Insulin Peptides. XIV. Synthetic Peptide Derivatives Related to the N-Terminus of the A Chain of Sheep Insulin (Positions 1–9)[1,2]

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Abstract: A synthesis is described of the methyl and ethyl ester of the nonapeptide N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine. Exposure of either of these esters to hydrazine produced the nonapeptide hydrazide. This derivative contains the nine amino acid residues found at the N-terminus of the A chain of sheep insulin.

In a previous communication[3] we have reported the synthesis of a partially protected nonapeptide with the carboxyl group of its C-terminal amino acid residue unprotected, which contains the amino acid sequence found at the amino-terminal end of the A chain of sheep insulin (positions 1–9). This nonapeptide fragment was subsequently condensed by the N,N^1-carbonyldiimidazole method with a dodecapeptide derivative containing the carboxyl-terminal amino acid sequence of the A chain of sheep insulin (positions 10–21) to give the protected form of that chain.[4] However, the over-all yield for the synthesis of the N-terminal partially protected nonapeptide fragment was not very satisfactory. Furthermore, we felt that alternative routes for synthesis of the protected sheep insulin A chain were desirable. Consequently, we decided to prepare the amino-terminal nonpeptide fragment by a different route and attempt its condensation with the carboxyl-terminal dodecapeptide subunit by another procedure.

Application of the azide method for coupling large peptide subunits gave very favorable results in our laboratory in the synthesis of the B chain of sheep insulin[5] and in the synthesis of the A and B chain of human insulin.[2,6] We have thus decided to use the azide procedure also for the coupling of the nonapeptide and dodecapeptide fragments in the construction of the protected form of the sheep insulin A chain. The details of this alternative synthesis are given in the following communication,[7] and the preparation of a key intermediate used for its execution is described in the present report. This intermediate is the hydrazide of N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine (XI), the nonapeptide subunit which contains the amino acid sequence found at the amino terminus of the A chain of sheep insulin.

The synthesis of this nonapeptide derivative, which is illustrated in Chart I, involves the condensation of an activated protected tetrapeptide subunit with a partially protected pentapeptide subunit. Thus coupling of N-carbobenzoxyglcyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamic acid azide (VIIIa) with L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine methyl ester (IVa) afforded the fully protected nonapeptide IX, from which, after removal of the γ-*t*-butyl ester group and exposure to hydrazine hydrate, the desired partially protected nonapeptide hydrazide XI was obtained.

Interaction of N-carbobenzoxy-L-valine *p*-nitrophenyl ester[8] with the product obtained by hydrogenolysis of N-carbobenzoxy-L-glutamic acid α-methyl γ-*t*-butyl ester[9] yielded N-carbobenzoxy-L-valyl-L-glutamic acid α-methyl γ-*t*-butyl ester (V) which after hydrogenolysis gave L-valyl-L-glutamic acid α-methyl γ-*t*-butyl ester (Va). The dipeptide N-carbobenzoxyglycyl-L-isoleucine hydrazide (VI), whose physical characteristics have been reported by Wang, *et al.*,[10] was prepared by treating N-carbobenzoxyglycine *p*-nitrophenyl ester[8] with L-isoleucine methyl ester and then exposing the resulting product to hydrazine hydrate. Conversion of this hydrazide to the corresponding azide (VIa) and coupling of the latter compound with Va yielded the crystalline tetrapeptide N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid α-methyl γ-*t*-butyl ester (VII) which by treatment with hydrazine afforded the crystalline hydrazide VIII.

The stepwise *p*-nitrophenyl ester method[11,12] was employed to prepare the protected pentapeptide IV. N-Carbobenzoxy-L-alanylglycine methyl ester (I) was decarbobenzoxylated with HBr in acetic acid, and the resulting derivative Ia was condensed with the *p*-nitrophenyl ester of N-carbobenzoxy-S-benzyl-L-cysteine[12] (Ib) to give the protected tripeptide II. Deblocking of II with HBr in acetic acid afforded IIa which was converted to the protected tetrapeptide III upon reaction with Ib. On exposure to HBr in acetic acid the blocked tetrapeptide III was converted to derivative IIIa bearing a free amino group which in turn was treated with the *p*-nitrophenyl ester of N-carbobenzoxy-L-glutamine[12] (IIIb) to give the protected pentapeptide IV. The latter compound and all

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(2) A preliminary report describing some of the results presented in this paper has appeared: P. G. Katsoyannis, A. Tometsko, and C. Zalut, *J. Am. Chem. Soc.*, **88**, 166 (1966).

