Insulin Peptides. XIX. The Synthesis of Two Nonapeptide Derivatives Related to the N Terminus of the B Chain of Insulin from Various Species (Positions 1–9)[1,2]

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Abstract: A synthesis is described of the hydrazides of *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine and *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine. These derivatives contain the nine amino acid residues found at the N terminus of the B chain of insulin from various species.

In previous reports[3,4] we have described the synthesis of polypeptide derivatives with amino acid sequences found at the carboxyl terminus of the B chain of insulin from various species. Specifically in the preceding paper[4] the synthesis of the C-terminal heneicosapeptide derivative is presented (positions 10–30). In the present paper we describe the synthesis of peptide derivatives with amino acid sequences found at the amino terminus of the insulin B chain. These derivatives include a fully protected and a partially protected nonapeptide fragment (positions 1–9).

The amino acid sequence at the amino terminus of the B chain of insulin from several species[5,6] is phenylalanylvalylasparaginylglutaminylhistidylleucylcysteinylglycylserine. The present study relates synthetic routes to the fully protected nonapeptide *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine hydrazide (X) and the partially protected derivative *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine hydrazide (XVII). Both these derivatives were used for the synthesis of the B chain of sheep[2,7] and human[8,9] insulin and eventually for the total synthesis of these proteins.[10]

The synthesis of these nonapeptide derivatives, which is summarized in Chart I, involves the condensation of the C-terminal tetrapeptide fragment with the respective N-terminal pentapeptide fragment. The common intermediate for the synthesis of the two nonapeptide derivatives, namely the C-terminal tetrapeptide *N*-benzyloxycarbonyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine methyl ester (III) was prepared stepwise starting with the dipeptide *N*-benzyloxycarbonylglycyl-L-serine methyl ester (I). The *p*-nitrophenyl ester method[11,12] was employed for the activation of the benzyloxycarbonylamino acids used at each synthetic step. Decarbobenzoxylation of the intermediate derivatives was effected by HBr in trifluoroacetic acid.

The stepwise *p*-nitrophenyl ester method was also employed for the construction of the N-terminal pentapeptide fragments *N*-benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidine benzyl ester (VII) and its partially protected methyl ester derivative XIV in which the histidine residue is unprotected. Decarbobenzoxylation of the intermediate protected peptides during the synthetic steps in the construction of VII was carried out by HBr in acetic acid and in the construction of XIV by catalytic hydrogenation.

On exposure to NaOH the protected pentapeptide VII is converted to the partially blocked derivative VIII, bearing a free carboxyl group. The latter product was subsequently condensed by the *N,N'*-dicyclohexylcarbodiimide method,[13] with L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine methyl ester, which was obtained by decarbobenzoxylation of the protected derivative III, to give the fully protected nonapeptide methyl ester IX. This coupling step is a point where racemization could have occurred. However, as is described in the Experimental Section, the hydrazide X which is derived from the protected nonapeptide IX is digested completely by aminopeptidase M (APM). This implies that the optical purity of the constituent amino acids was preserved during the synthesis of the nonapeptide derivative. For the synthesis of the nonapeptide derivative XVI, bearing an unprotected histidine residue, the pentapeptide ester XIV is converted to the hydrazide XV. The latter material is transformed to the corresponding azide by the Rudinger procedure,[14] and this in turn upon interaction with the product obtained by decarbobenzoxylation of the protected tetrapeptide III, affords the desired partially protected nonapeptide XVI. On exposure to hydrazine hydrate, the nonapeptide derivatives IX and XVI are converted to the respective hydrazides X and XVII. The chemical purity of these hydrazides was

