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The Synthesis of the Protected Octapeptide Derivatives with the Sequence of B^{23-30} Human Insulin Chain

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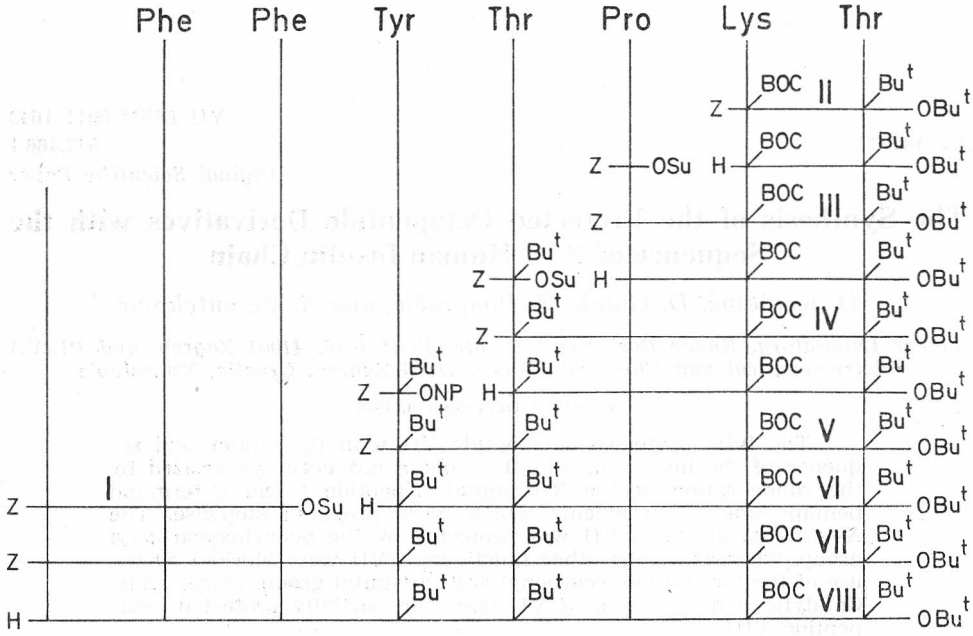
The fully protected octapeptide VII with the amino acid sequence of the human insulin B^{23-30} chain has been synthesized by the condensation of the *N*-terminal tripeptide I and *C*-terminal pentapeptide VI fragments which were prepared stepwise. The *N*-terminal group of VII was protected by the benzyloxycarbonyl group, whereas all the other functions of VII were blocked by the use of the *tert*-butyloxycarbonyl and *tert*-butyl group, respectively. Catalytic hydrogenation of VII gave the partially protected octapeptide VIII.

The synthesis of the protected octapeptide containing the amino acid sequence of the carboxyl terminal portion of the human insulin B chain has been reported from several laboratories[1–4]; the preparations differ in the pattern after which the peptide chain was built, as well as in the type and number of blocking groups. Recently, Obermeier and Geiger[4] described a new semi-synthesis of human insulin starting from porcine insulin; the procedure involves the use of a partially protected human insulin B^{23-30} octapeptide derivative in which the ε-amino group of lysine, the hydroxy group of tyrosine and the hydroxy- and carboxy-functions of the *C*-terminal threonine are protected by *tert*-butyloxycarbonyl- and *tert*-butyl-group, respectively. The authors give the overall scheme after which the peptide was prepared and claim that details of this synthesis are to be published in a separate paper.

We wish to report the preparation[5] of the fully protected human insulin B^{23-30} octapeptide derivative VII (Scheme 1.) which was obtained by coupling of the *N*-terminal tri- and *C*-terminal penta-peptide fragment (I and VI) through the active ester method. Product VII differs from the above peptide derivative in that the hydroxy group of the central threonyl residue (B^{27} of human insulin) has also been protected by a *tert*-butyl group.

The *C*-terminal pentapeptide fragment VI was synthesized by the stepwise approach: the benzyloxycarbonyl group was used for the protection of α-amino acid functions, and acylated amino acids were activated by conversion into the corresponding *N*-hydroxysuccinimidyl or *p*-nitrophenyl esters. The benzyloxycarbonyl group was removed from the protected intermediates II—V by catalytic hydrogenation without addition of the acid equivalent, and the resulting free bases were used immediately in the next condensation step. The crude intermediates showed on TLC (solvents *A* and *B*) one major and several very weak spots and were used in the next step without purification; samples of II—V



Scheme 1

were passed through silica gel columns with solvent *A* or *B* and then characterized.

