precipitated triethylamine hydrochloride was filtered off and washed with tetrahydrofuran (10 ml.). To the combined filtrates was added a solution of N-formyl-L-valine (4.35 g.) in tetrahydrofuran (25 ml.) followed by N,N'-dicyclohexylcarbodiimide (6.8 g.). The reaction mixture was stirred at 5° for 5 hr., at room temperature for 1 hr. and acidified with drops of acetic acid. The precipitated N,N'-dicyclohexylurea was filtered off (6.9 g., m.p. 234°) and the filtrate concentrated to 15 ml. *in vacuo* and mixed with cold water (150 ml.) containing acetic acid (1 ml.). The precipitated crystalline product was isolated by filtration, washed with water and recrystallized from 60% aqueous methanol; wt., 6.8 g. (56%), m.p. 126–128°. A sample for analysis was recrystallized from methanol; m.p. 128–129°, $[\alpha]^{27}D$ −50.0° (c = 1.46, dimethylformamide).

Anal. Calcd. for $C_{18}H_{26}O_4N_2S_2$: C, 54.2; H, 6.52; N, 7.0. Found: C, 54.1; H, 6.63; N, 6.8.

L-Valyl-S-benzylthiomethyl-L-cysteine Methyl Ester Hydrochloride.—A solution of N-formyl-L-valyl-S-benzylthiomethyl-L-cysteine methyl ester (1.6 g.) in methanol (30 ml.) and 1 *N* HCl (8 ml.) was refluxed for 1 hr. and the solvent removed under reduced pressure. The residue was dissolved in methanol, the solvent evaporated and this procedure was repeated three times. The solid residue then was dissolved in methanol (15 ml.). Addition of ether (100 ml.) caused the crystallization of the dipeptide ester hydrochloride; wt., 1.12 g. (70%), m.p. 161–162°, $[\alpha]^{27}D$ −4.9° (c = 1.12, dimethylformamide).

Anal. Calcd. for $C_{17}H_{27}O_3N_2S_2Cl$: C, 50.2; H, 6.68; N, 6.9. Found: C, 50.0; H, 6.72; N, 7.3.

Paper chromatography on Whatman #1 paper using the Partridge system revealed the presence of only one ninhydrin positive component with R_f = 0.74.

N-Carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine Methyl Ester.—A solution of N-carbobenzoxy-L-valine (3.8 g.) and triethylamine (2.1 ml.) in tetrahydrofuran (30 ml.) was cooled to −5° and isobutyl chlorocarbonate (2 ml.) added with stirring. After 10 minutes at this temperature a solution of S-benzylthiomethyl-L-cysteine methyl ester (prepared as described previously from 4.7 g. S-benzylthiomethyl-L-cysteine methyl ester hydrochloride, 2.1 ml. of triethylamine and 30 ml. of tetrahydrofuran) was added with stirring. After 30 minutes at −5° and 6 hr. at room temperature the reaction mixture was concentrated *in vacuo* to *ca.* 15 ml. and poured into cold water (300 ml.) containing concentrated HCl (2 ml.). The precipitated product was separated by filtration, washed with water, triturated with 5% aqueous $KHCO_3$, washed again with water and crystallized from 70% aqueous methanol; wt., 6.3 g. (83%), m.p. 112°, $[\alpha]^{30}D$ −28.8° (c = 1.05, dimethylformamide).

Anal. Calcd. for $C_{25}H_{32}N_2O_5S_2$: C, 59.50; H, 6.38; N, 5.55. Found: C, 60.0; H, 6.45; N, 5.55.

Decarbobenzoxylation of N-Carbobenzoxy-L-valyl-S-benzylthiomethyl-L-cysteine Methyl Ester. A. Treatment with HBr in Acetic Acid.—A 100 mg. sample of the protected dipeptide ester was added to 4 ml. of 2 *N* HBr in acetic acid. After 1 hr. at room temperature dry ether was added and the precipitate which formed was washed with ether. Paper chromatography of this product on Whatman #1 paper using the Partridge system revealed the presence of four ninhydrin-positive components with R_f's 0.38, 0.48, 0.60 and 0.77.