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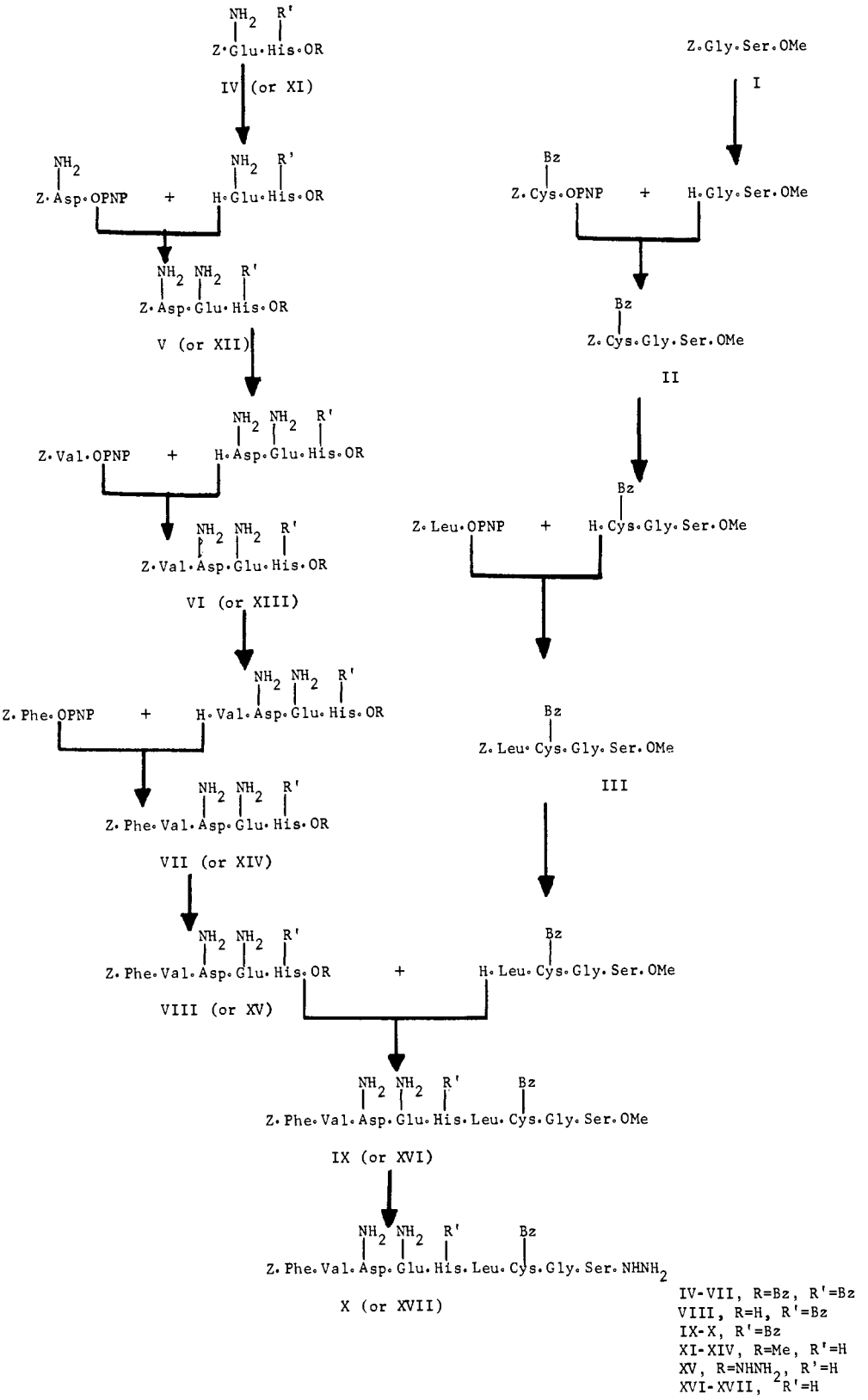
Chart I



established by elemental analysis and amino acid analysis of the respective acid hydrolysates. The stereochemical purity of both hydrazides was established by complete digestibility with APM.

Experimental Section

Melting points for all compounds were taken in capillary tubes and are not corrected.

For paper chromatography the protected peptides were deblocked by treatment with 2 *N* HBr in acetic acid, or in certain instances, as indicated in the experimental part, by HBr in trifluoroacetic acid or by catalytic hydrogenation and the resulting salts were chromatographed on Whatman No. 1 filter paper. R_f^1 values refer to the Partridge system;[15] R_f^2 values refer to the system[16] 1-butanol–pyridine–acetic acid–water, 30:30:6:24, and

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are expressed as a multiple of the distance traveled by a histidine marker; R_f[3] values refer to the system[17] 2-butanol–ammonia. Enzymatic analyses (APM) were performed according to the procedure of Pfleiderer, *et al.*[18] APM was purchased from Henley and Co., New York, N.Y. 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, Tex.), had been attached. Optical rotations were determined with a Zeiss photoelectric precision polarimeter. The following abbreviations are used: Z, benzyloxycarbonyl; Bz, benzyl; Me, methyl; PNP, *p*-nitrophenyl; DMF, dimethylformamide; DMSO, dimethyl sulfoxide.

