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## AMINO ACIDS AND PEPTIDES. XIV. SYNTHESIS OF A TETRAPEPTIDE SEQUENCE (A<sub>5</sub>-A<sub>8</sub>) OF GLUCAGON<sup>1</sup>

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### ABSTRACT

A synthesis of the tetrapeptide sequence A<sub>5</sub>-A<sub>8</sub> of glucagon is described that employs various blocking groups, coupling procedures, and routes.

The threonyl-phenylalanyl-threonyl-seryl sequence A<sub>5</sub>-A<sub>8</sub> in the hyperglycemic hormone, glucagon (Fòa and Galansino, 1962), has been prepared by three different groups within the past four years (Beyerman and Bontekoe, 1964; Schröder and Gibian, 1962; Wünsch and Wendleberger, 1964). In connection with our earlier work on protected glucagon fragments (Costopanagiotis, Handford, and Weinstein, 1968), there are given here some new approaches to the dipeptides N-benzyloxycarbonyl-L-threonyl-L-phenylalanine methyl ester (I) and N-benzyloxycarbonyl-L-threonyl-L-serine methyl ester (II), as well as the tetrapeptide N-benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine methyl ester (III).

N-Benzyloxycarbonyl-L-threonine (IV) was reacted with L-phenylalanine methyl ester (V) with the aid of either N,N'-dicyclohexylcarbodiimide (Sheehan and Hess, 1955) or 2-ethyl-5-phenylisoxazolium-3<sup>1</sup>-sulfonate (Woodward, Olofson, and Mayer, 1961) to afford the dipeptide I. Carbodiimide is favored in this case, as the isoxazolium agent provided an impure, gummy product, which required extensive recrystallization. An attempt to make N-benzyloxycarbonyl-L-threonine 2,4,5-trichlorophenyl ester (VI) yielded an unstable oil. If isolated, this particular compound would have been a valuable addition to the list of 2,4,5-trichlorophenyl esters (Pless and Boissonnas, 1963) that have been widely employed in recent years to facilitate the construction of peptides (Schröder and Lübke, 1964). A similar effort to obtain N-benzyloxycarbonyl-L-threonine *p*-nitrophenyl ester (VII) gave only a minor amount of the activated ester (Bodansky and Ondetti, 1966), while the alternative 2,4-dinitrophenyl ester (VIII) is apparently easily available by the same procedure (Rocchi, Marchiori, and Scoffone, 1963). No report has appeared to date on the preparation of N-benzyloxycarbonyl-L-threonine pentachlorophenyl ester (IX). The coupling of compound IV and L-serine methyl ester (X) with either N,N'-dicyclohexylcarbodiimide or 2-ethyl-5-phenylisoxazolium-3<sup>1</sup>-sulfonate produced dipeptide II. Here the isoxazolium salt gave the superior yield, in contrast with the observations described earlier for dipeptide I. The disparity may be due to a combination of both solvent (dichloromethane vs. acetonitrile) and temperature factors (0° vs. 10°C).

Treatment of dipeptide I with aqueous alkali or hydrazine furnished N-

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benzyloxycarbonyl-L-threonyl-L-phenylalanine (XI) and N-benzyloxycarbonyl-L-threonyl-L-phenylalanine hydrazide (XII), respectively. An azide coupling between hydrazide XII and L-threonyl-L-serine methyl ester (XIII), modified by the use of *t*-butyl nitrite with dimethylformamide as the solvent (Honz and Rudlinger, 1961; Schröder, 1964; Mazur and Schlatter, 1964), formed the desired tetrapeptide III. The same reaction, but with the use of sodium nitrite and hydrochloric acid-acetic acid (Greenstein and Winitz, 1961), gave tetrapeptide III in an equivalent amount. Alternatively, an azide coupling between hydrazide XII and 1-hydroxypiperidine (Handford, Jones, Young, and Johnson, 1965) afforded N-benzyloxycarbonyl-L-threonyl-L-serine 1-piperidyl ester (XIV). If the mineral acid was replaced by acetic acid, citric acid, or formic acid, no reaction occurred between hydrazide XII and 1-hydroxypiperidine. The activated ester XIV, on treatment with the amine XIII in the presence of one equivalent of acetic acid, yielded the tetrapeptide III. However, this coupling went only very slowly, and the resultant low yield may well have been caused by appreciable cyclization of the amine XIII to the corresponding diketopiperazine (XV). Finally, treatment of tetrapeptide III with hydrazine afforded N-benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine hydrazide (XVI).

