moval of the ethyl acetate, a crystalline residue remained which was recrystallized from aqueous acetic acid; wt. 16 g. (82%), m.p. 263–264°, $[\alpha]^{26}D$ −27° (*c* 1, dimethylformamide); R_f (hydrobromide) 0.85, single ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for $C_{45}H_{55}N_5O_7S$: C, 66.7; H, 6.86; N, 8.6. Found: C, 66.5; H, 6.61; N, 8.4.

The peptide hydrobromide which was obtained from the protected derivative was completely digested by LAP.

N-Carbobenzoxy-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (IV).—N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (13 g.) was suspended in acetic acid (60 ml.) and treated with 4 *N* HBr in acetic acid (60 ml.). After 1 hour at room temperature, dry ether (500 ml.) was added, the precipitate which formed was filtered, washed with ether, dried and dissolved in dimethylformamide (100 ml.). To this solution triethylamine (2.3 ml.) was added followed by N-carbobenzoxy-L-leucine *p*-nitrophenyl ester (6.1 g.). After 24 hours at room temperature, the yellow solution was diluted with 1 *N* NH_4OH (10 ml.), stirred for 1 hour and poured into ice-cold 1 *N* NH_4OH (300 ml.). The precipitated product was collected by filtration, washed with water, 1 *N* HCl and water and reprecipitated from aqueous acetic acid; wt. 12.6 g. (96%), m.p. 248–250°, $[\alpha]^{29}D$ −38.9° (*c* 1.03, dimethylformamide); R_f (hydrobromide) 0.84, single ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for $C_{44}H_{60}N_6O_8S$: C, 63.4; H, 7.25; N, 10.0. Found: C, 63.2; H, 7.14; N, 9.7.

N-Carbobenzoxy-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide Hydrate (V).—Compound IV (6 g.) was dissolved in 2 *N* HBr in acetic acid (60 ml.). After 1 hour at room temperature, dry ether was added. The precipitate was washed with ether, dried briefly over KOH and dissolved in dimethylformamide (50 ml.). Triethylamine (1 ml.) was added to this solution followed by N-carbobenzoxy-L-alanine *p*-nitrophenyl ester (2.4 g.). After 24 hours at room temperature, the reaction mixture was diluted with 1 *N* NH_4OH (200 ml.). The precipitated product was isolated by filtration, washed with water, 1 *N* HCl and water and reprecipitated from aqueous acetic acid; wt. 6 g. (90%), m.p. 259°, $[\alpha]^{29}D$ −41.7° (*c* 1, dimethylformamide); R_f (hydrobromide) 0.91, single ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for $C_{47}H_{65}N_7O_9S \cdot H_2O$: C, 61.2; H, 7.34; N, 10.7. Found: C, 61.2; H, 7.06; N, 10.8.

N-Carbobenzoxy-γ-benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (VI).—Compound V (3.7 g.) was suspended in acetic acid (10 ml.) and treated with 4 *N* HBr in acetic acid (10 ml.). After 1 hour, dry ether (200 ml.) was added, the precipitate which was formed was collected by filtration, washed with ether and dried over KOH *in vacuo*. This solid was dissolved in dimethylformamide (20 ml.); triethylamine (0.6 ml.) was added followed by N-carbobenzoxy-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (1.8 g.). After 24 hours the yellow solution was diluted with 1 *N* NH_4OH (2 ml.), stirred for 1 hour and poured into ice-cold 1 *N* NH_4OH (50 ml.). The precipitate was collected by filtration, washed with water, 1 *N* HCl and water and purified on reprecipitation from aqueous acetic acid; wt. 4 g. (97%), m.p. 254–260°, $[\alpha]^{26}D$ −27.9° (*c* 0.48, dimethylformamide); R_f (hydrobromide) 0.72, single ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for $C_{59}H_{78}N_8O_{12}S$: C, 63.1; H, 7.00; N, 10.0. Found: C, 62.9; H, 7.11; N, 10.0.