N-Benzyloxycarbonylglycyl-L-serine Methyl Ester (I). To a suspension of *N*-benzyloxycarbonylglycine[20] (24.8 g) and L-serine methyl ester hydrochloride (18 g) in methylene chloride (200 ml) containing triethylamine (16.7 ml) and cooled to 0° was added *N*,*N*′-dicyclohexylcarbodiimide (26.5 g). The reaction mixture was stirred at room temperature for 24 hr and the precipitated material was removed by filtration and washed with methylene chloride (60 ml). The combined filtrates were concentrated to dryness *in vacuo* and the resulting solid residue was washed with ether. This solid was extracted with boiling water (1000 ml) and upon concentration of the extract to two-thirds of its original volume *in vacuo* the desired product crystallized. Recrystallization from methanol–ether gave 27 g (77.5%) of product: mp 94–96°; lit.[21] mp 94–96°.

N-Benzyloxycarbonyl-S-benzyl-L-cysteinylglycyl-L-serine Methyl Ester (II). A solution of I (15.5 g) in methanol (150 ml) containing acetic acid (4 ml) was hydrogenated for 2 hr over 10% palladium/charcoal catalyst (1 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 (75 ml), cooled to 0°, triethylamine (3 ml) was added followed by *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester[12] (23.4 g). After 24 hr at room temperature the reaction mixture was poured into ethyl acetate (1000 ml) and water (150 ml). The organic layer was washed with 1 *N* NH_4OH, water, 1 *N* HCl, and water again. (In order to prevent precipitation of the product during the washing, DMF was added to the ethyl acetate solution.) Addition of ether to the ethyl acetate solution caused the crystallization of the product: 18.4 g (75%); mp 149–151°; $[\alpha]^{29}D$ −29.2° (*c* 1, DMF); after HBr in trifluoroacetic acid treatment, R_f^1 0.74, R_f^2 3.28 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{24}H_{29}N_3O_7S$: C, 57.2; H, 5.80; N, 8.3. Found: C, 57.3; H, 5.70; N, 8.1.

N-Benzyloxycarbonyl-L-leucyl-S-benzyl-L-cysteinylglycyl-L-serine Methyl Ester (III). Compound II (20.2 g) was dissolved in trifluoroacetic acid (60 ml) containing water (0.5 ml) and HBr was passed through the solution. After 1 hr at 15° the solvent was removed by evaporation *in vacuo* and the remaining oily product was dried by dissolving in methanol followed by concentration *in vacuo* and solidified by trituration with ether. To a solution of this solid in DMF (80 ml) containing triethylamine (5.6 ml) was added *N*-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (15.5 g). After 24 hr the reaction mixture was poured into ethyl acetate (1 l.) and water (200 ml). The organic layer was washed successively with 1 *N* NH_4OH, water, 1 *N* HCl, and water, and dried over $MgSO_4$. Concentration of the solution to one-half of its original volume and addition of ether (500 ml) resulted in the precipitation of the product: 20.5 g (85%); mp 139–141°; $[\alpha]^{27}D$ −25.5° (*c* 1.1, DMF); after HBr in trifluoroacetic acid cleavage, R_f^1 0.85; R_f^2 3.74 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{30}H_{40}N_4O_8S$: C, 58.4; H, 6.54; N, 9.1. Found: C, 58.6; H, 6.69; N, 9.0.

N-Benzyloxycarbonyl-L-glutaminyl-*im*-benzyl-L-histidine Benzyl Ester (IV). To a solution of *im*-benzyl-L-histidine benzyl ester dibenzenesulfonate[22] (14 g) in DMF (60 ml) cooled to 0° was added triethylamine (5.8 ml) followed by *N*-benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[12] (8.5 g). After 24 hr at room temperature, the reaction mixture was diluted with 1 *N* NH_4OH (5 ml), stirred for 0.5 hr, and then poured into ice-cold 0.1 *N* NH_4OH (600 ml). The precipitated product was collected by filtration, washed successively with 1 *N* NH_4OH and water, dried, and crystallized from 50% aqueous methanol: 11.2 g (88%); mp 169–171°; $[\alpha]^{28}D$ −25.7° (*c* 1, acetic acid); after HBr in acetic acid treatment, R_f^1 0.40.

Anal. Calcd for $C_{38}H_{35}N_5O_6$: C, 66.3; H, 5.90; N, 11.8. Found: C, 65.8; H, 6.12; N, 11.5.