Condensation of VI, previously purified over a silica gel column with solvent *B*, with the *N*-protected tripeptide *N*-hydroxysuccinimide ester I gave the fully protected octapeptide VII. Selective deblocking of VII by catalytic hydrogenation afforded the final product VIII in which all the functions, except the amino group of *N*-terminal glycine, are protected with the acid-labile groups. Octapeptide derivatives VII and VIII were obtained in good yields as crystalline compounds and were characterized by elemental- and amino acid--analysis, optical rotation and ir and NMR spectra.

EXPERIMENTAL

Melting points are uncorrected. The concentrations of solutions were carried out under reduced pressure in a rotary evaporator at minimum temperature. Column chromatography was done on silica gel (Merck, 0.02—0.2 mm). TLC on Kiesel G (Merck) plates was used to monitor the purity of all intermediates; compounds with peptide bonds were detected by the chlorine-starch-iodine method, compounds with free amino group with ninhydrin, and *N*-acylated compounds with ninhydrin, after spraying the plates with 5% HBr in acetic acid-ethanol (1 : 5) and heating. The solvent systems used: *A* benzene-ethyl acetate; *B* chloroform-methanol (proportions are given in the text). Optical rotations were determined at 20—25 °C. Proton NMR spectra were recorded on a Varian A-60A spectrometer with tetramethylsilane as the internal standard. Ir spectra were recorded on a Perkin-Elmer Model 137 spectrometer.

N-*Benzyloxycarbonyl*-O-tert-*butyl*-L-*tyrosine* p-*Nitrophenyl Ester*

The title compound was obtained in the standard way by condensation of *N*-benzyloxycarbonyl-*O*-*tert*-butyl-L-tyrosine[6] (2.65 g) and *p*-nitrophenol (1.10 g)

in the presence of dicyclohexylcarbodi-imide (DCC, 1.48 g) in ethyl acetate (25 ml). After removal of dicyclohexylurea (DCHU) and the solvent, the residue was crystallised from ethanol to give 2.47 g (70.3%) of the product with m. p. 85—86 °C. A second crystallisation afforded the analytical sample: m. p. 86—86.5 °C, $[\alpha]_D$ + 10.5° (c 1.0, dioxan). NMR data ($CDCl_3$): τ 1.75—3.25 (m, 13 H, Ph + 2 × C_6H_4), 4.62 (d, 1 H, J = 7.5 Hz, removed by D_2O exchange, NH), 4.88 (s, 2 H, PhCH_2O), 5.21 (q, 1 H, changes to triplet on deuteration, *CH* of Tyr), 8.69 (s, 9 H, Me_3COC).

Anal. $C_{27}H_{28}N_2O_7$ (492.53) calc'd.: C 65.85; H 5.73; N 5.69%
found: C 65.84; H 5.58; N 5.85%

N-*Benzyloxycarbonyl-glycyl*-L-*phenylalanyl*-L-*phenylalanine* N-*Hydroxysuccinimide Ester* (I)

To a cooled solution of *N*-benzyloxycarbonyl-glycyl-L-phenylalanyl-L-phenylalanine[7] (2.18 g) in dry dioxan (60 ml), *N*-hydroxysuccinimide (497 mg) was added followed by DCC (890 mg). After 12 h at room temperature, DCHU was filtered off, the filtrate was concentrated (0.1 Torr)* and the residue was crystallised from ethyl acetate-ether; yield: 1.82 g, 70.0%, m. p. 116—118 °C, $[\alpha]_D$ — 28.5° (c 1.0, MeOH). The compound decomposes on standing and should be used within several days.

Anal. $C_{32}H_{32}N_4O_8$ (600.60) calc'd.: C 63.99; H 5.37; N 9.33%
found: C 63.80; H 5.51; N 9.09%

N^2-*Benzyloxycarbonyl*-N^6-tert-*butyloxycarbonyl*-L-*lysyl*-O-tert-*butyl*-L-*-threonine* tert-*Butyl Ester* (II)