The heptapeptide amide hydrobromide which was formed from the carbobenzoxy derivative on exposure to HBr in acetic acid was fully digested with LAP.

N-Carbobenzoxy-L-leucyl-L-valine *p*-Nitrophenyl Ester (VIII).—To a precooled solution of N-carbobenzoxy-L-leucyl-L-valine (1.0 g.) in ethyl acetate (15 ml.) and tetrahydrofuran (5 ml.) was added *p*-nitrophenol (0.45 g.) followed by N,N′-dicyclohexylcarbodiimide (0.57 g.). After 30 minutes at 0° and 2 hours at room temperature the N,N′-dicyclohexylurea which was separated was filtered off and the filtrate concentrated to dryness *in vacuo*. On reprecipitation from ether–petroleum ether, 0.62 g. (47%) of crystalline product was obtained, m.p. 126°, $[\alpha]^{27}D$ +16.8° (*c* 0.53, dimethylformamide).

Anal. Calcd. for $C_{25}H_{31}N_3O_7$: C, 61.8; H, 6.42; N, 8.6. Found: C, 62.0; H, 6.42; N, 8.4.

N-Carbobenzoxy-L-leucyl-L-valyl-γ-benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide One and One-half Hydrate (IX).—N-Carbobenzoxy-γ-benzyl-L-glutamyl-L-alanyl-L-leucyl-L-tyrosyl-L-leucyl-L-valyl-S-benzyl-L-cysteinamide (0.3 g.) was dissolved in 2 *N* HBr in acetic acid (4 ml.). After 50 minutes at room temperature dry ether (50 ml.) was added and the precipitated material was isolated by filtration and washed thoroughly with dry ether and dried over KOH *in vacuo*. To a solution of this product in dimethylformamide (8 ml.), triethylamine (0.1 ml.) was added followed by N-carbobenzoxy-L-leucyl-L-valine *p*-nitrophenyl ester (0.12 g.). After 24 hours the reaction mixture was diluted with 1 *N* NH_4OH (0.5 ml.), stirred 30 minutes and poured into ice-cold 1 *N* NH_4OH (50 ml.). The precipitated product was collected by filtration, washed with 1 *N* NH_4OH, water, 1 *N* HCl and water again. On precipitation from dimethylformamide–water, 0.28 g. (91%) of product was obtained, m.p. 262–266°, $[\alpha]^{28}D$ −32.8° (*c* 0.18, dimethylformamide); R_f (hydrobromide) 0.93, single sharp ninhydrin- and tyrosine-positive spot.

Anal. Calcd. for $C_{70}H_{98}N_{10}O_{14}S \cdot 1.5\ H_2O$: C, 61.7; H, 7.39; N, 10.3. Found: C, 61.4; H, 7.22; N, 10.7.

Amino acid analysis by a Beckman–Spinco analyzer after acid hydrolysis showed the expected composition expressed in molar ratios: $glu_{0.96}ala_{1.0}val_{2.0}leu_{3.0}tyr_{0.78}$ (S-benzylcysteine present on paper chromatogram but not determined). The amino acid recovery was 96% of the theoretical value.

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[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNA.]

Insulin Peptides. VI. The Synthesis of a Partially Protected Nonapeptide Corresponding to the First Nine Amino Acid Residues of the A-Chain of Insulin[1]

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The partially protected nonapeptide N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine, containing the N-terminal sequence of the A-chain of sheep insulin, has been synthesized. The key step for the synthesis of this compound involved the condensation of an acyltetrapeptide subunit with a pentapeptide ester subunit. Both these subunits were synthesized by stepwise elongation of the peptide chain from the amino end.

Regeneration of insulin activity from the separated A- and B-chains or their benzyl derivatives has been accomplished independently by two groups of investigators.[2–5] This provides sufficient assurance that the eventual synthesis of insulin can be achieved if chemically synthesized A- and B-chains are available.