N-Benzyloxycarbonyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidine Benzyl Ester (V). A solution of IV (3.3 g) in acetic acid (10 ml) was treated with 4 *N* HBr in acetic acid (10 ml). After 35 min the reaction mixture was diluted with anhydrous ether (200 ml) and the precipitated product was filtered off, washed thoroughly with ether, and dried over KOH *in vacuo*. To a solution of this product in DMF (20 ml) made slightly alkaline with triethylamine (0.9–1.1 ml) was added *N*-benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[12] (2.2 g). After 24 hr at room temperature, the reaction mixture was diluted with 1 *N* NH_4OH (3 ml), stirred 1 hr, and poured into ice-cold 0.5 *N* NH_4OH (120 ml). The precipitated product was collected by filtration, washed thoroughly with 1 *N* NH_4OH and water, and dried. On reprecipitation from methanol–water (1:1), 2.5 g (63%) of product was obtained: mp 184–185°; $[\alpha]^{28}D$ −20.9° (*c* 1.5, acetic acid); after HBr in acetic acid treatment, R_f^1 0.36.

Anal. Calcd for $C_{37}H_{41}N_7O_8$: C, 62.4; H, 5.81; N, 13.8. Found: C, 62.1; H, 5.94; N, 13.8.

N-Benzyloxycarbonyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidine Benzyl Ester (VI). A solution of V (2.5 g) in acetic acid (7 ml) was treated with 4 *N* HBr in acetic acid (7 ml). After 35 min the reaction mixture was diluted with ether (150 ml) and the precipitated tripeptide ester hydrobromide was isolated by filtration, washed thoroughly with ether, and dried over KOH *in vacuo*. To a solution of this product in DMF (30 ml) neutralized with triethylamine (0.5–0.7 ml), *N*-benzyloxycarbonyl-L-valine *p*-nitrophenyl ester[23] (1.4 g) was added. After 24 hr the reaction mixture was diluted with 1 *N* NH_4OH (3 ml), stirred for 1 hr, and poured into ice-cold 0.5 *N* NH_4OH (150 ml). The precipitated protected tetrapeptide was filtered off and washed with 1 *N* NH_4OH and water. On reprecipitation from methanol–water (1:1) 1.4 g (48%) of product was obtained: mp 221–223°; $[\alpha]^{28}D$ −31.1° (*c* 0.9, acetic acid); after HBr in acetic acid treatment, R_f^1 0.39.

Anal. Calcd for $C_{42}H_{50}N_8O_9$: C, 62.2; H, 6.21; N, 13.8. Found: C, 62.3; H, 6.29; N, 14.0.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidine Benzyl Ester (VII). The protected tetrapeptide VI (19 g) was dissolved in acetic acid (100 ml) and treated with 4 *N* HBr in acetic acid (100 ml). After 35 min the reaction mixture was poured into anhydrous ether (1 l.) and the precipitated partially protected tetrapeptide hydrobromide was isolated by filtration, washed with ether, and dried over KOH *in vacuo*. To a solution of this material in DMF (200 ml), triethylamine (12.5 ml) was added followed by *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester[24] (9.7 g). The reaction mixture was stirred at room temperature for 24 hr, diluted with 1 *N* NH_4OH (20 ml), stirred for 30 min, and subsequently poured into ice-cold 0.5 *N* NH_4OH (1 l.). The precipitated protected pentapeptide was isolated by filtration, washed thoroughly with 0.5 *N* NH_4OH and water, and dried. This product was triturated with warm methanol (150 ml) and reprecipitated from dimethylformamide–water: 15.5 g (71%); mp 231–232°. A sample for analysis was reprecipitated from dimethyl sulfoxide–methanol: mp 232–233°; $[\alpha]^{28}D$ −27.3° (*c* 1, acetic acid); after HBr in acetic acid treatment, R_f^1 0.51.

Anal. Calcd for $C_{51}H_{59}N_9O_{10}$: C, 63.9; H, 6.20; N, 13.2. Found: C, 63.3; H, 6.23; N, 13.0.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidine (VIII). Finely powdered compound VII (7.2 g) was dissolved in DMF (200 ml) and to this solution, cooled to 0°, 1 *N* NaOH (11 ml) was added over a period of 1 hr. The reaction mixture was stirred for an additional hour at room temperature and subsequently was diluted with cold water (400 ml) and 1 *N* HCl (11.2 ml). The precipitated partially pro-

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tected pentapeptide was isolated by filtration, washed with water, and dried. Upon reprecipitation from dimethyl sulfoxide–water, 4.3 g (66%) of product was obtained: mp 223–225°; $[\alpha]^{29}D$ −3.4° (*c* 1, DMSO); after HBr in acetic acid treatment, R_f^1 0.42, R_f^2 2.9 × His; single ninhydrin-positive spot. Amino acid ratios after acid hydrolysis: $Glu_{1.0}Asp_{1.0}Val_{1.0}Phe_{1.0}$*im*-benzylhistidine$_{0.9}$.

