randerath, 1963) of the final product obtained showed the presence of DNP-Ala and no α-DNP-Lys. DNP analysis of the original poly-ε-benzyloxycarbonyllysine showed the presence of α-DNP-Lys exclusively.

Trialanyl-Insulin. A solution of 30 mg of insulin hydrochloride (Carpenter, 1958) and triethylamine (25 μl) in DMF (3 ml) was added to a suspension of Boc-Ala-PHMI (500 mg containing 0.7 mmole of alanine) in 3 ml of DMF. The mixture was stirred for 30 hr at room temperature, the polymer was filtered and washed twice with DMF (10 ml each washing), and the combined filtrates evaporated to dryness under high vacuum (30°). The residue was triturated with ether (dry and peroxide free), and the colorless powder obtained was dried over P_2O_5 and NaOH under high vacuum. It was then dissolved in 2 ml of anhydrous trifluoroacetic acid. The solution was kept for 2 hr at room temperature, the

trifluoroacetic acid was evaporated under high vacuum (20°), and the residue was dried over NaOH and P_2O_5. Purification of the crude product obtained was effected by its dissolution in 0.5 M acetic acid and chromatography on Sephadex G-25 column (eluted with 0.5 M acetic acid). The protein peak was concentrated by lyophilization to yield 22 mg of the alanylated insulin. The amino acid analysis, after total acid hydrolysis, revealed incorporation of three alanine residues per insulin molecule. A sample of the product was dinitrophenylated, the product hydrolyzed by acid and the hydrolysate examined by tlc (Randerath, 1963). The chromatogram revealed the presence of DNP-Ala but no traces of DNP-Gly or DNP-Phe (the two α-amino terminals of native insulin). Only small traces of ϵ-DNP-Lys could be detected.

Reaction between Z-Val-PHMI and H_2N-Val-OBzl at 70°. L-Valine benzyl ester was reacted with Z-L-Val-PHMI in a 1:2 molar ratio of amino acid to polymer according to the general procedure described above. The course of the reaction was followed by tlc. It was found that the coupling reaction reaches completion within 12–14 hr at room temperature. Z-Val-Val-OBzl was obtained in almost a quantitative yield: mp 114°, $[\alpha]^{23}_D$ −42.9° (*c* 1%, methanol). When the above reaction was carried out at 70° dipeptide formation was completed within 45–60 min (mp 114°, $[\alpha]^{23}_D$ −42.7° (*c* 1%, methanol)); Z-Val-Val-OBzl was also prepared by reacting Z-Val with Val-OBzl in ethyl acetate using DCC as a coupling reagent. The dipeptide derivative was crystallized from ethyl acetate–petroleum ether: mp 112–114°, $[\alpha]^{23}_D$ −40° (*c* 1%, methanol) (lit. (T. Sugimura and W. K. Paik, 1961, unpublished data cited in Greenstein and Winitz (1961)) mp 116°, $[\alpha]^{25}_D$ −44.3° (*c* 2%, methanol)). The three Z-Val-ValOBzl preparations were found to be identical on tlc using various solvent systems.

References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1964), *J. Amer. Chem. Soc. 86*, 1839.

Carpenter, F. H. (1958), *Arch. Biochem. Biophys. 78*, 539.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Fridkin, M., Patchornik, A., and Katchalski, E. (1966), *J. Amer. Chem. Soc. 88*, 3164.

Fridkin, M., Patchornik, A., and Katchalski, E. (1968), *J. Amer. Chem. Soc. 90*, 2953.

Fridkin, M., Patchornik, A., and Katchalski, E. (1971), *Proc. 10th Eur. Peptide Symp., Abano, Italy*, 164.

Goren, H. J., and Fridkin, M. (1971), *Proc. Can. Fed. Biol. Soc. 14*, 13.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, N. Y., John Wiley and Sons, p 1139.

Klausner, Y. S., and Wolman, Y. (1968), *J. Chromatogr. Sci. 38*, 152.

Laufer, D. A., Chapman, T. M., Marlborough, D. I., Vaidya, V. M., and Blout, E. R. (1968), *J. Amer. Chem. Soc. 90*, 2696.

Patchornik, A., Fridkin, M., and Katchalski, E. (1967), *Proc. 8th Eur. Peptide Symp.*, 92.

Randerath, K. (1963), Thin-Layer Chromatography, New York, N. Y., Academic Press, p 98–103.

Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H. (1967), *Bull. Chem. Soc. Jap. 40*, 2164.

Sklyarov, L. Y., Gorbunov, V. I., and Shchukina, L. A. (1966), *Zh. Obshch. Khim. 36*, 2220.

Wieland, T., and Birr, C. (1966), *Angew. Chem. 78*, 303.

Wieland, T., and Birr, C. (1967), *Chimia 21*, 581.

Wilchek, M., Sarid, S., and Patchornik, A. (1965), *Biochem. Biophys. Acta 104*, 616.