Insulin Peptides. XIII. The Synthesis of a Dodecapeptide Derivative Containing the C-Terminal Sequence of the A Chain of Sheep Insulin[1,2]

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Abstract: Syntheses are described of peptide derivatives related to the C-terminus of the A chain of sheep and human insulin. Thus the preparation is given of N-carbobenzoxy-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester and N-carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester. The former derivative contains the nine amino acid residues found at the C-terminus of the A chain of sheep and human insulin whereas the latter derivative contains the 12 amino acid residues corresponding to the C-terminal sequence of the A chain of sheep insulin.

This communication describes the synthesis of a dodecapeptide derivative which contains the amino acid sequence found at the carboxyl terminus of the A chain of sheep insulin and which has served as a key compound in the synthesis of that chain in our laboratory.[2] An intermediate of this dodecapeptide derivative, namely the carboxyl-terminal nonapeptide portion, has also served as an intermediate in the synthesis of the A chain of human insulin.[3]

The amino acid sequence at the carboxyl terminus of the A chain of sheep insulin (positions 10–21) as determined by Sanger and co-workers is valylcysteinylserylleucyltyrosylglutaminylleucylglutamylasparaginyltyrosylcysteinylasparagine.[4] The same amino acid-sequence but with an isoleucine residue replacing the valine residue represents the carboxyl-terminal portion of the A chain of human insulin.[5] The present study relates synthetic routes to the partially protected nonapeptide N-carbobenzoxy-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (VI), an intermediate in the synthesis of the A chain both of sheep and human insulin, and to the partially protected dodecapeptide N-carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (IX), an intermediate in the synthesis of the sheep insulin A chain. For the synthesis of these peptide derivatives the "fragment condensation" approach was employed.[6] Peptide subunits were prepared stepwise by adding on to the amino terminus of the chain and were then condensed to form larger peptide fragments. The carbobenzoxy group was used exclusively to protect the amino function of the amino acids which served as the "carboxyl components" in the stepwise addition. Activation of these carboxyl components was carried out by conversion to the corresponding *p*-nitrophenyl esters.[7,8] Activation, however, of the carboxyl components in the "fragment condensation" was carried out by the azide method and in one instance by the mixed anhydride method. Decarbobenzoxylation of the intermediate protected peptides during the various synthetic steps was effected mainly by treatment with HBr in acetic acid. HBr in trifluoroacetic acid was used to decarbobenzoxylate serine-containing peptides.[9] The over-all scheme which was employed for the synthesis of the protected nonapeptide (VI) and the protected dodecapeptide (IX) is summarized in Chart I.

N-Carbobenzoxy-L-asparagine *p*-nitrobenzyl ester, which was prepared by a slight modification of our original procedure[10] in 72% yield, was decarbobenzoxylated and then condensed with N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester[8] to give the crystalline N-carbobenzoxy-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (I). After splitting the carbobenzoxy group from I, the resulting compound Ia was coupled either with N-carbobenzoxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester[8] to give the crystalline tripeptide N-carbobenzoxy-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (II) or with N-carbobenzoxy-L-asparaginyl-L-tyrosine[10] (Ib) to yield the tetrapeptide N-carbobenzoxy-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (III). The mixed anhydride method was employed to effect the latter coupling. Decarbobenzoxylation of the protected tetrapeptide III yielded the derivative IIIa bearing a free amino group which was treated with the *p*-nitrophenyl ester of N-carbobenzoxy-γ-benzyl-L-glutamic acid[11] (IIIb) to give the pentapeptide N-carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (IV). Preliminary experiments showed that the protected pentapeptide IV, on exposure to HBr in acetic