Peptide chemistry probably has not developed to the level of sophistication necessary to cope with the synthesis of large protein molecules.[6] However, the present synthetic methodology and the development of purification techniques has undoubtedly paved the

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way for the synthesis of polypeptides of the size of the insulin chains.[6,7]

A program has therefore been initiated in this Laboratory directed toward the synthesis of the two chains of sheep insulin which eventually can lead us to the total synthesis of this protein hormone.[8]

In previous communications[9-12] we have reported the synthesis of protected peptides containing amino acid sequences found in the C-terminal and middle sections of both chains of sheep insulin. In the present communication we describe the synthesis of the partially protected nonapeptide N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine (XI). The sequence corresponding to this peptide is found in the N-terminal portion of the A-chain of sheep insulin.

In the synthesis of the nonapeptide, which is illustrated in Chart I, the "fragment condensation" approach was employed.[6] It involved the coupling of a partially protected tetrapeptide subunit with a partially protected pentapeptide subunit. Thus condensation of N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid γ-*tert*-butyl ester (IV) with L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (IX) by the carbodiimide method[13] yielded the fully protected nonapeptide X from which, on partial saponification, the desired partially protected nonapeptide XI was obtained. The tetrapeptide subunit IV and the pentapeptide subunit IX were synthesized by stepwise elongation of the peptide chain from the amino end. The carbobenzoxy[14] group and in one instance the *p*-nitrocarbobenzoxy[15] group were used for the synthesis of the appropriate acylamino acids which served as the "carboxyl components" in the various synthetic steps. Activation of the carboxyl components was carried out exclusively by conversion to the corresponding *p*-nitrophenyl esters.[16] In the stepwise synthesis of the pentapeptide subunit IX decarbobenzoxylation of the various intermediate peptide derivatives was effected by treatment with HBr in acetic acid. In the synthesis of the tetrapeptide subunit IV, however, decarbobenzoxylation of the intermediate blocked peptides was carried out by catalytic hydrogenation. Deblocking through hydrogenolysis in the latter case was necessary because of the presence of the highly labile *tert*-butyl ester group[17] on the C-terminal glutamic acid residue.

The chemical purity of the intermediate peptides was ascertained by elemental analysis and by paper chromatography of the deblocked derivatives. In the latter case the chromatograms exhibited sharp single spots indicating the presence of single homogeneous components. The chemical purity of the final product was established by elemental analysis, paper chromatography and amino acid analysis of an acid hydrolysate by the Stein and Moore technique.[18] In the latter case the constituent amino acids, with the exception of S-benzylcysteine, were obtained in the proportions required by theory and with an average recovery of 86%. S-Benzylcysteine was not determined. Its presence in the hydrolysate was demonstrated by paper chromatography (R_f = 0.70 in the Partridge[19] system), but it was not eluted after 20-hr. chromatography on the long (150 cm.) column of a Beckman-Spinco amino acid analyzer. However, chromatography of the hydrolysate on the short column (15 cm.) of the analyzer, which is routinely used for the determination of the basic amino acids,[18] revealed the presence of a ninhydrin-positive peak appearing after 36 ml. of effluent and tentatively identified as S-benzylcysteine.

The stereochemical homogeneity of the partially protected nonapeptide XI was established by incubation of the deblocked derivative with leucine aminopeptidase followed by paper chromatography. Chromatograms of the digest indicated the presence of ninhydrin-positive spots with R_f values corresponding only to the expected amino acids. This suggests that the digestion was complete and implies that the optical purity of the constituent amino acids was preserved during the synthetic processes.[7]

An alcoholic solution of N-carbobenzoxy-L-glutamic acid α-ethyl-γ-*tert*-butyl ester[20,21] was hydrogenated over palladium and the resulting product was treated with N-carbobenzoxy-L-valine *p*-nitrophenyl ester[22] to yield the crystalline N-carbobenzoxy-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (I) in 79% yield. The protected tripeptide N-carbobenzoxy-L-leucyl-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (II) was readily prepared in crystalline form and in 92% yield by the coupling of N-carbobenzoxy-L-isoleucine *p*-nitrophenyl ester[16] with the product which was obtained by catalytic hydrogenation of the protected dipeptide I. The same pattern was employed for the conversion of the protected tripeptide II to the tetrapeptide N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (III). The *p*-nitrophenyl ester of N-*p*-nitrocarbobenzoxyglycine which was used for this conversion was prepared in the usual manner.[16]