(3) P. G. Katsoyannis, K. Fukuda, and A. Tometsko, *ibid.*, **85**, 1681 (1963).

(4) P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *ibid.*, **85**, 2863 (1963).

(5) P. G. Katsoyannis, K. Fukuda, A. Tometsko, K. Suzuki, and M. Tilak, *ibid.*, **86**, 930 (1964).

(6) P. G. Katsoyannis, A. Tometsko, J. Ginos, and M. Tilak, *ibid.*, **88**, 164 (1966).

(7) P. G. Katsoyannis, A. Tometsko, C. Zalut, and K. Fukuda, *ibid.*, **88**, 3625 (1966).

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

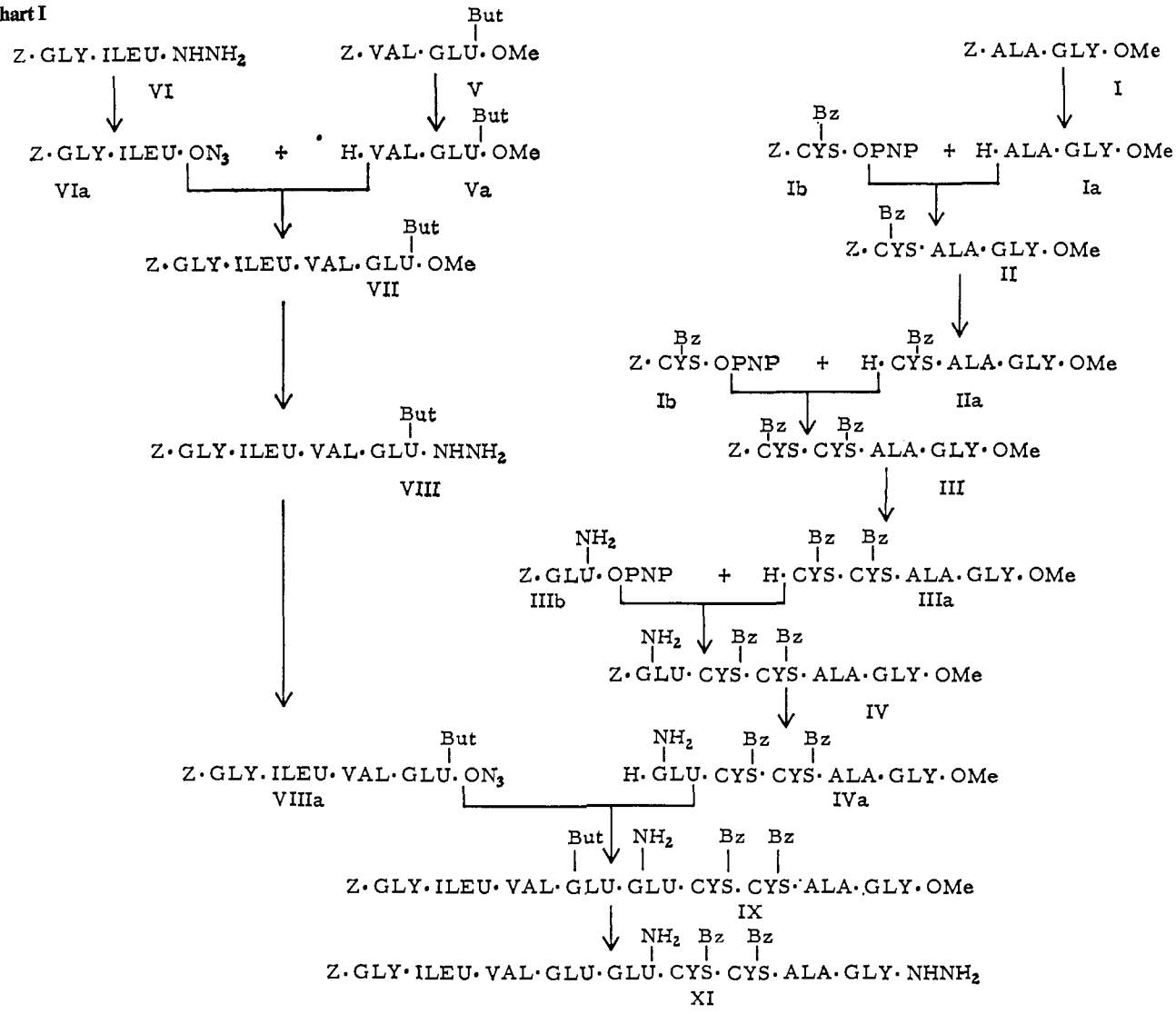
(9) R. Schwyzer and H. Kappeler, *ibid.*, **44**, 1991 (1961).