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(2) A preliminary report describing part of the results presented in this paper has appeared: P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *J. Am. Chem. Soc.*, **85**, 2863 (1963).
(3) P. G. Katsoyannis, A. Tometsko, and C. Zalut, *ibid.*, **88**, 166 (1966).
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(5) D. S. H. W. Nicol and L. Smith, *Nature*, **181**, 483 (1960).
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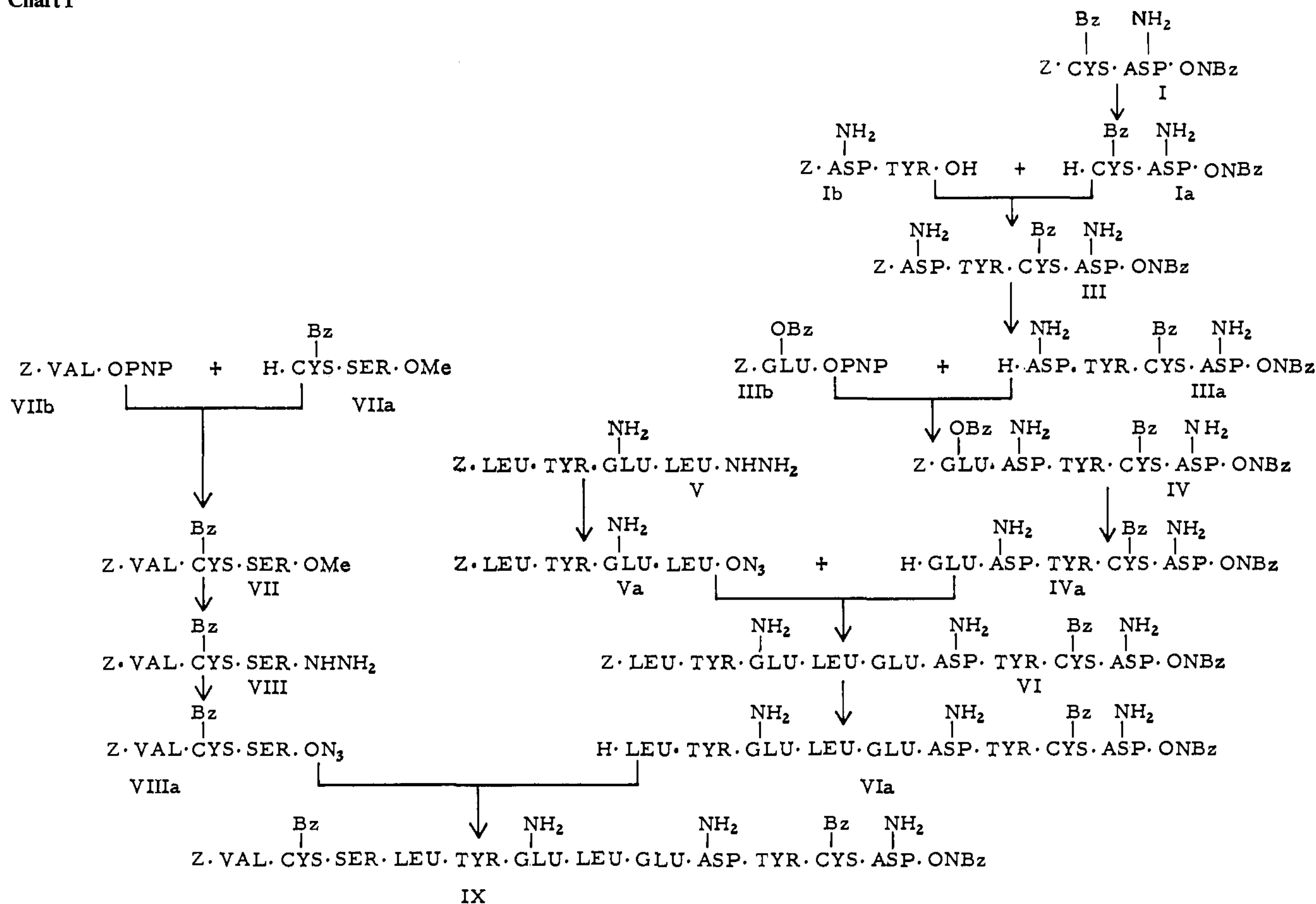
(7) M. Bodanszky, *Nature*, **175**, 685 (1955).
(8) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
(9) S. Guttman and R. A. Boissonnas, *Helv. Chim. Acta*, **41**, 1852 (1958).
(10) P. G. Katsoyannis and K. Suzuki, *J. Am. Chem. Soc.*, **83**, 4057 (1961).
(11) M. Goodman and K. C. Steuben, *ibid.*, **81**, 3980 (1959).

Chart I



acid for a short time (30 min or less), gave rise to two components, as judged by paper chromatography of the deblocked product. This prompted us to investigate the stability of the γ-benzyl ester group in the glutamic acid residue to the action of HBr in acetic acid. As model compounds we used γ-benzyl-L-glutamate[12] and N-carbobenzoxy-γ-benzyl-L-glutamate.[12] Each of these model compounds was treated with 2 N HBr in acetic acid for a specified time, precipitated with ether, and chromatographed on paper in the usual manner. The results of this study are shown in Table I. As can be seen on exposure to 2 N HBr in acetic acid, the γ-benzyl ester group is split from both model compounds although somewhat faster from the carbobenzoxylated derivative. Even then, however, quantitative cleavage occurs only after exposure for 1 hr to the cleaving reagent. In view of this finding we treated the pentapeptide derivative IV with 2 N HBr in acetic acid for 1 hr and we thus obtained the chromatographically pure partially deblocked derivative IVa. This in turn was coupled with the tetrapeptide azide Va, which was prepared from the corresponding hydrazide V in the usual manner, to give the nonapeptide N-carbobenzoxy-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine p-nitrobenzyl ester (VI).