B. Treatment with HBr in Acetic Acid in the Presence of Diethylphosphite and Methyl Ethyl Sulfide.—A 100 mg. sample of the dipeptide ester was added in a mixture containing 2 ml. of 4 *N* HBr in acetic acid, 1 ml. of diethylphosphite and 1 ml. of methyl ethyl sulfide. After 1 hr. a mixture of ethyl acetate–petroleum ether (1:1) was added and the product which separated as a heavy oil was washed with ether. Paper chromatography of this product, carried out as described in A, revealed the presence of one main ninhydrin-positive component with R_f = 0.76 and traces of two other ninhydrin-positive components with R_f's 0.42 and 0.54.

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

Insulin Peptides. II. Synthesis of a Protected Pentapeptide Containing the C-Terminal Sequence of the A-Chain of Insulin[1]

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RECEIVED APRIL 11, 1961

A protected pentapeptide containing the C-terminal sequence of the A-chain of insulin, namely N-carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, has been prepared. Stepwise elongation of the peptide chain from the carboxyl toward the amino end using the *p*-nitrophenyl esters of the appropriate N-carbobenzoxyamino acids was employed mainly in the synthesis of this compound and its intermediates.

In the preceding paper[3] we reported the synthesis of certain peptides containing amino acid sequences found in the intra-chain ring region of the structure of the A-chain of sheep insulin postulated by Sanger, *et al.*[4]

In connection with our synthetic studies on peptides with amino acid sequences found in insulin, we would like to report the synthesis of the protected pentapeptide N-carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester. The sequence corresponding to this peptide is found in the C-terminal portion of the A-chain of insulin. For the synthesis of this pentapeptide and its intermediates the conventional methods of peptide chemistry were employed.

Stepwise elongation of the peptide chain from the carboxyl toward the amino end was the principal approach used. The appropriate N-carbobenzoxy amino acids served as the "carboxyl component" and they were activated by conversion to the corresponding *p*-nitrophenyl esters.[5] Since the use of HBr in acetic acid was unavoidable for the decarbobenzoxylation of the intermediate cysteine-containing peptides, the carboxyl group of the C-terminal amino acid, asparagine, was protected by conversion to its *p*-nitrobenzyl ester.

(1) This work was supported by a Senior Research Fellowship (SF-151) from the Public Health Service and by a grant (A-3067) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, for which we wish to express our appreciation.

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This group has been shown[6] to be relatively stable to the action of HBr in acetic acid.

N-Carbobenzoxy-L-asparagine[7] was treated with *p*-nitrobenzyl chloride in dimethylformamide in the presence of triethylamine to give N-carbobenzoxy-L-asparagine *p*-nitrobenzyl ester. Decarbobenzoxylation of this ester on exposure to HBr in acetic acid[8] and coupling of the resulting product with N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine *p*-nitrophenyl ester yielded the crystalline N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester.

N-Carbobenzoxy-L-asparagine *p*-nitrophenyl ester[5] was condensed with tyrosine ethyl ester to give N-carbobenzoxy-L-asparaginyl-L-tyrosine ethyl ester which on saponification gave N-carbobenzoxy-L-asparaginyl-L-tyrosine.

Decarbobenzoxylation of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester was effected by the action of HBr in acetic acid. The resulting product was coupled either with N-carbobenzoxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester[5] to give the tripeptide N-carbobenzoxy-O-benzyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester or with N-carbobenzoxy-L-asparaginyl-L-tyrosine to yield the tetrapeptide N-carbobenzoxy-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester. The latter coupling was effected using either the method of mixed anhydrides[9] or the carbodiimide procedure.[10] The same tetrapeptide also was prepared, in a higher yield, by coupling N-carbobenzoxy-L-asparagine *p*-nitrophenyl ester with the product obtained by the exposure of N-carbobenzoxy-O-benzyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester to HBr in acetic acid.

Decarbobenzoxylation of N-carbobenzoxy-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester by HBr in acetic acid and coupling of the resulting product with N-carbobenzoxy-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester[11] yielded the protected pentapeptide, N-carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester.

