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SOLID-PHASE SYNTHESIS OF TWO GLYCOPEPTIDES CONTAINING THE AMINO ACID SEQUENCE 5 TO 9 OF SOMATOSTATIN*

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ABSTRACT

2,3,4,6-Tetra-*O*-acetyl-1-*N*-[*N*-(*tert*-butyloxycarbonyl)-L-aspart-4-oyl]-D-glucopyranosylamine and 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(*tert*-butyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-D-glucopyranosylamine were introduced, respectively, by the solid-phase procedure in the amino acid sequence 5 to 9 of somatostatin. The two resulting glycopeptides β-D-Glc*p*-(1→4)- and β-D-Glc*p*NAc-(1→4)-Asn-Phe-Phe-Trp-Lys-OH were homogeneous on examination by t.l.c. and l.c., and their structures were confirmed by m.s. of the *N*-acetyl, permethyl derivatives.

INTRODUCTION

The carbohydrate substituents of the glycoprotein hormones have specific functions, one of which is to impart an extended biological half-life to the parent molecule[1]. The overall objective of synthesizing longer-acting analogs of somatostatin[2] (SS) (Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) suggested that the incorporation of a sugar moiety in the molecule might increase the biological half-life of the native molecule. Since the Asn-5 residue of somatostatin may be substituted, and even deleted, without complete loss of biological activity[3,4], this residue is the logical position for introducing a sugar substituent.

In the *N*-glycoproteins containing the 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-D-glucopyranosylamine residue, so far, only 2-acetamido-2-deoxy-D-glucose has been found[5]. Although the chemical synthesis of a few glycopeptides has been reported[6], all of them were synthesized by classical peptide methods. In order

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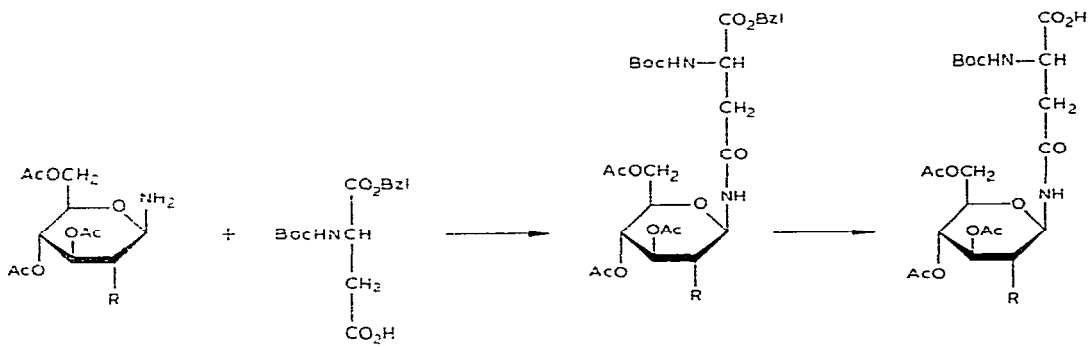
to verify the stability of the 2-acetamido-2-deoxy-1-*N*-(L-aspart-4-oyl)-D-glucopyranosylamine linkage under the conditions of solid-phase peptide synthesis[7], we first attempted the synthesis of glycopeptides **8** and **9** containing a β-D-glucopyranosyl and a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue, respectively, coupled to Asn-5 of the amino acid sequence 5 to 9 (Asn-Phe-Phe-Trp-Lys-OH) of somatostatin. These two glycopeptides also served as model compounds for t.l.c. and l.c. comparison of the enzyme digest of the two corresponding glycosylated somatostatin, β-D-Glc*p*-(1→4)-Asn-5-somatostatin and β-D-Glc*p*NAc-(1→4)-Asn-5-somatostatin, which upon trypsin treatment specifically released **8** and **9**, respectively.