Table I

Compound	Time of exposure (min) to 2 N HBr in CH_3COOH	Paper chromatography of the deblocked product — R_f^1 Major component	R_f^1 Minor component	R_f^2 Major component	R_f^2 Minor component
Glutamic acid	...	0.24	None	1.05	...
	10	0.24	None	1.05	None
γ-Benzyl-L-glutamate, mp 178°	...	0.70	None	3.90	None
	10	0.24	0.7	1.05	3.90
	30	0.24	0.7	1.05	3.90
	60	0.24	0.7 (trace)	1.05	3.90 (trace)
N-Carbobenzoxy-γ-benzyl-L-glutamate, mp 76°	10	0.24	0.7	1.05	3.90
	30	0.24	0.7 (trace)	1.05	3.90 (trace)
	60	0.24	None	1.05	None

(12) W. E. Hanby, S. G. Waley, and J. Watson, *J. Chem. Soc.*, 3239 (1950).

Interaction of the *p*-nitrophenyl ester VIIb of N-carbobenzoxy-L-valine[13] with S-benzyl-L-cysteinyl-L-serine methyl ester VIIa, which was obtained from its carbobenzoxy derivative[14] by treatment with HBr in trifluoroacetic acid, afforded N-carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-serine methyl ester (VII). Reaction of VII with hydrazine hydrate yielded the tripeptide hydrazide VIII which was subsequently converted to the respective azide VIIIa. The final step consisted in the interaction of the tripeptide azide VIIIa with the partially protected nonapeptide VIa which was obtained by decarbobenzoxylation of the protected derivative VI. The product of this reaction, the desired dodecapeptide N-carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (IX), was obtained in 84% yield.

The chemical purity of this final product was established by elemental analysis, paper chromatography of the decarbobenzoxylated derivative, and amino acid analysis of an acid hydrolysate. For establishing the stereochemical homogeneity the protected dodecapeptide was decarbobenzoxylated by treatment with HBr in trifluoroacetic acid and the partially protected derivative was digested with leucine aminopeptidase (LAP). Analysis of the digest by the automatic analyzer showed that the dodecapeptide derivative was completely digested by the enzyme and thus demonstrated that the optical purity of the constituent amino acids was preserved during the various synthetic steps.

Experimental Section[15]

Capillary melting points were determined for all compounds and are corrected.

For paper chromatography the protected peptides were deblocked by treatment with 2 *N* HBr in acetic and in certain instances, as indicated in the experimental part, by HBr in trifluoroacetic acid and the resulting hydrobromides were chromatographed on Whatman No. 1 filter paper. R_f^1 values refer to the Partridge system;[16] R_f^2 values refer to the system[17] 1-butanol-pyridine-acetic acid-water, 30:20:6:24, and are expressed as a multiple of the distance traveled by a histidine marker. Enzymatic analysis (LAP) was performed according to the procedure of Hofmann, *et al.*,[18] using a commercial crystalline enzyme (Worthington). The amino acid analyses of acid hydrolysates were carried out according to the method of Moore, Spackman, and Stein[19] with a Beckman-Spinco amino acid analyzer, Model 120B, to which a digital readout system, Model CRS-10AB (Infotronics Corp., Houston, Texas), has been attached. Optical rotations were determined with a Zeiss photoelectric precision polarimeter.

N-Carbobenzoxy-L-asparagine *p*-Nitrobenzyl Ester. A solution of N-carbobenzoxy-L-asparagine (21.2 g) and *p*-nitrobenzyl chloride (20.6 g) in DMF (60 ml) containing triethylamine (10.2 ml) was heated at 65° for 4 hr, cooled at room temperature, and then poured into cold 1 *N* $KHCO_3$ (500 ml). The precipitated material was isolated by filtration, washed with water and cold methanol (100 ml), and dried. After trituration with ethyl acetate and recrystallization from aqueous methanol 22.5 g (72%) of product was obtained; mp 166–168°.