(10) T. Wang, J. Hsu, W. Chang, L. Cheng, C. Hsing, A. Chi, T. Loh, C. Li, P. Shi, and Y. Yieh, *Sci. Sinica* (Peking), **13**, 2030 (1964).

(11) M. Bodanszky, *Nature*, **175**, 685 (1955).

(12) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

Chart I



the protected intermediate peptides were obtained in crystalline form and in yields ranging from 75 to 97%.

Removal of the carbobenzoxy group from the protected pentapeptide IV and coupling of the ensuing product IVa with the tetrapeptide azide VIIIa, which in turn was prepared from the corresponding hydrazide VIII, gave the fully protected nonapeptide methyl ester IX in 83% yield. Starting with the protected pentapeptide ethyl ester[3] and following the same route we obtained the fully protected nonapeptide ethyl ester (X) in 73% yield. The γ-*t*-butyl ester group from the glutamic acid residue in IX and X was removed by exposing these compounds to trifluoroacetic acid.[13,14] Interaction of either one of the partially protected nonapeptides thus obtained with hydrazine hydrate yielded the desired nonapeptide hydrazide XI in 73% yield.

Experimental Section[15]

N-Carbobenzoxy-L-alanylglycine Methyl Ester (I). To a solution of glycine methyl ester hydrochloride (12.5 g) and triethylamine (14 ml) in DMF (150 ml) was added N-carbobenzoxy-L-alanine *p*-nitrophenyl ester[16] (32 g). After stirring for 24 hr the reaction mixture was poured into ethyl acetate (800 ml) and water (200 ml). The organic layer was separated, washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water, and dried over $MgSO_4$. The crystalline product formed on removal of the ethyl acetate *in vacuo* was recrystallized from ethyl acetate–petroleum ether; wt, 19.6 g (75%); mp, 99°; $[\alpha]^{27}D$ −1.9° (*c* 1, DMF); R_f^1 0.42; R_f^2 3.3 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{14}H_{18}N_2O_5$: C, 57.1; H, 6.12; N, 9.5. Found: C, 57.2; H, 6.14; N, 9.5.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-alanylglycine Methyl Ester (II). N-Carbobenzoxy-L-alanylglycine methyl ester (I, 39 g) was dissolved in methanol (400 ml) containing concentrated HCl (11 ml) and hydrogenated for 2 hr over 10% palladium–charcoal catalyst (6 g). The catalyst was filtered off, and the filtrate was evaporated to dryness *in vacuo*. The remaining product was dried by the addition of methanol followed by evaporation under reduced pressure. To a solution of the residue in DMF (500 ml) triethylamine (18 ml) was added followed by N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester (58.3 g). After 24 hr at room temperature the reaction mixture was poured into cold 1 *N* NH_4OH (1000 ml). The crystalline precipitate was collected by filtration, washed successively with 1 *N* NH_4OH (until free of the yellow color), water, 1 *N* HCl, and water, and dried; wt, 58.8 g (96%); mp, 180°. A sample for analysis was recrystallized from ethyl acetate; melting point unchanged; $[\alpha]^{27}D$ −17.4° (*c* 1, DMF); R_f^1 0.74; R_f^2 4.9 × His; single ninhydrin-positive spot.