N-Benzyloxycarbonyl-*O*-*tert*-butyl-L-threonine *tert*-butyl ester[8] (3.83 g, 10.5 mmol) was hydrogenated for 2 h over 10% palladium-on-charcoal (400 mg) in methanol (40 ml), the catalyst was removed by centrifugation, the supernatant concentrated to dryness, and the residue was dried by the addition of methanol followed by evaporation. To the solution of this residue in *N*,*N*-dimethylformamide (DMF, 5 ml) was added at 0 °C N^2-benzyloxycarbonyl-N^6-*tert*-butyloxycarbonyl-L-lysine *p*-nitrophenyl ester[9] (5.27 g, 10.5 mmol) in DMF (5 ml). After 24 h at 0 °C and 5 h at room temperature, the reaction mixture was diluted with cold water (30 ml) and extracted with ethyl acetate. The combined extracts were washed successively with saturated aqueous sodium chloride, 1 M ammonium hydroxide, water, 10% aqueous citric acid and water, dried (Na_2SO_4) and concentrated. The remaining viscous oil (5.76 g, 92.5%) was used directly in the next step.

A sample (300 mg) of II was passed through a silicagel column with benzene, benzene-ethyl acetate (2 : 1) and (1 : 1) as the eluents; the last solvent eluted II (260 mg) as a colourless syrup, $[\alpha]_D$ + 6.7° (c 1.0, $CHCl_3$). NMR data ($CDCl_3$): τ 2.68 (s, 5 H, Ph), 4.90 (s, 2 H, PhCH_2O), 8.56, 8.58 (2 s, unresolved, 9 H + 9 H, Me_3COOC and Me_3COCON), 8.86 (s, Me_3COC + downfield peak of *Me*CH doublet).

Anal. $C_{31}H_{51}N_3O_8$ (593.74) calc'd.: C 62.70; H 8.66; N 7.08%
found: C 62.79; H 8.75; N 7.32%

N-*Benzyloxycarbonyl*-L-*prolyl*-N^6-*tert-butyloxycarbonyl*-L-*lysyl*-O-*tert-butyl*-L-*threonine* tert-*Butyl Ester* (III)

The fully protected dipeptide II (5.70 g, 9.62 mmol) was deblocked by catalytic hydrogenation as described above. The oily residue was dissolved in DMF (5 ml), and to this solution *N*-benzyloxycarbonyl-L-proline *N*-hydroxysuccinimide ester[10] (3.33 g, 9.62 mmol) in DMF (5 ml) was added at 0 °C. After 24 h at 0 °C and 5 h at room temperature, cold water was added, and the mixture was extracted with ethyl acetate. The combined extracts were washed with saturated aqueous sodium chloride, 10% aqueous citric acid, water, saturated aqueous sodium hydrogen carbonate and water, dried and concentrated. The remaining syrup (6.20 g, 93.0%) was used directly in the next step.

* Torr = 133.322 Pa

A sample (485 g) of crude III was passed through a silica gel (10 g) column with benzene-ethyl acetate (1 : 2) to give the product which still contained (TLC, solvent *A*, 2 : 1) traces of impurities; the latter were removed from a solution of ethyl acetate by addition of a few drops of petroleum ether. After centrifugation the supernatant was evaporated to dryness, and the residue was dissolved in chloroform; subsequent addition of petroleum ether at 0 °C deposited III as colourless crystals, m. p. 106—107 °C, $[\alpha]_D$ — 38.6° (c 1.7, $CHCl_3$). NMR data ($CDCl_3$): τ 2.48—2.73 (m, 5 H, Ph), 4.82 (s, 3 H, PhCH_2OCON + NH), 8.50, 8.56, 8.80 (3 s, unresolved, 30 H, Me_3COOC, Me_3COCON, Me_3COC + MeCH doublet masked by the Me_3C singlets).

Anal. $C_{36}H_{58}N_4O_9$ (690.85) calc'd.: C 62.58; H 8.46; N 8.11%
found: C 62.74; H 8.70; N 8.29%

N-*Benzyloxycarbonyl*-O-tert-*butyl*-L-*threonyl*-L-*prolyl*-N^6-tert-*butyloxycarbonyl*-L-*lysyl*-O-tert-*butyl*-L-*threonine* tert-*Butyl Ester* (IV)

The fully protected tripeptide III (6.20 g, 8.95 mmol) was deblocked by catalytic hydrogenation as described above. The oily residue was dissolved in DMF (5 ml), and to this solution *N*-benzyloxycarbonyl-*O*-*tert*-butyl-L-threonine *N*-hydroxysuccinimide ester[11] (3.63 g, 8.95 mmol) in DMF (5 ml) was added at 0 °C. After 24 h at 0 °C and 5 h at room temperature, water was added, and the reaction mixture was further processed as described for III. After removal of the solvent, the product was obtained as a solid which was used directly in the next step.