(13) B. Iselin, W. R. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, 40, 373 (1957).

(14) St. Guttmann and R. A. Boissonnas, *ibid.*, 43, 201 (1960).

(15) The following abbreviations are used: DMF = dimethylformamide; DMSO = dimethyl sulfoxide; Z = carbobenzoxy; Bz = benzyl; PNP = *p*-nitrophenyl; Me = methyl; N_3 = azide; NBz = *p*-nitrobenzyl.

(16) S. M. Partridge, *Biochem. J.*, 42, 238 (1948).

(17) S. G. Waley and G. Watson, *ibid.*, 55, 328 (1953).

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N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (I). A suspension of N-carbobenzoxy-L-asparagine *p*-nitrobenzyl ester (24.2 g) in acetic acid (120 ml) was treated with 4 *N* HBr in acetic acid (120 ml). After 2 hr at room temperature, the reaction mixture was poured into ether (800 ml) and the precipitated hydrobromide was filtered off, washed with ether, and dried over KOH *in vacuo*. To a solution of this product in DMF (100 ml), triethylamine (10 ml) was added, followed by N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester (28 g). After 24 hr at room temperature the reaction mixture was diluted with 1 *N* NH_4OH (5 ml), stirred for 30 min, and mixed with ethyl acetate (800 ml) and water (100 ml). The organic layer was separated and washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and finally with water. Following the final washing the product started crystallizing out. After 1 hr the product was filtered off and washed with cold ethyl acetate (25 ml). The combined filtrates were concentrated to a small volume (80 ml) and another crop of crystalline product was collected. The combined crystalline solid was triturated with methanol (250 ml) and reprecipitated from a solution in dimethylformamide upon addition of water; wt, 25.7 g (72%); mp 177–179°; $[\alpha]^{28}D$ −32.6° (*c* 1.0, DMF); R_f^1 0.76; R_f^2 6.0 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{29}H_{30}N_4O_8S$: C, 58.6; H, 5.08; N, 9.4. Found: C, 58.7; H, 5.25; N, 9.4.

N-Carbobenzoxy-O-benzyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (II). A suspension of I (1.78 g) in acetic acid (5 ml) and 4 *N* HBr in acetic acid (5 ml) was stored at room temperature for 1 hr. The resulting solution was then poured into ether, and the precipitated hydrobromide was filtered off, washed with ether, and dried over KOH *in vacuo*. This product was dissolved in DMF (10 ml), triethylamine (0.6 ml) was added, and finally N-carbobenzoxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester (1.5 g). After 24 hr the reaction mixture was diluted with 1 *N* NH_4OH (1 ml), stirred for 30 min and poured into cold 1 *N* NH_4OH (100 ml). The precipitated product was filtered off, washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water, and crystallized from aqueous acetic acid; wt, 1.5 g (62%); mp 209–211°; $[\alpha]^{28}D$ −27.3° (*c* 1.0, DMF); R_f^1 0.82; single ninhydrin-positive spot.

Anal. Calcd for $C_{45}H_{45}N_5O_{10}S$: C, 60.7; H, 5.34; N, 8.2. Found: C, 60.4; H, 5.34; N, 8.1.

N-Carbobenzoxy-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (III). A suspension of I (5.9 g) in acetic acid (15 ml) was treated with 4 *N* HBr in acetic acid (15 ml). After 1 hr at room temperature the resulting solution was poured into dry ether, and the precipitated hydrobromide was isolated by filtration, washed with ether, and dried over KOH *in vacuo*. This product was dissolved in DMF (10 ml) containing triethylamine (1.5 ml) and stirred 10 min, and the precipitated triethylamine hydrobromide was filtered off. The filtrate was then added to the mixed anhydride prepared as follows. A solution of N-carbobenzoxy-L-asparaginyl-L-tyrosine (4.3 g) in tetrahydrofuran (25 ml) and DMF (8 ml) containing triethylamine (1.4 ml) was cooled to −10° and isobutyl chlorocarbonate (1.2 ml) dissolved in tetrahydrofuran (3 ml) was added. After 15 min the solution of S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, prepared as described above, was added. The reaction mixture was stirred at 0° for 24 hr and then poured into 1 *N* HCl (200 ml). The precipitated product was isolated by filtration, washed successively with water, 1 *N* $KHCO_3$, and water, and reprecipitated from aqueous acetic acid; wt, 6.5 g (75%); mp 218–220°; $[\alpha]^{28}D$ −34.6° (*c* 1.1, DMF); R_f^1 0.65; R_f^2 5.8 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{42}H_{45}N_7O_{12}S$: C, 57.8; H, 5.20; N, 11.2. Found: C, 57.8; H, 4.98; N, 11.5.