#### EXPERIMENTAL DATA

Melting points are uncorrected. Infrared (potassium bromide) and ultraviolet (methanol) measurements were made on a Perkin-Elmer 421 and Cary 14 spectrophotometers. Thin-layer chromatograms employed silica gel G as the support, methanol-chloroform (1:9) as the eluent, and iodine vapor for detection purposes. Evaporations were performed at water-pump pressure in a rotary evaporator at minimum temperature; high-boiling solvents were removed at reduced pressure (0.2–0.5 mm.). Acetonitrile and dimethylformamide were spectroscopic quality and light petroleum had a b.p. 30–60°. Magnesium sulfate was generally used for drying purposes.

##### *N*-Benzyloxycarbonyl-L-Threonyl-L-Phenylalanine Methyl Ester (I)

N,N'-Dicyclohexylcarbodiimide (13.80 g, 0.0669 mole) was added to a stirred dichloromethane (100 ml) solution at 0°C of N-benzyloxycarbonyl-L-threonine (17.54 g, 0.0667 mole); mp 103.0–104.5°;  $[\alpha]^{26.5}_D -5.6^\circ$  (*c* 4, acetic acid) and L-phenylalanine methyl ester (13.11 g, 0.0732 mole), freshly prepared by evaporating a dried ethereal extract of basified (K<sub>2</sub>CO<sub>3</sub>) aqueous solution of L-phenylalanine methyl ester hydrochloride (20.00 g, 0.0929 mole; mp 161.5–163.0°;  $[\alpha]^{27.0}_D -3.6^\circ$  (*c* 5.03, water). The solution was stirred at 10° for 24 hrs, then at room temperature for 24 hrs. The reaction mixture was filtered, evaporated, and the oily residue was partitioned between water and ethyl acetate. The organic phase was washed with 5% sodium bicarbonate solution, water, 5% hydrochloric acid, water and brine, then dried and evaporated to leave a solid. The product was crystallized from ethyl acetate-*n*-hexane to give N-benzyloxycarbonyl-L-threonyl-L-phenylalanine methyl ester (22.05 g, 80%); mp 106°–107.5°, [lit., (Schröder and Gibian; Beyerman and Bontekoe) 106°, 107°];  $[\alpha]^{26}_D +3^\circ$  (*c* 1, acetic acid), [lit., (Schröder and Gibian)  $[\alpha]^{22}_D +5.0^\circ$  (*c* 1, acetic acid)]; *R*<sub>f</sub> 0.68 (acetone);  $\nu_{\max}$  3400 broad (OH), 3295 (NH), 3060 and 3030 (CH, aromatic), 2970 (CH, alkyl), 1735–1645 very broad (C=O and (COHN), 1525 broad (CONH), 1238 and 1210 (CO), 1110 and 1035 (OH), and 698 (Ph) cm<sup>-1</sup>;  $\lambda_{\max}$  242, 247, 252, 258, 261 (shoulder), 264 and 267 m $\mu$  ( $\epsilon$  171, 214, 285, 360, 270, 289 and 179).

The dipeptide was prepared alternatively by the use of 2-ethyl-5-phenylisoxazolium-3'-sulfonate (28%).