A sample of IV (360 mg) was passed through a silica gel column with benzene and then benzene-ethyl acetate (2 : 1); the latter solvent eluted IV (300 mg) as a solid, $[\alpha]_D$ — 30.0° (c 1.0, $CHCl_3$).

Anal. $C_{44}H_{73}N_5O_{11}$ (848.06) calc'd.: C 62.31; H 8.68; N 8.26%
found: C 62.52; H 8.80; N 8.46%

N-*Benzyloxycarbonyl*-O-tert-*butyl*-L-*tyrosyl*-O-tert-*butyl*-L-*threonyl*-L-*prolyl*-N^6-tert-*butyloxycarbonyl*-L-*lysyl*-O-tert-*butyl*-L-*threonine* tert-*Butyl Ester* (V)

The fully protected tetrapeptide IV (3.95 g, 4.65 mmol) was deblocked by catalytic hydrogenation as described above. The oily residue was dissolved in DMF (5 ml), and to this solution *N*-benzyloxycarbonyl-*O*-tert-butyl-L-tyrosine *p*-nitrophenyl ester (2.29 g, 4.65 mmol) in DMF (5 ml) was added at 0 °C. After 48 h at 0 °C and 5 h at room temperature water was added, and the reaction mixture was further processed as described for the preparation of II. After removal of the solvent, the product was obtained as a solid, m. p. 77—79 °C, $[\alpha]_D$ — 13.2° (c 1.4, $CHCl_3$), yield: 4.44 g, 89.7%.

A sample (80 mg) of V was passed through a silica gel column (5 g) with benzene, benzene-ethyl acetate (1 : 1) and (1 : 2); the last solvent eluted V as a chromatographically homogeneous syrup which solidified on drying over phosphorous pentoxide.

Anal. $C_{57}H_{90}N_6O_{13}$ (1,067.34) calc'd: C 64.14; H 8.50; N 7.88%
found: C 63.93; H 8.50; N 8.01%

O-tert-*Butyl*-L-*tyrosyl*-O-tert-*butyl*-L-*threonyl*-L-*prolyl*-N^6-tert-*butyloxycarbonyl*-L-*lysyl*-O-tert-*butyl*-L-*threonine* tert-*Butyl Ester* (VI)

The crude, fully protected pentapeptide V (3.57 g, 3.35 mmol) was deblocked by catalytic hydrogenation as described above, and, after the removal of catalyst and solvent, the free base (3.05 g, 97.0%) was obtained as a solid. A portion of this material (600 mg) was passed through a silica gel (20 g) column with solvent *B* (18 : 1) to give chromatographically homogeneous VI (510 mg), m. p. 87—89 °C, $[\alpha]_D$ —31.3° (c 1.1, $CHCl_3$). NMR data ($CDCl_3$): τ 3.02 (m, 4 H, $J_{AA'} = J_{XX'} = 9.0$ Hz, CH_2—C_6H_4—O), 8.57, 8.60 (2 s, unresolved, Me_3COOC, Me_3COCON), 8.70, 8.77, 8.86

(3 s, unresolved, $Me_3COC_6H_4$, 2 × Me_3COC and 2 × *Me*CH doublets masked by Me_3C singlets).

Anal. $C_{49}H_{84}N_6O_{11}$ (933.25) calc'd.: C 63.06; H 9.07; N 9.01%
found: C 63.33; H 8.87; N 9.27%

N-*Benzyloxycarbonyl-glycyl*-L-*phenylalanyl*-L-*phenylalanyl*-O-tert-*butyl*-L-*tyrosyl*-O-tert-*butyl*-L-*threonyl*-L-*prolyl*-N^6-tert-*butyloxycarbonyl*-L-*lysyl*-O-tert-*butyl*-L-*threonine* tert-*Butyl Ester* (VII)