Exposure of the fully protected tetrapeptide III to dilute alkali led to the preferential hydrolysis of the α-ethyl ester function of the C-terminal glutamic acid residue and yielded the partially protected tetrapeptide N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid γ-*tert*-butyl ester (IV) in 76% yield.

The protected dipeptide N-carbobenzoxy-L-alanylglycine ethyl ester[23] (V) was obtained in crystalline form and in 80% yield by the interaction of N-carbobenzoxy-L-alanine *p*-nitrophenyl ester[24] with glycine ethyl ester. Removal of the carbobenzoxy group with HBr in acetic acid and coupling the resulting product with N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester[16] afforded the crystalline tripeptide N-carbobenzoxy-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (VI) in 72% yield. The protected tetrapeptide N-carbobenzoxy-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (VII) was prepared in 82% yield by a similar series of reactions and it was converted to the protected pentapeptide VIII in 77% yield by decarbobenzoxylation and condensation of the ensuing product with N-carbobenzoxy-L-glutamine *p*-nitrophenyl ester.[16]

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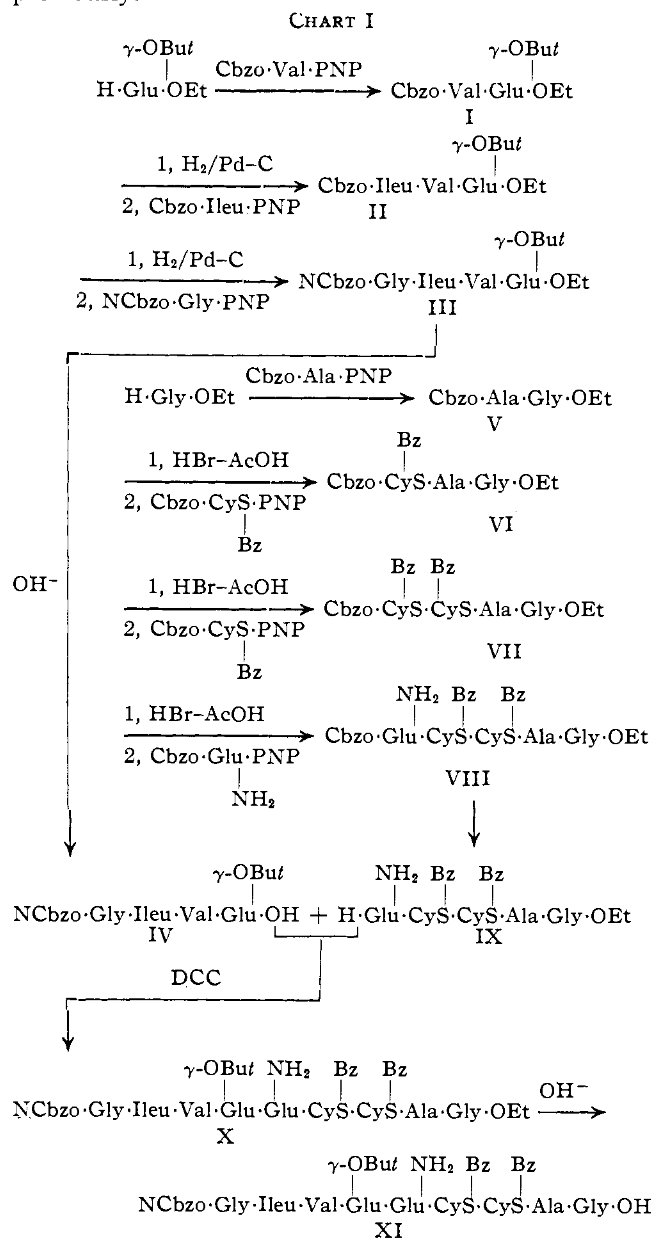
For the synthesis of the fully protected nonapeptide X, N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid γ-*tert*-butyl ester (IV) was condensed by the carbodiimide method with L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester. The latter compound was obtained from its carbobenzoxy derivative VIII on exposure to HBr in acetic acid. Selective hydrolysis of the ethyl ester function of the C-terminal amino acid residue of the fully protected nonapeptide X was effected by treatment with dilute sodium hydroxide and the final product N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*tert*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine (XI) was obtained in 87% yield. The chemical and stereochemical homogeneity of this nonapeptide was discussed previously.