##### *N*-Benzyloxycarbonyl-L-Threonyl-L-Serine Methyl Ester (II)

To a stirred slurry of 2-ethyl-5-phenylisoxazolium-3'-sulfonate (1.269 g, 0.00500 mole) in acetonitrile at 0° was added N-benzyloxycarbonyl-L-threonine (1.316 g,

0.00500 mole), followed by tri-*n*-butylamine (0.927 g, 0.00500 mole) in acetonitrile (2 ml). After 3 hrs, L-serine methyl ester hydrochloride (0.934 g, 0.00600 mole) was added, followed by tri-*n*-butylamine (1.110 g, 0.00600 mole), and the solution was allowed to warm to room temperature and stirred for another 24 hrs. The solvent was removed and the oily residue was partitioned between water and ethyl acetate. The organic phase was washed with 5% sodium bicarbonate solution, water, 5% hydrochloric acid, water and brine, then dried and evaporated to leave a white solid. This material was crystallized from ethyl acetate-*n*-hexane to afford N-benzyloxycarbonyl-L-threonyl-L-serine methyl ester as white needles (1.043 g, 59%); mp 136–137.5° [lit., (Schröder and Gibian; Beyerman and Bontekoe; Hofmann, Haas, Smithers, and Zanetti) 131°, 137°, 136°];  $[\alpha]^{28}_D +14.9^\circ$  (*c* 1, pyridine);  $[\alpha]^{22.2}_D +9.6^\circ$  (*c* 1, dimethylformamide); [lit., (Schröder and Gibian)  $[\alpha]^{22}_D +13.1^\circ$  (pyridine); (Beyerman and Bontekoe; Hofmann, Haas, Smithers, and Zanetti)  $[\alpha]_D +10.0^\circ$ ,  $+7.6^\circ$  (dimethylformamide)];  $R_f$  0.42;  $\nu_{\max}$  3420 (OH), 3280 (NH), 3090, 3065 and 3025 (CH, aromatic), 2965 (CH, alkyl), 1742 (C=O), 1628 and 1547 (CONH), 1242 and 1215 (CO), 1120 and 1052 (OH), and 697 (Ph)  $\text{cm}^{-1}$ ;  $\lambda_{\max}$  242, 247, 252, 257, 262, 264 and 267  $\text{m}\mu$  ( $\epsilon$  103, 143, 201, 248, 200, 218, and 154). The dipeptide was prepared alternatively by the use of N,N'-dicyclohexylcarbodiimide (45%).

#### *N*-Benzyloxycarbonyl-L-Threonyl-L-Phenylalanine (XI)

To a stirred solution of N-benzyloxycarbonyl-L-threonyl-L-phenylalanine methyl ester (4.145 g, 0.0100 mole) in dioxane (30 ml) was added dropwise 0.2 N sodium hydroxide solution (50 ml). After 2.5 hrs, the solution was acidified with 5% hydrochloric acid (10 ml); then the solvent was removed and the residue was distributed between ethyl acetate and water. The organic phase was washed with saturated sodium bicarbonate solution, water, and brine, then dried and evaporated to afford starting dipeptide ester (0.970 g) identical with an authentic sample on the basis of mixed mp and  $R_f$  comparisons. The sodium bicarbonate extract was rewashed with ethyl acetate, then acidified with hydrochloric acid (5%) to precipitate the crude product. Crystallization from methanol-water and recrystallization from ethyl acetate-*n*-hexane yielded N-benzyloxycarbonyl-L-threonyl-L-phenylalanine as white needles (2.160 g, 71%); mp 156–157°, [lit., (Beyerman and Bontekoe) 151–152°];  $[\alpha]^{26.5}_D +18.2^\circ$  (*c* 1, dimethylformamide), [lit., (Beyerman and Bontekoe)  $[\alpha]^{20}_D +19^\circ$  (*c* 2, dimethylformamide)];  $R_f$  0.00;  $\nu_{\max}$  3395 broad (OH), 3325 broad (NH), 3060 and 3025 (CH, aromatic), 2965 (CH, alkyl), 1715 broad (C=O), 1650 and 1520 broad (CONH), 1230 broad (CO), 1065 broad (OH) and 695 (Ph)  $\text{cm}^{-1}$ ;  $\lambda_{\max}$  242, 247, 252, 257, 261 shoulder, 264 and 267  $\text{m}\mu$  ( $\epsilon$  163, 200, 272, 352, 264, 287 and 181).