Anal. Calcd for $C_{44}H_{53}N_9O_{10}$: C, 60.9; H, 6.14; N, 14.5. Found: C, 60.2; H, 6.34; N, 14.0.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine Methyl Ester (IX). *N*-Benzyloxycarbonyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine methyl ester (4.2 g) was dissolved in trifluoroacetic acid (15 ml) and HBr was passed through this solution at 5–10°. After 1 hr the solution was concentrated to dryness *in vacuo*. The remaining product was dried by the addition of methanol followed by evaporation under reduced pressure. The residue was triturated with ether and the resulting solid tetrapeptide hydrobromide was filtered off, washed with ether, and dried over KOH *in vacuo*. This solid was dissolved in DMF (35 ml) containing triethylamine (0.9 ml) and the resulting mixture was added to a suspension of the partially protected pentapeptide VIII (4 g) in DMF (200 ml). To the reaction mixture cooled to 0° was then added *N,N'*-dicyclohexylcarbodiimide (3 g). After 60 hr at room temperature the precipitated dicyclohexylurea was removed by filtration and the filtrate was diluted with saturated $KHCO_3$ (30 ml), stirred 30 min, and then mixed with cold water (600 ml). The precipitated product was isolated by centrifugation, washed with water and 50% aqueous DMF (500 ml), and dried. Upon trituration with hot methanol (500 ml), 4.2 g (70%) of the protected nonapeptide was obtained: mp 229–231°. A sample for analysis was reprecipitated from dimethyl sulfoxide–water: mp 232–234°; $[\alpha]^{27}D$ −25.2° (*c* 1.05 DMSO); after HBr in trifluoroacetic acid treatment, R_f^1 0.81, R_f^2 3.6 × His, R_f^3 0.95; single ninhydrin-positive spot. Amino acid analysis of the protected derivative after acid hydrolysis showed the following composition expressed in molar ratios: $Asp_{1.0}Glu_{1.0}Ser_{0.7}Gly_{0.9}Val_{1.0}Leu_{0.9}Phe_{1.0}$*S*-benzylcysteine$_{0.9}$. (*im*-Benzylhistidine was not determined.)

Anal. Calcd for $C_{66}H_{85}N_{13}O_{15}S$: C, 59.5; H, 6.43; N, 13.7. Found: C, 59.1; H, 6.78; N, 13.5.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine Hydrazide. (X). A suspension of finely powdered compound IX (9.3 g) in DMF (250 ml) was stirred at 45° for 1 hr and then mixed with hydrazine hydrate (8.5 ml). After 24 hr stirring at room temperature the reaction mixture was cooled to 0° and mixed with ice-cold water (600 ml). The precipitated product was isolated by centrifugation, washed with water and 50% aqueous methanol, and finally triturated with hot methanol (100 ml): 8.6 g (92%); mp 238–240°. A sample for analysis was reprecipitated from dimethyl sulfoxide–water: mp 241–243°; $[\alpha]^{28}D$ −36.7° (*c* 1.3, acetic acid); R_f^1 0.91; single spot (chlorine test).

Anal. Calcd for $C_{65}H_{83}N_{15}O_{14}S$: C, 58.6; H, 6.43; N, 15.8. Found: C, 58.1; H, 6.62; N, 15.6.

Amino acid analysis of an acid hydrolysate of the protected nonapeptide hydrazide showed the following composition expressed in molar ratios: $Asp_{1.1}Glu_{1.1}Ser_{0.8}Gly_{1.0}Val_{1.1}Leu_{1.0}Phe_{1.1}$-*im*-benzylhistidine$_{0.9}$ *S*-benzylcysteine$_{0.8}$.