CHART I



Bu*t*: *tert*-butyl
Cbzo: carbobenzoxy
NCbzo: *p*-nitrocarbobenzoxy
Bz: benzyl
PNP: *p*-nitrophenyl ester
DCC: N,N′-dicyclohexylcarbodiimide

Experimental

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

For paper chromatography the protected peptides were deblocked either with HBr in acetic acid or by catalytic hydrogenation and chromatographed on Whatman No. 1 filter paper at room temperature. The Partridge[19] or the 2-butanol–ammonia system[25] was employed for the development of the chromatograms; the location of the peptides was revealed either by the ninhydrin or the chlorine method.[26]

N-Carbobenzoxy-L-valyl-L-glutamic Acid α-Ethyl-γ-*tert*-butyl Ester (I).—N-Carbobenzoxy-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (3.4 g.) was dissolved in ethanol (50 ml.) and hydrogenated for 2 hours in the presence of 10% palladium–charcoal catalyst (1 g.). The catalyst was filtered off and the filtrate was concentrated to dryness. To a solution of the residue in dimethylformamide (40 ml.) N-carbobenzoxy-L-valine *p*-nitrophenyl ester (3.5 g.) was added followed by a few drops of triethylamine to ensure basicity. After 40 hours at room temperature the yellow reaction mixture was poured into ice-cold 1 *N* Na_2CO_3 (400 ml.). The white precipitate was collected by filtration, washed with 1 *N* Na_2CO_3, water, 1 *N* acetic acid and water, and extracted into ethyl acetate (50 ml.). The organic layer was washed successively with 1 *N* Na_2CO_3, water, 1 *N* acetic acid and water and evaporated to dryness. The remaining oily product was precipitated from ethanol–water (1:2) and then crystallized from ethyl acetate–petroleum ether; wt. 3.6 g. (79%), m.p. 67°. For analysis a sample was recrystallized from ethyl acetate–petroleum ether; m.p. 70–71°, $[\alpha]^{26}D$ −26.7° (*c* 1, ethanol).

Anal. Calcd. for $C_{24}H_{36}N_2O_7$: C, 62.0; H, 7.83; N, 6.0. Found: C, 62.0; H, 7.69; N, 6.0.

For paper chromatography a sample was decarbobenzoxylated by catalytic hydrogenation; R_f^{19} 0.85, R_f^{25} 0.90, sharp single spot (ninhydrin or chlorine test).

N-Carbobenzoxy-L-isoleucyl-L-valyl-L-glutamic Acid α-Ethyl-γ-*tert*-butyl Ester (II).—Compound I (7 g.) was dissolved in ethanol (150 ml.) and hydrogenated for 2 hours over 10% palladium–charcoal catalyst (2 g.). The catalyst was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in dimethylformamide (130 ml.) containing a few drops of triethylamine. To this solution N-carbobenzoxy-L-isoleucine *p*-nitrophenyl ester (7 g.) was added. After 24 hours at room temperature the yellow solution was poured into ice-cold 1 *N* Na_2CO_3 (1000 ml.) and stirred for 25 minutes. The precipitated product was isolated by filtration, washed thoroughly with water and dissolved in methylene chloride (50 ml.). The organic layer was washed with 1 *N* acetic acid and water, dried with $MgSO_4$ and evaporated to dryness *in vacuo*. The product was thus obtained in crystalline form; wt. 7.95 g. (92%), m.p. 180–184°. For analysis a sample was recrystallized from methylene chloride–ether; m.p. 186–189°, $[\alpha]^{26}D$ −18.5° (*c* 1, chloroform).