#### *N*-Benzyloxycarbonyl-L-Threonyl-L-Phenylalanine Hydrazide (XII)

To a solution of N-benzyloxycarbonyl-L-threonyl-L-phenylalanine methyl ester (17.120 g, 0.0413 mole) in methanol (150 ml) at reflux was added dropwise 95% hydrazine (6 ml, 0.180 mole). After 1 hr, the hot solution was allowed to stand for 3 hrs, then the crystalline product was collected, washed with hot methanol and dried overnight. Recrystallization from methanol gave N-benzyloxycarbonyl-L-threonyl-L-phenylalanine hydrazide (12.86 g, 75%), mp 207–208° [lit., (Schröder and Gibian) 201–203°];  $[\alpha]^{27}_D -27.8^\circ$  (*c* 1, 1 N hydrochloric acid);  $[\alpha]^{28}_D -2.2^\circ$  (*c* 1, dimethylformamide);  $[\alpha]^{30}_D -23.5^\circ$  (*c* 1, acetic acid); [lit., (Schröder and Gibian)  $[\alpha]^{22}_D -23.6^\circ$  (*c* 1, acetic acid)];  $R_f$  0.59 (methanol);  $\nu_{\max}$  3380 (OH), 3290 (NH), 3060 and 3040 (CH, aromatic), 2965 (CH, alkyl), 1710–1643 broad (C=O and CONH), 1525 broad (CONH), 1258 and 1238 (CO), 1043 (OH), and 695 (Ph)  $\text{cm}^{-1}$ ;  $\lambda_{\max}$  247, 252, 257, 262 shoulder, 264 and 267  $\text{m}\mu$  ( $\epsilon$  223, 251, 323, 250, 253 and 161).

*N*-Benzyloxycarbonyl-L-Threonine-L-Phenylalanine l-Piperidyl Ester (XIV)

To a solution of *N*-benzyloxycarbonyl-L-threonine-L-phenylalanine hydrazide (1.660 g., 0.00401 mole) in dimethylformamide (15 ml) at  $-40^{\circ}$  was slowly added 2.90 N hydrochloric acid in tetrahydrofuran (5.52 ml, 0.0160 mole hydrochloric acid), followed by *t*-butyl nitrite (0.56 ml, 0.00480 mole). After 30 mins, triethylamine (2.24 ml, 0.0160 mole) and then l-hydroxypiperidine (0.808 g, 0.00800 mole) in dimethylformamide (5 ml) were added and the solution was allowed to stand for 1 hr at  $-40^{\circ}$  and 48 hrs at  $0^{\circ}$ . The solvent was removed and the residue was distributed between ethyl acetate and water. The organic phase was washed with 0.5 M citric acid, water, saturated sodium bicarbonate solution, water, and brine, dried, and evaporated to a small bulk. The addition of diisopropyl ether until the solution became opalescent at room temperature caused the separation of crystalline, analytically pure *N*-benzyloxycarbonyl-L-threonine-L-phenylalanine l-piperidyl ester (1.138 g, 59%); mp  $141.5-142.5^{\circ}$ ;  $[\alpha]^{26}_D +26.4^{\circ}$  (*c* 1, chloroform);  $[\alpha]^{26}_D +1.4^{\circ}$  (*c* 1, ethyl acetate);  $R_f$  0.72;  $\nu_{\max}$  3500 broad (OH), 3300 (NH), 3060 and 3045 (CH, aromatic), 2945 (CH, alicyclic and alkyl), 1751 (C=O, piperidyl ester), 1720-1655 broad (C=O and CONH), 1515 broad (CONH), 1240 broad (CO), 1030 (OH), and 700 (Ph)  $\text{cm}^{-1}$ ;  $\lambda_{\max}$  247, 252, 257, 262 shoulder, 264 and 267  $\mu$  ( $\epsilon$  302, 358, 421, 312, 329 and 203).

Anal. Calcd. for  $\text{C}_{26}\text{H}_{33}\text{N}_3\text{O}_6$  (483.6); C, 64.58; H, 6.88; N, 8.69. Found: C, 64.67; H, 6.82; N, 8.63.