For evaluation of stereochemical homogeneity a sample of the decarbobenzoxylated nonapeptide was digested with APM. Amino acid analysis of the digest by the automatic analyzer gave the following amino acid composition expressed in molar ratios: $Gly_{0.9}Val_{1.1}Leu_{0.9}Phe_{1.1}$*im*-benzylhistidine$_{0.8}$*S*-benzylcysteine$_{0.7}$. Glutamine, asparagine, and serine emerge at the same position on the chromatogram and were not determined.

N-Benzyloxycarbonyl-L-glutaminyl-L-histidine Methyl Ester (XI). A suspension of L-histidine methyl ester dihydrochloride (25 g) in methylene chloride (200 ml) saturated with NH_3 was stirred for 0.5 hr at room temperature. The precipitated NH_4Cl was filtered off and the filtrate was concentrated to dryness *in vacuo* to yield L-histidine methyl ester; wt 17.3 g (98%). To a solution of the free base in a mixture consisting of methylene chloride (200 ml) and ethyl acetate (100 ml) was added *N*-benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester (40 g) followed by triethylamine (0.2 ml). The reaction mixture was stirred at room temperature for 48 hr. During this period the mixture thickened considerably and it was necessary to dilute it with additional amounts of methylene chloride (300 ml) and ethyl acetate (100 ml). The precipitated product was isolated by filtration and washed twice with cold methylene chloride and then with ether. This material was subsequently reprecipitated twice from methanol–ether to yield the analytically pure product: 36 g (84%); mp 195°; $[\alpha]^{27}D$ −35.6° (*c* 1.17, 1 *N* HCl) [lit.[25] mp 174°; $[\alpha]^{23}D$ −32.4° (1.17, 1 *N* HCl)]; after hydrogenolysis in the presence of HCl, R_f^1 0.2, R_f^2 1.6 × His: single ninhydrin-positive spot.

Anal. Calcd for $C_{20}H_{25}N_5O_6$: C, 55.7; H, 5.84; N, 16.2. Found: C, 55.9; H, 5.74; N, 16.1.

N-Benzyloxycarbonyl-L-asparaginyl-L-glutaminyl-L-histidine Methyl Ester (XII). A solution of XI (28.5 g) in methanol (400 ml) containing concentrated HCl (11 ml) was hydrogenated for 2 hr over 10% palladium/charcoal catalyst (3 g). The catalyst was filtered off and the filtrate concentrated to dryness *in vacuo*. The residue was dried by the addition of methanol followed by evaporation and solidified by trituration with ether: 24.3 g (97%). To a solution of this solid in DMF (200 ml) cooled to 0°, triethylamine (14 ml) was added followed by *N*-benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester (26.3 g). The reaction was allowed to proceed at room temperature for 48 hr during which time an additional amount of DMF (160 ml) and triethylamine (4.8 ml) was added to the reaction mixture. Subsequently the reaction mixture was poured into ether (1.5 l.). The precipitated product was collected by filtration and washed thoroughly with ether. This material was suspended in boiling methanol (1.8 l.) and the suspension was allowed to cool to room temperature. The precipitated product was collected and triturated once more with methanol (1 l.): 32.2 g (86%); mp 195–197°. A sample for analysis was reprecipitated from dimethylformamide–methanol: mp 204–207°; $[\alpha]^{26}D$ −13.9° (*c* 1, formic acid); [lit.[26] mp 198–201°; $[\alpha]^{22}D$ −13° (*c* 1, formic acid)]; after hydrogenolysis in the presence of acetic acid, R_f^1 0.12, R_f^2 1.2 × His; single ninhydrin-positive spot.

Anal. Calcd for $C_{24}H_{31}N_7O_8$: C, 52.8; H, 5.73; N, 18.0. Found: C, 52.8; H, 5.85; N, 17.8.

N-Benzyloxycarbonyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidine Methyl Ester (XIII). A suspension of XII (29 g) in methanol (400 ml) containing concentrated HCl (7.4 ml) was hydrogenated for 6 hr over 10% palladium/charcoal catalyst (3 g). The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was dried by the addition of methanol followed by evaporation and solidified by washing with ether and drying over P_2O_5 and KOH *in vacuo:* 22 g (100%). To a solution of this product in DMF (350 ml) cooled to 0°, *N*-benzyloxycarbonyl-L-valine *p*-nitrophenyl ester (16.8 g) was added followed by triethylamine (10 ml). The reaction was allowed to proceed at room temperature for 48 hr, and during this period additional amounts of DMF (250 ml) and of triethylamine (2.8 ml) were added. The reaction mixture was then poured into ice-cold 0.5 *N* NH_4OH (1.5 l.) saturated with NaCl. The precipitated product was isolated by filtration and washed successively with water, methanol, and ether: 26 g (90%); mp 234–235°. A sample for analysis was reprecipitated from dimethyl sulfoxide–methanol: mp 236°; $[\alpha]D$ −32.3° (*c* 1, formic acid) [lit.[26] mp 219–221°; $[\alpha]^{22}D$ −34° (*c* 1, formic acid)]; after hydrogenolysis in the presence of acetic acid, R_f^1 0.26, R_f^2 1.7 × His; single ninhydrin-positive spot. Amino acid ratios after acid hydrolysis: $His_{1.0}Asp_{1.0}Glu_{1.0}Val_{1.0}$.