The chemical purity of the synthetic peptides was established by elemental analysis of the protected derivatives and by paper chromatography of the deblocked compounds. In the latter case, sharp single spots were obtained indicating the presence of a single component. The optical homogeneity of these peptides was established[12] by deblocking one of the intermediates and the final product and subsequently digesting the resulting compounds with leucine aminopeptidase (LAP). In both cases paper chromatographic analyses of the LAP digests revealed the presence of ninhydrin positive components with R_f values corresponding to the expected amino acids only. The absence of ninhydrin-positive components with R_f values corresponding to any of the intermediates suggests that digestion was complete. This implies that no racemization of the constituent amino acids had occurred during the synthetic processes.[13]

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Experimental

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

For paper chromatography, the protected amino acids and peptides were deblocked by treatment with HBr in acetic acid in the usual manner.[8] The resulting hydrobromides were chromatographed on paper, Whatman No. 1, using the Partridge system[14] and the R_f values are reported. Whatman No. 1 paper and the Partridge system also were used for the paper chromatography of the LAP-digests.

N-Carbobenzoxy-L-asparagine *p*-Nitrobenzyl Ester.—A solution of N-carbobenzoxy-L-asparagine (10.6 g.), triethylamine (9 ml.) and *p*-nitrobenzyl chloride (10.3 g.) in dimethylformamide (35 ml.) was heated at 65° for 3 hr., cooled at room temperature and poured into cold 0.2 *M* aqueous $KHCO_3$ (250 ml.). The precipitated product was filtered off, washed with water and cold methanol, dissolved in a warm mixture of methanol (500 ml.) and water (50 ml.) and treated with Norit A. The decolorized warm solution was mixed with hot water (200 ml.) and cooled. The precipitated crystalline product was isolated by filtration and dried; wt. 5.1 g. (32%); m.p. 167–168°, $[\alpha]^{28}D$ −63.3° (*c* = 0.98, dimethylformamide); R_f = 0.62.

Anal. Calcd. for $C_{19}H_{19}O_7N_3$: C, 56.9; H, 4.77; N, 10.3. Found: C, 57.0; H, 4.75; N, 10.5.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine *p*-Nitrophenyl Ester.—To a cold solution of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine[8] (16.0 g.) and *p*-nitrophenol (5.8 g.) in ethyl acetate (60 ml.) was added a solution of N,N′-dicyclohexylcarbodiimide (8.5 g.) in ethyl acetate (15 ml.). The reaction mixture was stirred for 30 minutes at 0° and for 3 hr. at room temperature. The precipitated N,N′-dicyclohexylurea was filtered off and washed with ethyl acetate. The combined filtrates were concentrated to a small volume *in vacuo* and then mixed with hot ethanol (150 ml.). After cooling the precipitated crystalline product was isolated by filtration, washed with cold ethanol and dried; wt. 11.9 g. (57%), m.p. 105–107°, $[\alpha]^{28}D$ −39.8° (*c* = 1.03, dimethylformamide).

Anal. Calcd. for $C_{24}H_{21}O_8N_3S$: C, 56.9; H, 4.13; N, 8.3. Found: C, 56.5; H, 4.23; N, 8.1.

N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester.—N-Carbobenzoxy-L-asparagine *p*-nitrobenzyl ester (7.0 g.) was suspended in acetic acid (20 ml.) and treated with 4 *N* HBr in acetic acid (20 ml.). After 2 hr. at room temperature dry ether (250 ml.) was added and the precipitated product was filtered off, washed with ether and dried over KOH *in vacuo*. This product was dissolved in dimethylformamide (90 ml.) and triethylamine (5.9 ml.) and the precipitated triethylamine hydrobromide was filtered off. To the filtrate N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteine *p*-nitrophenyl ester (8.4 g.) was added, the reaction mixture stored at room temperature for 24 hr. and subsequently poured into a 5% aqueous solution of $KHCO_3$ (200 ml.). The precipitated product was filtered off, washed successively with water, 1 *N* HCl and water and crystallized from dimethylformamide by addition of water; wt., 8.8 g. (80%), m.p. 220–223°. A sample for analysis was recrystallized twice from the same solvent; m.p. 225–227°, $[\alpha]^{28}D$ − 37.9° (*c* = 1.13, dimethylformamide); R_f = 0.65.

Anal. Calcd. for $C_{29}H_{31}O_{10}N_5S$: C, 54.4; H, 4.89; N, 11.1. Found: C, 54.2; H, 4.82; N, 11.0.