*N*-Benzyloxycarbonyl-L-Threonyl-L-Phenylalanyl-L-Threonyl-L-Serine Methyl Ester (III)

To a solution of *N*-benzyloxycarbonyl-L-threonyl-L-phenylalanine hydrazide (3.320 g, 0.00800 mole) in dimethylformamide (30 ml) cooled to  $-40^{\circ}$  was added slowly 3.11 N hydrochloric acid in tetrahydrofuran (10.3 ml, 0.0320 mole hydrochloric acid), followed by *t*-butyl nitrite (1.11 ml, 0.00960 mole). After 30 mins, the azide solution was treated with triethylamine (5.65 ml, 0.0404 mole), and immediately afterwards with a solution of L-threonyl-L-serine methyl ester hydroacetate in dimethylformamide (40 ml), which was freshly prepared by the hydrogenolysis of *N*-benzyloxycarbonyl-L-threonyl-L-serine methyl ester (2.980 g, 0.00840 mole) in methanol (25 ml) in the presence of acetic acid (0.48 ml, 0.00840 mole) and 10% palladium on charcoal (0.084 g) for 3 hrs at room temperature, removing the catalyst and solvent, and dissolving the white, crystalline residue in dimethylformamide with gentle warming. The coupling reaction was allowed to proceed for 1 hr at  $-40^{\circ}$  and 48 hrs at  $0^{\circ}$ , the solvent was removed and the residue was dissolved in methanol-water (15:1.5). On storage at  $0^{\circ}$ , this solution deposited small, white needles (3.394 g, 68%), which were recrystallized from methanol-water (9:1) to yield *N*-benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine methyl ester monohydrate as fern-shaped needle clusters; mp  $200-200.5^{\circ}$  [lit., (Beyerman and Bontekoe)  $199^{\circ}$ ];  $[\alpha]^{30}_D -18.6^{\circ}$  (*c* 2, acetic acid);  $[\alpha]^{26}_D -2.9^{\circ}$  (*c* 1.0 dimethylformamide); [lit., (Beyerman and Bontekoe)  $[\alpha]^{21}_D -4^{\circ}$  (*c* 2.8 dimethylformamide)];  $R_f$  0.46; 0.69 (methanol);  $\nu_{\max}$  3400-3270 broad (OH and NH), 3060 and 3045 (CH, aromatic) 2970 (CH, alkyl), 1740-1645 very broad (C=O and CONH), 1520 broad (CONH), 1225 broad (CO), 1070 broad (OH) and 697 (Ph)  $\text{cm}^{-1}$ ;  $\lambda_{\max}$  247, 252, 257, 261 shoulder, 264 and 267  $\mu$  ( $\epsilon$  264, 339, 427, 295, 338 and 218).

The tetrapeptide was prepared alternatively by the use of sodium nitrite-hydrochloric acid-acetic acid (68%) and the l-piperidyl ester (16%).

*N*-Benzyloxycarbonyl-L-Threonyl-L-Phenylalanyl-L-Threonyl-L-Serine Hydrazide (XVI)

To a hot solution of *N*-benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine methyl ester monohydrate (2.48 g, 0.00414 mole) in methanol

(50 ml) was added 95% hydrazine (0.68 ml, 0.0200 mole). After 48 hrs at room temperature, the gelatinous mixture was chilled at 0° for 15 hrs, then filtered and washed with chilled methanol and ether to afford a white powder. The solid separated from ethanol to yield N-benzoyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine hydrazide (2.41 g, 97%); mp 201–202°;  $[\alpha]_{25}^{28} D -20.0^\circ$  (c, 0.69, acetic acid);  $R_f$  0.00; 0.65 (n-butanol-water-acetic acid (62:26:12)). The analytical sample was dried at 110° *in vacuo* over phosphorus pentoxide for 3 days; routine drying overnight at room temperature *in vacuo* gave a product that possessed a high nitrogen value, presumably due to absorbed hydrazine.

Anal. Calcd. for  $C_{28}H_{38}N_6O_9$  (602.6); C, 55.80; H, 6.36; N, 13.95. Found: C, 55.51; H, 6.32; N, 13.71.

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