Anal. Calcd. for $C_{30}H_{47}N_3O_8$: C, 62.4; H, 8.20; N, 7.3 Found: C, 62.3; H, 8.32; N, 7.3.

For paper chromatography the product was decarbobenzoxylated by catalytic hydrogenation; R_f^{19} 0.89, R_f^{25} 0.94 single spot (ninhydrin or chlorine test).

N-*p*-Nitrocarbobenzoxyglycine *p*-Nitrophenyl Ester.—To a cold solution of N-*p*-nitrocarbobenzoxyglycine[27] (2.54 g.) in dioxane (20 ml.) and ethyl acetate (10 ml.), *p*-nitrophenol (1.7 g.) was added followed by N,N′-dicyclohexylcarbodiimide (2.1 g.). After 1 hour the mixture was allowed to come to room temperature and was held there for 16 hours. The N,N′-dicyclohexylurea which separated was removed by filtration. Addition of petroleum ether to this filtrate caused the precipitation of the product. On reprecipitation from ethyl acetate–petroleum ether the material crystallized; wt. 3.2 g. (85%), m.p. 138°.

Anal. Calcd. for $C_{16}H_{13}N_3O_8$: C, 51.2; H, 3.49; N, 11.2. Found: C, 50.9; H, 3.78; N, 11.2.

N-*p*-Nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic Acid α-Ethyl-γ-*tert*-butyl Ester (III).—N-Carbobenzoxy-L-isoleucyl-L-valyl-L-glutamic acid α-ethyl-γ-*tert*-butyl ester (0.78 g.) was dissolved in dioxane (15 ml.) and hydrogenated for 2 hours in the presence of 10% palladium–charcoal catalyst (0.4 g.). The catalyst was filtered off and the filtrate evaporated *in vacuo*. To a solution of the residue in dimethylformamide (25 ml.) N-*p*-nitrocarbobenzoxyglycine *p*-nitrophenyl ester (0.62 g.) was added followed by a few drops of triethylamine to ensure basicity. After 48 hours the reaction mixture was poured into ice-cold 0.4 *N* acetic acid (120 ml.) and stirred for 20 minutes. The precipitate which was formed was separated by filtration, washed with water and dried. When reprecipitated from methylene chloride–ether the product was obtained in crystalline form; wt. 0.66 g. (72%), m.p. 183–186°, $[\alpha]^{26}D$ −32° (*c* 1, chloroform).

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Anal. Calcd. for $C_{32}H_{49}N_5O_{11}$: C, 56.5; H, 7.27; N, 10.3. Found: C, 56.7; H, 7.23; N, 10.2.

For paper chromatography the protected peptide was deblocked by catalytic hydrogenation; R_f[19] 0.81, R_f[25] 0.88, single spot (ninhydrin and chlorine test).

N-*p*-Nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic Acid *γ-tert*-Butyl Ester (IV).—To a solution of the fully protected tetrapeptide III (0.34 g.) in dioxane (8 ml.) and water (2 ml.) 1 *N* NaOH (0.65 ml.) was added. After 2.5 hours at room temperature the solution was poured into water (60 ml.) containing 1 *N* acetic acid (1 ml.). The precipitated product was isolated by filtration, washed with water and dried; wt. 0.25 g. (76%), m.p. 176–179°, $[\alpha]^{26}D$ −7.7° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{30}H_{45}N_5O_{11}$: C, 55.3; H, 6.96; N, 10.7. Found: C, 55.3; H, 7.11; N, 10.4.