Anal. Calcd for $C_{29}H_{40}N_8O_9$: C, 54.3; H, 6.25; N, 17.4. Found: C, 54.1; H, 6.32; N, 17.2.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidine Methyl Ester (XIV). A suspension of XIII (15 g) in methanol (400 ml) containing concentrated HCl (4.2 ml) and water (2 ml) was hydrogenated for 5 hr over 10% palladium/charcoal catalyst (2.5 g). The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The residue was dried by the addition of methanol followed by evaporation: 13.5 g (100%). To a solution of this material in DMF (180 ml) cooled to 0° was added *N*-benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (10.3 g) followed by triethylamine (4 ml). The reaction was allowed to proceed at room temperature for 48 hr and during this period additional amounts of DMF (80 ml) and triethylamine (2.5 ml) were added. The reaction mixture was subsequently poured into ice-cold 0.5 *N* NH_4OH (600 ml) saturated with NaCl. The precipitated product was collected by filtration and washed successively with 0.5 *N* NH_4OH, water, methanol, and ether. Upon reprecipitation from dimethyl sulfoxide–methanol 15 g (82%) of product was obtained: mp 239–240°. A sample for analysis was reprecipitated from the same solvent system: mp

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241–243°; $[\alpha]^{26}D$ −26.4° (*c* 1, acetic acid [lit.[26] mp 230–233; $[\alpha]^{22}D$ −27° (*c* 0.5, formic acid)]; after hydrogenation in methanol containing acetic acid, R_f^1 0.32, R_f^2 2.35 × His; single ninhydrin-positive spot. Amino acid ratios after acid hydrolysis are: $His_{1.0}Asp_{1.0}Glu_{1.0}Val_{1.0}Phe_{1.0}$.

Anal. Calcd for $C_{38}H_{49}N_9O_{10}$: C, 57.6; H, 6.23; N, 15.9. Found: C, 57.8; H, 6.50; N, 15.8.

After catalytic hydrogenation and digestion with leucine amino peptidase,[27] amino acid ratios are: $His_{1.0}Val_{1.1}Phe_{1.1}$. Glutamine and asparagine emerge at the same position and were not determined.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidine Hydrazide (XV). To a suspension of XIV (5 g) in DMF (200 ml) containing hexamethylphosphoramide (10 ml) which was heated to 60° for a few minutes and then cooled to 40° was added hydrazine hydrate (10 ml). The resulting solution was stirred at 40° for 5 hr and at room temperature for 25 hr. During this period the reaction mixture thickened considerably and it was necessary to dilute it with methanol (220 ml). The precipitated hydrazide was subsequently filtered off and washed successively with methanol and water and dried: 4.8 g (96%); mp 245°. A sample for analysis was reprecipitated from dimethyl sulfoxide–methanol: mp 248–250°; $[\alpha]^{27}D$ −18.6° (*c* 1, DMSO) [lit.[26] mp 238–240°; $[\alpha]^{22}D$ −36° (*c* 0.5, formic acid)].