(13) L. A. Carpino, *J. Am. Chem. Soc.*, 79, 98 (1957).

(14) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, *Angew. Chem.*, 72, 915 (1960).

(15) The analytical procedures used were those described in the preceding paper (XIII; *J. Am. Chem. Soc.*, 88, 5618 (1966)). R_f^1 refers to the Partridge system and R_f^2 to the pyridine system (see paper XIII). The following abbreviations are used: Z = carbobenzoxy; Bz = benzyl; But = γ-*t*-butyl; PNP = *p*-nitrophenyl; Me = methyl; DMF = dimethylformamide; DMSO = dimethyl sulfoxide.

(16) M. Goodman and K. C. Stueben, *J. Am. Chem. Soc.*, 81, 3980 (1959).

Anal. Calcd for $C_{24}H_{29}N_3O_6S$: C, 59.1; H, 5.95; N, 8.6. Found: C, 59.1; H, 5.99; N, 8.8.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Methyl Ester (III). A suspension of II (24.3 g) in acetic acid (75 ml) was treated with 4 *N* HBr in acetic acid (75 ml). After 1 hr the reaction mixture was poured into anhydrous ether (1000 ml). The precipitated product was filtered off, washed with ether, dried over KOH *in vacuo*, and dissolved in DMF (200 ml). To this solution triethylamine (11 ml) was added followed by N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester (21.4 g). After 24 hr the reaction mixture was poured into cold 1 *N* NH_4OH (800 ml). The precipitated crystalline product was filtered off, washed successively with 1 *N* NH_4OH, water, and 1 *N* HCl, and dried; wt, 30 g (96%); mp, 194–195°. The melting point remained unchanged on recrystallization from dimethylformamide–water; $[\alpha]^{27}D$ −29.9° (*c* 1, DMF); R_f^1 0.88; R_f^2 5.4 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{34}H_{40}N_4O_7S_2$: C, 60.0; H, 5.88; N, 8.2. Found: C, 59.8; H, 6.10; N, 8.3.

N-Carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Methyl Ester (IV). Compound III (13.6 g) was suspended in acetic acid (35 ml) and 4 *N* HBr in acetic acid (35 ml). After 1 hr the resulting solution was poured into anhydrous ether (500 ml). The precipitated hydrobromide was isolated by filtration, washed with ether, and dried over KOH *in vacuo*. This material was dissolved in DMF (165 ml), and triethylamine (4.3 ml) was added followed by N-carbobenzoxy-L-glutamine *p*-nitrophenyl ester (7.4 g). The reaction mixture was stirred for 24 hr and then poured into cold 1 *N* NH_4OH (300 ml). The crystalline precipitate was filtered off, washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water, and dried; wt, 14.1 g (97%); mp 248–250°. For analysis a sample was recrystallized from dimethylformamide–water; melting point unchanged; $[\alpha]^{27}D$ −34.2° (*c* 1, DMF); R_f^1 0.78; R_f^2 4.9 × His; single ninhydrin-positive spot; amino acid ratios in acid hydrolysate: $Glu_{1.0}$-$Gly_{1.0}Ala_{1.1}$S-benzylcysteine$_{2.0}$.

Anal. Calcd for $C_{39}H_{48}N_6O_9S_2$: C, 57.9; H, 5.94; N, 10.4. Found: C, 57.9; H, 6.00; N, 10.4.