N-Carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (IV). N-Carbobenzoxy-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (12 g) was dissolved in acetic acid (80 ml) and treated with 4 *N* HBr in acetic acid (80 ml). After 1 hr at room temperature the resulting solution was poured into dry ether, and the precipitated product was filtered off, washed with ether, and dried over KOH *in vacuo*. To a solution of this material in DMF (90 ml) containing triethylamine (2.4 ml) was added N-carbobenzoxy-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (6.48 g). After 24 hr the reaction mixture was poured into cold 1 *N* $KHCO_3$ (600 ml). The precipitated product was collected, washed successively with water, 1 *N* HCl, and water, and reprecipitated from 50% aqueous acetic acid; wt, 12 g (82%); mp 219–222°; $[\alpha]^{27}D$ −42.2° (*c* 0.96, DMF); after treatment for 1 hr with HBr in acetic acid, R_f^1 0.58; R_f^2 4.3 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{54}H_{58}N_{8}O_{15}S$: C, 59.4; H, 5.35; N, 10.3. Found: C, 59.8; H, 5.44; N, 10.5.

N-Carbobenzoxy-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucine Hydrazide (V). N-Carbobenzoxy-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucine methyl ester[20] (15 g) was dissolved in warm methanol (200 ml), hydrazine hydrate (15 ml) added, and the mixture refluxed for 2 hr and stirred at room temperature for 24 hr. The precipitated crystalline product was collected by filtration and washed with methanol and ether; wt, 10.9 g (70%); mp 246–249°; $[\alpha]^{27}D$ −29.4° (*c* 1.0, DMF).

Anal. Calcd for $C_{34}H_{49}N_{7}O_{8}$: C, 59.7; H, 7.22; N, 14.3. Found: C, 59.4; H, 7.19; N, 14.4.

N-Carbobenzoxy-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (VI). A suspension of IV (8 g) in acetic acid (35 ml) was treated with 4 *N* HBr in acetic acid (35 ml). After 1 hr the resulting solution was poured into ether (600 ml). The precipitated, partially protected pentapeptide hydrobromide was isolated by filtration, washed with ether, and dried over KOH *in vacuo*. To a cooled (0°) solution of this material in DMF (40 ml) containing triethylamine (3 ml) was added the tetrapeptide azide prepared as follows: V (6.2 g) was dissolved in a mixture of acetic acid (50 ml), DMF (80 ml), and 2 *N* HCl (10 ml). This solution was cooled to −10° and $NaNO_2$ (692 mg) dissolved in cold water (3 ml) was slowly added. The reaction mixture was stirred at −10° for 10 min, and then was poured into cold saturated NaCl (400 ml). The precipitated azide was filtered off, washed successively with ice-cold water, 1 *N* $NaHCO_3$, and water, and dried for 2 hr at 0° under vacuum over P_2O_5. This azide was then added to the pentapeptide derivative prepared as described previously. After 24 hr at 0° the reaction mixture was poured into a solution consisting of methanol (300 ml), water (400 ml), and 1 *N* HCl (1 ml). The precipitated product was collected by filtration, washed successively with 50% aqueous methanol and anhydrous methanol, and dried; wt, 8.7 g (78%); mp 242–243°. A sample for analysis was precipitated from dimethylformamide–water; melting point unchanged; $[\alpha]^{28}D$ −46.2° (*c* 1, DMF).

Anal. Calcd for $C_{73}H_{91}N_{13}O_{21}S$: C, 57.7; H, 6.00; N, 12.0; O, 22.15. Found: C, 57.7; H, 6.31; N, 11.6; O, 22.11.

The nonapeptide derivative was decarbobenzoxylated on exposure to 2 *N* HBr in acetic acid and chromatographed on paper; R_f^1 0.86; R_f^2 4.7 × His; single ninhydrin-positive spot. Amino acid analysis of an acid hydrolysate of the protected nonapeptide showed the following composition expressed in molar ratios: $Asp_{2.00}Glu_{2.00}Leu_{2.00}Tyr_{1.6}$S-benzylcysteine$_{0.80}$.