To a solution of the purified pentapeptide base VI (632 mg, 0.68 mmol) in DMF (8 ml) the tripeptide active ester I (448 mg, 10% excess) was added at 0 °C. After 24 h at 0 °C and 5 h at room temperature cold water (20 ml) was added, the mixture was extracted with ethyl acetate, and the combined extracts were further processed as described for III. After removal of the solvent, the product was obtained as a solid foam (938 mg, 97.5%), m. p. 124—144 °C which on TLC (solvent *B*, 18 : 1) showed a major ($R_f \sim 0.6$) and several very weak spots. This material was passed through a silica gel (25 g) column with solvent *B* (18 : 1) to give chromatographically homogeneous VII (750 mg, 78.1%), m. p. 153—154 °C, $[\alpha]_D$ — 31.5° (c 1.1, $CHCl_3$). ν_{max}^{KBr} 3370 s (NH), 3000 s (Me, CH_2, CH), 1660 and 1520 vs (amide I and II), 1450 s, 1400 m and 1370 s (Me_3C), 1160 s (C—O—C), 740 m and 700 s cm^{-1}. NMR data ($CDCl_3$): τ 2.58—3.00 (m, 19 H, 3 × Ph + C_6H_4), 8.56, 8.58, 8.83 (3 s, unresolved, 51 H, Me_3COOC, Me_3COCON, 3 × Me_3COC and 2 × *Me*CH doublets masked by Me_3C singlets). Amino acid analysis after acidic hydrolysis, expressed in molar ratio: $Gly_{1.0}$ $Phe_{2.0}$ $Tyr_{0.8}$ $Thr_{1.9}$ $Pro_{1.0}$ $Lys_{1.1}$.

Anal. $C_{77}H_{111}N_9O_{16}$ (1,418.73) calc'd.: C 65.18; H 7.89; N 8.89%
found: C 65.43; H 7.79; N 9.04%

Glycyl-L-*phenylalanyl*-L-*phenylalanyl*-O-tert-*butyl*-L-*tyrosyl*-O-tert-*butyl*-L-*threonyl*-L-*prolyl*-N^6-tert-*butyloxycarbonyl*-L-*lysyl*-O-tert-*butyl*-L-*threonine* tert-*Butyl Ester* (VIII)

The fully protected octapeptide VII (518 mg, 0.366 mmol) was hydrogenated for 2 h over 10% palladium-on-charcoal (300 mg) in methanol-DMF (4 : 1, 20 ml) containing acetic acid (0.5 ml). The catalyst was centrifuged off, the supernatant concentrated, and to a solution of the residue in ethyl acetate petroleum ether was added at 0 °C. The precipitated octapeptide VIII (423 mg, 90%) had m. p. 137—140 °C (sinterring at 118 °C), $[\alpha]_D$ — 40.7° (c 1.1, $CHCl_3$) and was chromatographically homogeneous (solvent *B*, 18 : 1). ν_{max}^{KBr} 3500 s and 3380 s (NH), 1645 vs and 1520 s (amide I and II), 1450 m, 1400 m and 1370 m (Me_3C), 1160 s, 740 m and 695 m cm^{-1}. NMR data ($CDCl_3$): τ 2.50—2.73 (m, 14 H, 2 × Ph + C_6H_4), 8.50, 8.67 and 8.80 (3 s, Me_3COOC, Me_3COCON and 3 × Me_3COC + 2 × *Me*CH doublets masked by Me_3C singlets).

Anal. $C_{69}H_{105}N_9O_{14}$ (1,284.60) calc'd.: C 64.51; H 8.24; N 9.81%
found: C 64.27; H 8.45; N 9.92%

A sample (10 mg) of VIII was deblocked by exposure to trifluoroacetic acid (1 ml) at 0 °C for 30 min; this material was completely digested by bacterial protease (Sigma).

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SAŽETAK

Sinteza zaštićenih derivata oktapeptida čija sekvenca odgovara lancu B^{23-30} humanog insulina

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Potpuno zaštićeni oktapeptid VII sintetiziran je kondenzacijom *N*-terminalnog tripeptida s *C*-terminalnim pentapeptidom metodom aktivnog estera. U spoju VII *N*-terminalna amino funkcija glicina zaštićena je s benziloksikarbonil skupinom, ε-amino skupina lizina s *tert*-butiloksikarbonil skupinom, dok su hidroksi funkcije tirozina i dva treonina te karboksi skupina *C*-terminalnog treonina blokirana s *tert*-butil skupinama. Katalitičkim hidriranjem VII dobiven je *N*-terminalno slobodni oktapeptid VIII u kojemu su sve ostale funkcije zaštićene skupinama koje su labilne u kiselom mediju. Sinteza *C*-terminalnog pentapeptid intermedijera izvedena je kondenzacijom po principu »glave na rep« preko aktivnih estera *N*-benziloksikarbonil-amino kiselina. Intermedijeri I—VI i produkti VII i VIII su potpuno karakterizirani.

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