The protected peptide was treated with HBr in acetic acid and the resulting peptide ester hydrobromide (R_f = 0.65) was digested with LAP. Paper chromatography of the digest revealed the presence of two main ninhydrin-

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positive components with R_f 0.71 and 0.18, identical with the R_f's of authentic samples of S-*p*-nitrobenzyl-L-cysteine and L-asparagine, respectively. A trace of a third ninhydrin-positive component with R_f identical with that of aspartic acid (0.22) also was present.[15]

N-Carbobenzoxy-O-benzyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester.—N-Carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (6.16 g.) was suspended in a mixture of acetic acid (30 ml.) and 4 *N* HBr in acetic acid (30 ml.). After 2 hr. at room temperature dry ether (400 ml.) was added and the precipitated hydrobromide was isolated by filtration, washed with ether and dried over KOH *in vacuo*. The dried product was dissolved in dimethylformamide (60 ml.), triethylamine (4 ml.) was added, and finally N-carbobenzoxy-O-benzyl-L-tyrosine *p*-nitrophenyl ester[5] (4.7 g.). After 24 hr. at room temperature the reaction mixture was poured into 5% aqueous $KHCO_3$ (350 ml.). The precipitated product was filtered off, washed successively with 1 *N* $KHCO_3$, water, 1 *N* HCl, and water and dried; wt. 7.2 g. (90%), m.p. 209–211°. A sample for analysis was reprecipitated from acetic acid by addition of water; the m.p. remained unchanged; $[\alpha]^{28}D$ −28.5° (*c* = 1.08, dimethylformamide); R_f = 0.81.

Anal. Calcd. for $C_{45}H_{44}O_{12}N_6S$: C, 60.5; H, 4.97; N, 9.4. Found: C, 60.5; H, 5.18; N, 9.7.

N-Carbobenzoxy-L-asparaginyl-L-tyrosine Ethyl Ester.—A solution of tyrosine ethyl ester hydrochloride (13.2 g.) in dimethylformamide (48 ml.) was treated with triethylamine (7.3 ml.) and the precipitated triethylamine hydrochloride was filtered off. To the filtrate N-carbobenzoxy-L-asparagine *p*-nitrophenyl ester (15.9 g.) was added, the reaction mixture stirred at room temperature for 2 hr. and poured into water (500 ml.). After storing several hours in the refrigerator the precipitated product was filtered off and crystallized from aqueous ethanol; wt. 13.8 g. (77%), m.p. 175°, $[\alpha]^{28}D$ + 0.005° (*c* = 1.01 dimethylformamide); R_f = 0.59. The same compound also was prepared in 24% yield by the mixed anhydride method using isovaleryl chloride.

Anal. Calcd. for $C_{23}H_{27}N_3O_7$: C, 60.4; H, 5.94; N, 9.2. Found: C, 60.7; H, 5.96; N, 9.0.

Carbobenzoxy-L-asparaginyl-L-tyrosine.—To a suspension of N-carbobenzoxy-L-asparaginyl-L-tyrosine ethyl ester (9.16 g.) in ethanol (20 ml.) was added 2 *N* NaOH (22 ml.). After 30 minutes the resulting solution was acidified with 6 *N* HCl and concentrated to one half of its volume *in vacuo*. The product which was precipitated upon cooling was filtered off, washed with water and recrystallized from methanol by the addition of water; wt. 6.75 g. (80%), m.p. 206°, $[\alpha]^{28}D$ + 16.6° (*c* = 1.0, dimethylformamide).

Anal. Calcd. for $C_{21}H_{23}N_3O_7$: C, 58.7; H, 5.39; N, 9.8. Found: C, 58.6; H, 5.45; N, 9.5.

N-Carbobenzoxy-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester. A. Mixed Anhydride Procedure.—A suspension of N-carbobenzoxy-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (0.62 g.) in acetic acid (2 ml.) was treated with 4 *N* HBr in acetic acid (2 ml.). After 2 hr. at room temperature dry ether was added and the precipitated hydrobromide was isolated by filtration, washed with ether and dried over KOH *in vacuo*. This product was dissolved in dimethylformamide (2 ml.) containing triethylamine (0.4 ml.), stirred 10 minutes and the precipitated triethylamine hydrobromide was filtered off. The filtrate was added to the mixed anhydride prepared as follows: A solution of N-carbobenzoxy-L-asparaginyl-L-tyrosine (0.41 g.) and triethylamine (0.14 ml.) in dimethylformamide (5 ml.) was cooled to −5° and isobutyl chlorocarbonate (0.12 ml.) was added. After 30 minutes the solution of S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, prepared as described above, was added. The reaction mixture was stirred at −5° for 1 hr. and after storing overnight at room temperature was poured into cold *N* HCl (50 ml.). The precipitated product was filtered off, washed successively with water, 1 *N* $KHCO_3$ and water and reprecipitated from aqueous acetic acid; wt. 0.40 g. (47%), m.p. 213–216°, $[\alpha]^{28}D$ −44.9° (*c* = 1.04, dimethylformamide); R_f = 0.58.