N-Carbobenzoxy-L-valyl-L-glutamic Acid α-Methyl γ-*t*-Butyl Ester (V). N-Carbobenzoxy-L-glutamic α-methyl γ-*t*-butyl ester (28 g) was dissolved in methanol (400 ml) and hydrogenated for 2 hr in the presence of 10% palladium–charcoal catalyst (6.5 g). The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The oily residue (15.9 g) was dissolved in DMF (100 ml) and to this solution N-carbobenzoxy-L-valine *p*-nitrophenyl ester (26.8 g) was added followed by a few drops of triethylamine to ensure basicity. After 3 days at room temperature the reaction mixture was poured into ethyl acetate (1000 ml) and 1 *N* NH_4OH (200 ml). The organic layer was washed successively with water, 1 *N* NH_4OH, water, 1 *N* acetic acid, and water, and dried. After removing the ethyl acetate the remaining product was crystallized from aqueous methanol; wt, 30.6 g (94%); mp 76–78°[17]; $[\alpha]^{27}D$ −5.8° (*c* 1, DMF), −30.5° (*c* 1, methanol) (lit.[18] mp 90°; $[\alpha]^{25}D$ −29.5° (*c* 1, methanol)); after hydrogenolysis: R_f^1 0.65; R_f^2 4.04 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{23}H_{34}N_2O_7$: C, 61.3; H, 7.55; N, 6.2. Found: C, 61.0; H, 7.70; N, 6.2.

N-Carbobenzoxyglycyl-L-isoleucine Hydrazide (VI). To a solution of L-isoleucine methyl ester hydrochloride (21.2 g) in DMF (100 ml) containing triethylamine (16 ml) was added N-carbobenzoxyglycine *p*-nitrophenyl ester (38.7 g). After 24 hr the reaction mixture was poured into ethyl acetate (800 ml) and water (200 ml). The organic layer was washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water, dried, and concentrated to dryness *in vacuo*. A solution of the remaining oily product (38 g) in methanol (150 ml) and hydrazine hydrate (12 ml) was refluxed for 4 hr and stirred at room temperature for 24 hr. The precipitated crystalline hydrazide was filtered off and washed with cold methanol; wt, 18 g, mp 196–197° (lit.[10] mp 198–200°). An additional amount of hydrazine hydrate (2 ml) was added to the combined filtrates from which, after 24 hr, a second crop of crystalline hydrazide was obtained; wt, 14 g; mp 195°. Total yield was 32 g (84%).

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic Acid α-Methyl γ-*t*-Butyl Ester (VII). A solution of V (9 g) in methanol (200 ml) was hydrogenated for 2 hr over 10% palladium–charcoal catalyst (2.5 g). The catalyst was filtered off and the filtrate was concentrated to dryness. The remaining oily product was dissolved in ethyl acetate (80 ml), cooled to 0°, and then mixed with a solution of the dipeptide azide prepared as follows: VI (7.5 g) was dissolved in DMF (50 ml) containing 2 *N* HCl (25 ml). After cooling this solution to −15°, $NaNO_2$ (1.66 g) dissolved in cold water (4 ml) was added. The reaction mixture was stirred at −15° for 5 min and then poured into precooled ethyl acetate (150 ml). The organic layer was washed quickly with ice-cold water, 1 *N* $NaHCO_3$, and water, dried over $MgSO_4$, and added to the solution of the amino component which was prepared as described previously. The reaction mixture was stirred at 0° for 48 hr. The precipitated crystalline tetrapeptide derivative was isolated by filtration and recrystallized from aqueous methanol; wt, 9.1 g (73%); mp 171–174°; $[\alpha]^{27}D$ −13.2° (*c* 1, DMF) (lit.[18] mp 202–203°; $[\alpha]^{25}D$ −13.2° (*c* 2, DMF)); after hydrogenolysis: R_f^1 0.86; R_f^2 4.81 × His.

Anal. Calcd for $C_{31}H_{48}N_4O_9$: C, 60.0; H, 7.74; N, 9.0. Found: C, 59.8; H, 7.86; N, 9.5.

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamic Acid Hydrazide (VIII). A solution of VII (8.7 g) in methanol (300 ml) was treated with hydrazine hydrate (15 ml). After 48 hr at room temperature the precipitated crystalline tetrapeptide hydrazide was filtered off, washed with methanol, and recrystallized from dimethylformamide–water; wt, 5.7 g (65%); mp 243–244°; $[\alpha]^{27}D$ −14° (*c* 1, DMF) (lit.[18] mp 243–244°; $[\alpha]^{25}D$ −13.8° (*c* 1, DMF)).