N-Carbobenzoxy-L-alanylglycine Ethyl Ester (V).—To a solution of glycine ethyl ester (5.56 g.) and triethylamine (5.6 ml.) in dimethylformamide (30 ml.) was added N-carbobenzoxy-L-alanine *p*-nitrophenyl ester (13.4 g.). After 24 hours at room temperature, the reaction mixture was poured into ethyl acetate (600 ml.) and water (200 ml.). The ethyl acetate layer was extracted successively with 1 *N* NH_4OH, water, 1 *N* HCl, water, and dried. On removal of the ethyl acetate *in vacuo* a crystalline residue remained; wt. 9.8 g. (80%), m.p. 97–98°. Recrystallization from aqueous ethanol gave 9.3 g. (76%), m.p. 98–99°, lit.[23] m.p. 100°.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-alanylglycine Ethyl Ester (VI).—N-Carbobenzoxy-L-alanylglycine ethyl ester (3.18 g.) was suspended in acetic acid (5 ml.) and treated with 4 *N* HBr in acetic acid (10 ml.). After 1 hour at room temperature the reaction mixture was poured into anhydrous ether (300 ml.). The precipitate was filtered, washed with ether and dried over KOH *in vacuo*. This solid was dissolved in dimethylformamide (20 ml.), triethylamine (1.4 ml.) was added, and the triethylamine hydrobromide was filtered off. To the filtrate N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester (4.4 g.) was added, and the resulting solution was left standing at room temperature for 24 hours. The yellow solution was then poured into a mixture of ethyl acetate (300 ml.) and water (100 ml.). The organic layer was extracted successively with 1 *N* NH_4OH, water, 1 *N* HCl, water and dried. Concentration of the solvent to a small volume and cooling of the solution afforded a solid which was crystallized from aqueous acetic acid; wt. 3.59 g. (72%), m.p. 156–157°, $[\alpha]^{27}D$ −16.2° (*c* 1, dimethylformamide), R_f[19] (hydrobromide) 0.75.

Anal. Calcd. for $C_{25}H_{31}N_3O_6S_1$: C, 59.8; H, 6.23; N, 8.4. Found: C, 59.8; H, 6.17; N, 8.3.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Ethyl Ester (VII).—N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (2.52 g.) was suspended in acetic acid (5 ml.) and treated with 4 *N* HBr in acetic acid (5 ml.). After 1 hour at room temperature the reaction mixture was poured into anhydrous ether (300 ml.). The precipitate formed was collected by filtration, washed with ether, and dried over KOH *in vacuo*. This material was dissolved in dimethylformamide (15 ml.) to which triethylamine (0.7 ml.) was added. The triethylamine hydrobromide was filtered off, and N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester (2.10 g.) was added to the filtrate. After 24 hours at room temperature the reaction mixture was poured into ice-cold 1 *N* NH_4OH (100 ml.). The precipitate was filtered off, washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, water and dried; wt. 3.1 g., m.p. 187–190°. After reprecipitation from aqueous acetic acid 2.6 g. (82%) of product was obtained, m.p. 188–190°, $[\alpha]^{26}D$ −29.3° (*c* 1, dimethylformamide), R_f[19] (hydrobromide) 0.86, single ninhydrin-positive spot.

Anal. Calcd. for $C_{35}H_{42}N_4O_7S_2$: C, 60.50; H, 6.09; N, 8.0. Found: C, 59.9; H, 6.30; N, 8.0.

N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Ethyl Ester (VIII).—N-Carbobenzoxy-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (7 g.) was suspended in 2 *N* HBr in acetic acid (50 ml.). After 1 hour at room temperature the reaction mixture was poured into cold dry ether (200 ml.). The precipitated product was isolated by filtration, washed with anhydrous ether, and dried over KOH *in vacuo*. This product was dissolved in dimethylformamide (45 ml.) and triethylamine (1.5 ml.) was added. The triethylamine hydrobromide was filtered off and N-carbobenzoxy-L-glutamine *p*-nitrophenyl ester (3.50 g.) was added to the filtrate. After standing overnight at room temperature the solution was poured into cold 1 *N* NH_4OH (200 ml.). The precipitate was filtered off and washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, water and dried. On reprecipitation from aqueous acetic acid, 6 g. (77%) of product was obtained, m.p. 250–252° dec., $[\alpha]^{27}D$ −31° (*c* 0.2, dimethylformamide), R_f[19] (hydrobromide) 0.84, single ninhydrin-positive spot.