Anal. Calcd. for $C_{42}H_{44}O_{14}N_8S$: C, 55.0; H, 4.84; N, 12.2. Found: C, 54.8; H, 4.98; N, 12.1.

B. Carbodiimide Procedure.—To a cold solution of S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (prepared as described previously from its N-carbobenzoxy derivative (0.4 g.)) and carbobenzoxy-L-asparaginyl-L-tyrosine (0.21 g.) in dimethylformamide (4 ml.) was added a solution of N,N′-dicyclohexylcarbodiimide (0.13 g.) in dimethylformamide (3 ml.). After 24 hr. the precipitated N,N′-dicyclohexylurea was filtered off and the filtrate was added to cold *N* HCl (50 ml.). The precipitated product subsequently was treated as described in A; wt. 0.1 g., m.p. 213–216°.

C. *p*-Nitrophenyl Ester Procedure.—N-Carbobenzoxy-O-benzyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (7.21 g.) was suspended in 2 *N* HBr in acetic acid (80 ml.). After 1 hr. at room temperature dry ether (400 ml.) was added and the precipitate which was formed was filtered off, washed with ether and dried over KOH *in vacuo*. This product was dissolved in dimethylformamide (60 ml.), triethylamine (3 ml.) added, followed by N-carbobenzoxy-L-asparagine *p*-nitrophenyl ester (2.82 g.). After 24 hr. at room temperature the reaction mixture was poured into cold 1 *N* $KHCO_3$ (300 ml.), and the precipitated product was isolated by filtration and washed successively with water, 1 *N* HCl, and water; wt. 6.0 g., m.p. 206–210°. On reprecipitation from aqueous acetic acid, 4.7 g. (75%) was obtained, m.p. 213–216°.

N-Carbobenzoxy-γ-benzyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-Nitrobenzyl Ester.—N-Carbobenzoxy-L-asparaginyl-L-tyrosyl-S-*p*-nitrobenzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (1.7 g.) was suspended in acetic acid (7.5 ml.) and treated with 4 *N* hydrobromic acid in acetic acid (7.5 ml.). After 1.5 hr. at room temperature dry ether was added and the precipitated product was filtered off, washed with ether and dried over KOH *in vacuo*. This material was dissolved in dimethylformamide (10 ml.), triethylamine (0.27 ml.) added and the precipitated triethylamine hydrobromide filtered off. N-Carbobenzoxy-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester (0.62 g.) was added to the filtrate and after 24 hr. at room temperature the reaction mixture was poured into 1 *N* $KHCO_3$ (100 ml.). The precipitated product was filtered off, washed successively with water, 1 *N* HCl and water, and dried; wt. 1.77 g., m.p. 204–207°. After reprecipitation from aqueous acetic acid 1.0 g. (47.5%) of pentapeptide was obtained; m.p. 218–219°, $[\alpha]^{28}D$ −33.3° (*c* = 1.06, dimethylformamide); R_f = 0.62.

Anal. Calcd. for $C_{54}H_{57}O_{17}N_9S$: C, 57.1; H, 5.06; N, 11.1. Found: C, 56.5; H, 5.08; N, 11.1.

The protected pentapeptide was deblocked by treatment with HBr in acetic acid and the resulting peptide ester hydrobromide (R_f = 0.62) was digested with LAP. Paper chromatography of the digest exhibited five ninhydrin-positive spots with R_f's 0.26, 0.46, 0.71, 0.18 and 0.22, identical with the R_f's of authentic samples of glutamic acid, tyrosine, S-*p*-nitrobenzyl-L-cysteine, asparagine and aspartic acid,[14] respectively.

Acknowledgments.—The enzymatic analyses reported in this work were performed by Mrs. C. Yanaihara to whom we wish to express our appreciation.

(15) This is in accordance with the findings of R. L. Hill and E. L. Smith [*J. Biol. Chem.*, **228**, 577 (1957)] who reported that during LAP digestion of the A and B chains of insulin some asparagine was hydrolyzed to aspartic acid.