Anal. Calcd for $C_{30}H_{48}N_6O_8$: C, 58.1; H, 7.74; N, 13.6. Found: C, 57.7; H, 7.96; N, 14.0.

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Methyl Ester (IX). A suspension of IV (1 g) in acetic acid (10 ml) was treated with 4 *N* HBr in acetic acid (10 ml). After 1 hr the reaction mixture was poured into anhydrous ether (250 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 (20 ml) triethylamine (0.5 ml) was added followed by the tetrapeptide azide prepared as follows. A suspension of VIII (1 g) in DMF (25 ml) was cooled to −15° (Dry Ice–acetone) and brought into solution by the addition of 2 *N* HCl (2 ml). To this solution $NaNO_2$ (110 mg) in water (1 ml) was added. After 5 min at −15° the reaction mixture was poured into cold (0°) half-saturated NaCl (200 ml), and the precipitated tetrapeptide azide was isolated by filtration, washed with cold water, and dried at 0° for 1 hr over P_2O_5 *in vacuo*. This azide was then added to the solution of the amino component IVa which was prepared as described previously. The reaction mixture was stirred at 0° for 48 hr and then poured into methanol (200 ml). The precipitated product was isolated by filtration washed with methanol and water, and dried; wt, 1.3 g (83%); mp, 265–267° dec. A sample for analysis was reprecipitated from dimethylformamide–water; mp 268–269° dec; $[\alpha]^{27}D$ −32.2° (*c* 1, DMSO); R_f^1 0.74; R_f^2 4.3 × His; amino acid ratios in acid hydrolysate $Glu_{2.1}Gly_{2.1}Ala_{1.0}Val_{0.8}Ile_{0.7}$S-benzylcysteine$_{1.9}$.

Anal. Calcd for $C_{61}H_{86}N_{10}O_{15}S_2$: C, 58.0; H, 6.81; N, 11.1. Found: C, 58.0; H, 6.73; N, 11.2.

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Ethyl Ester (X). Starting with N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine ethyl ester (8 g) and VIII (7.6 g) and following the procedure described for the preparation of compound IX, 9 g (73%) of the nonapeptide ethyl ester (X) were obtained; mp 274° dec; $[\alpha]^{22}D$ −29.6° (*c* 1, DMSO); R_f^1 0.86; R_f^2 4.1 × His; amino acid ratios in acid hydrolysate: $Glu_{2.0}Gly_{2.0}Ala_{1.0}Val_{0.7}Ile_{0.6}$S-benzylcysteine$_{1.8}$.

Anal. Calcd for $C_{62}H_{88}N_{10}O_{15}S_2$: C, 58.3; H, 6.89; N, 11.0. Found: C, 58.5; H, 7.03; N, 11.2.

N-Carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-alanylglycine Hydrazide (XI). Compound IX (3 g) was dissolved in trifluoroacetic acid (30 ml). After 30 min this solution was diluted with anhydrous ether (200 ml) and the precipitated product was collected by filtration, washed with ether, and dried over KOH *in vacuo*. A solution of this solid in DMF (250 ml) containing hydrazine hydrate (10 ml) was stirred for 24 hr at 42° and for 48 hr at 25°. The reaction mixture was then poured into water (600 ml) and the pH of the mixture adjusted to 6 with acetic acid. The

(17) In the original synthesis of this product mp 91–93° was obtained (see ref 2). However, in all the subsequent preparations mp 76–78° was obtained. While this manuscript was in preparation we noticed that Kung, *et al.* (*Sci. Sinica* (Peking), **15**, 222 (1966)), have obtained for the same compound mp 76–78°.

(18) H. Zahn, H. Bremer, N. Sroka, and J. Meienhofer, *Z. Naturforsch.*, **20b**, 646 (1965).

precipitated hydrazide was isolated by filtration, washed with water, dried, and reprecipitated from dimethyl sulfoxide–water; wt, 2.2 g (73%); mp 275° dec; $[\alpha]^{27}D$ −27.5° (*c* 1, DMSO).