Anal. Calcd. for $C_{40}H_{50}N_6O_9S_2$: C, 58.4; H, 6.12; N, 10.2. Found: C, 58.2; H, 6.17; N, 10.2.

N-*p*-Nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-*γ-tert*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Ethyl Ester (X).—N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (0.5 g.) was suspended in 2 *N* HBr in acetic acid (4 ml.). After 1 hour at room temperature dry ether was added. The precipitate was isolated by filtration and washed thoroughly with dry ether. This solid was dissolved in cold (0°) dimethylformamide (40 ml.); triethylamine (0.16 ml.) was added followed by N-*p*-nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid *γ-tert*-butyl ester (0.44 g.) and N,N′-dicyclohexylcarbodiimide (0.25 g.). After 15 minutes the mixture was allowed to come to room temperature and was held there for 2 days. During this period the mixture was turned to a gelatinous mass. Acetic acid (0.1 ml.) was then added and the gelatinous mass was mixed with methanol (200 ml.). After standing overnight at 5° the precipitated product was filtered off and washed with methanol; wt. 0.41 g. (52%), m.p. 268° dec. For analysis a sample was reprecipitated from dimethyl sulfoxide–dioxane; m.p. 270–272°, $[\alpha]^{27}D$ −27.1° (*c* 0.51, dimethyl sulfoxide).

Anal. Calcd. for $C_{62}H_{87}N_{11}O_{17}S_2$: C, 56.3; H, 6.63; N, 11.6. Found: C, 56.1; H, 6.56; N, 11.4.

N-*p*-Nitrocarbobenzoxyglycyl-L-isoleucyl-L-valyl-*γ-tert*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine (XI).—Compound X (55 mg.) was dissolved in dimethyl sulfoxide (3 ml.) and 0.1 *N* NaOH (0.5 ml.) was added. The reaction mixture was allowed to stand at room temperature for 3 hours then cooled to 0° and mixed with water (25 ml.) and 0.1 *N* HCl (0.55 ml.). The reaction mixture was stirred at 0° for 1 hour and the precipitated product was filtered off, washed with water and dried; wt. 47 mg. (87%), m.p. 254–259° dec., $[\alpha]^{26}D$ −27.5° (*c* 0.32, dimethyl sulfoxide).

Anal. Calcd. for $C_{60}H_{83}N_{11}O_{17}S_2$: C, 55.6; H, 6.46; N, 11.9. Found: C, 55.0; H, 6.55; N, 11.6.

Amino acid analysis of an acid hydrolysate by a Beckman–Spinco analyzer showed the expected composition expressed in molar ratios: $glu_{2.0}gly_{2.0}ala_{1.0}val_{0.9}ileu_{0.85}(NH_3)_{1.4}$(S-benzylcysteine present on a paper chromatogram but not determined). The average amino acid recovery was 86% of theory.

For paper chromatography and enzymatic analysis the fully protected nonapeptide was deblocked on exposure to HBr in acetic acid and the resulting hydrobromide was converted to the free base with NH_4OH.

Paper chromatography of this material using the formic acid system[28] exhibited a single spot, revealed either by ninhydrin or the chlorine test; R_f (formic acid system) 0.84.

The deblocked nonapeptide was digested with LAP. Paper chromatography of the digest revealed the presence of seven ninhydrin-positive components with R_f's 0.70, 0.63, 0.50, 0.28, 0.25, 0.21 and 0.23, identical with the R_f's of authentic samples of S-benzylcysteine, isoleucine, valine, alanine, glutamic acid, glutamine and glycine, respectively.

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