Anal. Calcd for $C_{37}H_{49}N_{11}O_9$: C, 56.1; H, 6.23; N, 19.5. Found: C, 56.0; H, 6.43; N, 19.4.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-S-benzyl-L-cysteinylglycyl-L-serine Methyl Ester (XVI). To a solution of compound XV (790 mg) in a mixture of DMSO (6 ml) and DMF (6 ml) was added 8.3 *N* HCl in freshly distilled tetrahydrofuran (0.5 ml). To this solution cooled to −20° (Dry Ice–acetone), *tert*-butylnitrite (0.14 ml) was added and the reaction was allowed to proceed for 6 min. To the reaction mixture, cooled to −30°, was then added a solution of L-leucyl-*S*-benzyl-L-cysteinylglycyl-L-serine methyl ester hydrobromide, prepared from its *N*-benzyloxycarbonyl derivative (1.3 g) as described previously, in the synthesis of compound IX, in DMF (4 ml) containing triethylamine (0.28 ml). After 24 hr at 2° and 24 hr at room temperature the reaction mixture (its pH being kept at about 8.0 by the addition of triethylamine) was diluted with methanol (15 ml) and its pH was adjusted to 6 with acetic acid After 30 min the precipitated material was removed by filtration: 22 mg. Amino acid analysis of an acid hydrolysate showed the following composition expressed in molar ratios: $His_{0.9}Asp_{1.0}Ser_{0.2}Glu_{1.0}Gly_{0.2}Val_{1.0}Leu_{0.2}Phe_{1.0}$*S*-benzylcysteine$_{0.16}$ indicating that this material is predominantly unreacted pentapeptide derivative. The above filtrate was diluted further with methanol (80 ml) and allowed to stand at room temperature overnight. The precipitated product was isolated by filtration and washed with methanol and ether: 737 mg (59%); mp 239–240°. A sample for analysis was reprecipitated from dimethyl sulfoxide–methanol: melting point unchanged; $[\alpha]^{27}D$ −27.7° (*c* 1, DMSO); after HBr in trifluoroacetic acid treatment, R_f^1 0.62, R_f^2 4.2 × His.

Anal. Calcd for $C_{59}H_{79}N_{13}O_{15}S$: C, 57.0; H, 6.41; N, 14.7. Found: C, 57.2; H, 6.32; N, 14.7.

Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $His_{1.0}Asp_{1.0}Ser_{0.8}Glu_{1.0}Gly_{0.9}Val_{1.0}Leu_{0.9}Phe_{1.0}$*S*-benzylcysteine$_{0.7}$.

N-Benzyloxycarbonyl-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-L-histidyl-L-leucyl-S-benzyl-L-cysteinylglycyl-L-serine Hydrazide (XVII). The protected nonapeptide ester XVI (20 g) was dissolved in DMF (600 ml) and treated with hydrazine hydrate (40 ml). After 96 hr at room temperature, the reaction mixture was diluted with ice-cold water (4 l.) and the precipitated hydrazide was filtered quickly and washed with water. On reprecipitation from dimethyl sulfoxide–water the product was obtained in analytically pure form: 16 g (80%); mp 250° dec; $[\alpha]^{27}D$ −29° (*c* 1, DMSO).

Anal. Calcd for $C_{58}H_{79}N_{15}O_{14}S$: C, 56.1; H, 6.41; N, 16.9. Found: C, 55.8; H, 6.56; N, 16.5.

Amino acid analysis after acid hydrolysis showed the following composition expressed in molar ratios: $His_{1.0}Asp_{1.0}Ser_{0.8}Glu_{1.0}Gly_{1.0}Val_{1.0}Leu_{1.0}Phe_{1.0}$*S*-benzylcysteine$_{0.9}$. For evaluation of stereochemical homogeneity a sample of the decarbobenzoxylated (HBr in trifluoroacetic acid) derivative was digested with APM. Amino acid analysis of the digest gave the following amino acid composition expressed in molar ratios: $His_{1.0}Gly_{0.8}Val_{1.1}Leu_{0.9}Phe_{1.1}$*S*-benzylcysteine$_{0.7}$. Glutamine, asparagine, and serine emerge at the same position on the chromatogram and were not determined.

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Insulin Peptides. XX. The Synthesis of a Heneicosapeptide Derivative Corresponding to the C-Terminal Sequence of the Human Insulin B Chain[1,2]

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Abstract: The synthesis of a heneicosapeptide derivative embodying the amino acid sequence found at the carboxyl terminus of the human insulin B chain is described. The desired product was obtained by interaction of the C-terminal hexadecapeptide fragment with the azide of the N-terminal nonapeptide fragment.

In one of the preceding papers we have reported the synthesis of a partially protected heneicosapeptide embodying the amino acid sequence found at the terminus of the B chain of bovine, sheep, or porcine insulin.[3] In the present paper we describe the synthesis of a heneicosapeptide derivative with the amino acid sequence corresponding to the carboxyl terminal portion of the human insulin B chain. The two heneicosapeptide derivatives differ only in the C-terminal amino acid residue; instead of alanine present in the bovine (sheep

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