Anal. Calcd for $C_{56}H_{78}N_{12}O_{14}S_2$: C, 55.7; H, 6.47; N, 13.9. Found: C, 55.3; H, 6.78; N, 13.7.

This hydrazide was also prepared in 73% yield if instead of the nonapeptide methyl ester the corresponding ethyl ester (compound X) was used.

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Insulin Peptides. XV. The Synthesis of the A Chain of Sheep Insulin and Its Combination with Synthetic or Natural B Chain to Produce Insulin[1,2]

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Abstract: The A chain of sheep insulin has been synthesized and isolated in a highly purified form as the S-sulfonated derivative. The key intermediate in this synthesis was the partially protected heneicosapeptide that embodies the amino acid sequence of the sheep insulin A chain. This intermediate was prepared by three routes: (a) the carbonyldiimidazole coupling of the N-terminal nonapeptide subunit with the C-terminal dodecapeptide subunit, (b) the azide coupling of a differently blocked derivative of the N-terminal nonapeptide fragment with the C-terminal dodecapeptide fragment, and (c) most efficiently by the azide coupling of the N-terminal tetrapeptide subunit with the C-terminal heptadecapeptide subunit. The partially protected heneicosapeptide obtained by any one of these procedures was converted to the A chain S-sulfonate by removing the protecting groups and sulfitolyzing the ensuing product. Purification of the crude material was accomplished by chromatography on Sephadex. The purified A chain S-sulfonate thus obtained was homogeneous as judged by paper chromatography, paper electrophoresis, and amino acid analysis and optically pure as judged by complete digestion with leucine aminopeptidase. The synthetic product was compared with natural bovine A chain S-sulfonate as to electrophoretic mobility in two pH values, paper chromatography in two solvent systems, chromatographic pattern on Sephadex G-50, and infrared pattern. In all these comparisons the synthetic sheep A chain S-sulfonate and the natural bovine A chain S-sulfonate exhibited an identical behavior. The synthetic A chain was converted to its sulfhydryl form and combined with the natural bovine B chain S-sulfonate. Upon this combination hybrid insulin (one chain synthetic, the other chain natural) was produced in yields ranging from 30 to 38% of theory based on the amount of the B chain S-sulfonate used. Finally, the sulfhydryl form of the synthetic A chain was combined with a synthetic preparation of sheep insulin B chain S-sulfonate. As a result of this combination all-synthetic sheep insulin was produced in yields ranging from 4.7 to 5.7%.

Pioneering studies by Sanger and co-workers, which marked the beginning of a new era in the structural chemistry of proteins, have led to the elucidation of the complete amino acid sequence and subsequently the over-all structure of insulin from various species.[3] Structure I was postulated for sheep insulin.

It may be pointed out that this protein is made up of two polypeptide chains, the A chain containing 21 amino acid residues and the B chain containing 30 amino acid residues, linked together by two disulfide bridges. In addition there is an intra-chain disulfide bridge in the A chain.

Concurrently with the strides in structural protein analysis, there has been a comparable development in the synthetic field.[4] Impressive advances in peptide synthetic methodology, in purification techniques, and in methods of assessing chemical and stereochemical homogeneity have made the synthesis of large polypeptide chains attainable.[5] On this basis, we undertook the synthesis of insulin, making the assumption that air oxidation of a mixture of the sulfhydryl forms of chemically synthesized A and B chains should generate insulin.[5,6] This assumption was verified with natural insulin chains even before the completion of our synthetic work. Dixon and Wardlaw[7] and shortly thereafter Du, *et al.*,[8] reported the cleavage of insulin to its two chains by oxidative sulfitolysis, namely by treatment with sodium sulfite in the pres-

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(2) Preliminary report of part of the results described in this paper has appeared: P. G. Katsoyannis, A. Tometsko, and K. Fukuda, *J. Am. Chem. Soc.*, **85**, 2863 (1963).

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