N-Carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-serine Methyl Ester (VII). N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-serine methyl ester (7.75 g) was dissolved in trifluoroacetic acid (30 ml) and HBr was passed through the solution for 45 min at 0°. Addition of dry ether to the reaction mixture caused the peptide ester hydrobromide to precipitate as a heavy oil. The ether was decanted and the residue was dried over KOH *in vacuo*. To a solution of this material in DMF (30 ml) containing triethylamine (3.8 ml) was added N-carbobenzoxy-L-valine *p*-nitrophenyl ester (5.45 g). After 24 hr the reaction mixture was diluted with 1 *N* NH_4OH (2 ml), stirred for 30 min, and poured into cold 1 *N* NH_4OH (200 ml). The precipitated product was collected by filtration, washed successively with 1 *N* NH_4OH, 1 *N* HCl, and water, and crystallized from methanol–water; wt, 4.0 g (50%); mp 199°; $[\alpha]^{28}D$ −29.1° (*c* 0.94, DMF); after HBr in trifluoroacetic acid treatment: R_f^1 0.82; R_f^2 5.8 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{27}H_{35}N_{3}O_{7}S$: C, 59.4; H, 6.64; N, 7.7. Found: C, 59.1; H, 6.37; N, 7.6.

N-Carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-serine Hydrazide (VIII). To a solution of VII (8 g) in methanol (700 ml) was added hydrazine hydrate (14 ml). After 48 hr at room temperature the precipitated hydrazide was isolated by filtration, washed with methanol, and reprecipitated from dimethylformamide–ether; wt, 7.4 g (92%); mp, 228–230°; $[\alpha]^{29}D$ −19.6° (*c* 0.58, DMF).

Anal. Calcd for $C_{26}H_{35}N_{5}O_{6}S$: C, 57.2; H, 6.46; N, 12.8. Found: C, 57.0; H, 6.37; N, 12.6.

N-Carbobenzoxy-L-valyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester (IX). A suspension of the nonapeptide derivative (VI, 4 g) in acetic acid (30 ml) was diluted with 4 *N* HBr in acetic acid (30 ml). After 1 hr the resulting solution was poured into dry ether (600 ml) and the precipitated hydrobromide of the partially protected nonapeptide was collected by filtration, washed with ether, and dried over KOH *in vacuo*. To a cooled (0°) solution of this product in DMF (50 ml) triethylamine (1.5 ml) was added followed by the tripeptide azide prepared as follows: VIII (2.4 g) was dissolved in a mixture of acetic acid (70 ml), DMF (30 ml), and 2 *N* HCl (6 ml). After cooling this solution to −10°, $NaNO_2$ (320 mg) dissolved in cold water (2 ml) was added. The reaction mixture was stirred at −10° for 10 min and then poured into an ice-cold saturated solution of NaCl (350 ml). The precipitated azide was filtered off, washed successively with cold (0°) water, 1 *N* $NaHCO_3$, and water, and dried for 1 hr at 0° over P_2O_5 *in vacuo*. The tripeptide azide was then added to the solution of the nonapeptide derivative which was prepared as described previously. The reaction mixture was stirred for 24 hr at 0° and then poured into 1 *N* HCl (400 ml). The precipitated product was filtered off, washed with water, and triturated with hot methanol; wt, 4.2 g (84%); mp 265° dec. A sample for analysis was precipitated from dimethylformamide–water; $[\alpha]^{28}D$ −27.5° (*c* 1, DMSO).

Anal. Calcd for $C_{91}H_{116}N_{16}O_{25}S_2$: C, 57.6; H, 6.12; N, 11.8. Found: C, 58.0; H, 6.57; N, 11.4.

For paper chromatography a sample of the dodecapeptide derivative was decarbobenzoxylated on exposure to HBr in trifluoroacetic acid; R_f^1 0.76, R_f^2 4.1 × His; single ninhydrin-positive spot. Amino acid analysis of an acid hydrolysate of the protected dodecapeptide by the automatic analyzer showed the following composition expressed in molar ratios: $Asp_{2.0}Glu_{2.0}Leu_{2.0}Ser_{0.80}Val_{0.90}Tyr_{1.6}$S-benzylcysteine$_{1.6}$.

For evaluation of stereochemical homogeneity a sample of the decarbobenzoxylated dodecapeptide was digested with LAP. Amino acid analysis of the digest by the automatic analyzer gave the following composition expressed in molar ratios: $Glu_{1.1}Leu_{1.8}Val_{1.0}Tyr_{1.8}$S-benzylcysteine$_{2.1}$. Glutamine, asparagine, and serine emerge at the same position on the chromatogram and were not determined.

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