RESULTS AND DISCUSSION

The synthesis of compounds similar to 2,3,4,6-tetra-*O*-acetyl-1-*N*-[*N*-(*tert*-butyloxycarbonyl)-L-aspart-4-oyl]-D-glucopyranosyl (**5**) and 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(*tert*-butyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-D-glucopyranosylamine (**6**) but having a *N*-benzyloxycarbonyl protecting group, easily removed by hydrogenation, has been previously described[6]. The introduction of glycoasparagine derivatives into peptides by solid-phase methodology required an acid-labile, N-protecting group, such as *N*-(*tert*-butyloxycarbonyl), that would not be cleaved during the hydrogenolysis of the benzyl ester group, and yet be readily removed by an anhydrous acid, such as trifluoroacetic acid. The starting materials for the synthesis of **5** and **6** were the corresponding 2,3,4,6-tetra-*O*-acetyl-[8] and 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromides[9], which, by treatment with sodium azide, gave the corresponding azides[10–12]. Hydrogenation of the azides with Adams' catalyst yielded the corresponding amines[13,14] **1** and **2**. The next step involved a



1 R = OAc
2 R = NHAc

3 R = OAc
4 R = NHAc

5 R = OAc
6 R = NHAc

7 Asn—Phe—Phe—Trp—Lys—OH

8 β-D-Glc*p*-(1→4)—Asn—Phe—Phe—Trp—Lys—OH

9 β-D-Glc*p*NAc-(1→4)—Asn—Phe—Phe—Trp—Lys—OH

condensation with 1-benzyl *N*-(*tert*-butyloxycarbonyl)-L-aspartate. In the synthesis of the corresponding *N*-(benzyloxycarbonyl) derivative, the method used by the previous investigators involved condensation in the presence of dicyclohexylcarbodiimide (DCC) with yields ranging between 40 to 65%. In the present work, **3** and **4** were obtained in 72–82% yields by the active ester method[15] using 1 equiv. of DCC in the presence of an excess (1.5 equiv.) of 1-hydroxybenzotriazole. The resulting compounds obtained were hydrogenated in the presence of 10% palladium-on-charcoal to afford the acids **5** and **6**, respectively. N.m.r. data confirmed the β-D configuration of these compounds ($J_{1,2}$ 8.5–9 Hz): **5** and **6** were found to be homogeneous in t.l.c. and l.c.

The synthesis of peptide **7** and glycopeptides **8** and **9** by the solid-phase procedure involved successive steps that have already been described[16]. Coupling of 2-*N*-(*tert*-butyloxycarbonyl)-6-*N*-(2-chlorobenzyloxycarbonyl)-L-lysine to the chloromethylated resin was performed by the Monahan and Gillon procedure[17]. The appropriate Boc-amino acids were coupled in the presence of DCC for 2 h, except for the glycoasparagines **5** and **6**; which were coupled by the dicyclohexyl carbodiimide 1-hydroxybenzotriazole method, and asparagine which was coupled overnight as the *p*-nitrophenyl ester. After cleavage of the peptide from the resin by hydrofluoric acid[18,19], peptides **8** and **9** were first *O*-deacetylated by a saturated solution of ammonia in methanol, and then purified by partition chromatography[20] on Sephadex

TABLE I

YIELDS[a] AND PROPERTIES OF **7**, **8**, AND **9**

Properties	*Compound*		
	7	**8**	**9**
Yield[a] (%)	40	36	40
$[\alpha]_D^{23}$[b]	−0.5	−0.5	−0.5
T.l.c.[c] (A)	0.53	0.45	0.46
(B)	0.55	0.50	0.52
L.c.[d] (min)	9.5	7.2	6.85
(minim. purity, %)	98	>99	>99
Amino acid and amino sugar analysis[e]			
Lys	1.02	(1.00)	0.97
Asp	1.01	1.03	1.05
Phe	(2.00)	2.00	2.15
Trp	0.93	0.93	0.94
GlcN			(1.00)
NH_3	1.20	1.00	1.30

[a]Yields were calculated on the basis of moles of peptide obtained after final purification relative to the total amount of starting Boc-amino acid on the resin. [b]Conc. 1, 1% acetic acid. [c]R_F values were determined on Eastman No. 13191 silica gel sheets. [d]For methodology, see Experimental section. [e]Peptides were hydrolyzed with 4M methanesulfonic acid for 20 h at 110°, and then analyzed with a Beckman 119 amino acid analyzer.

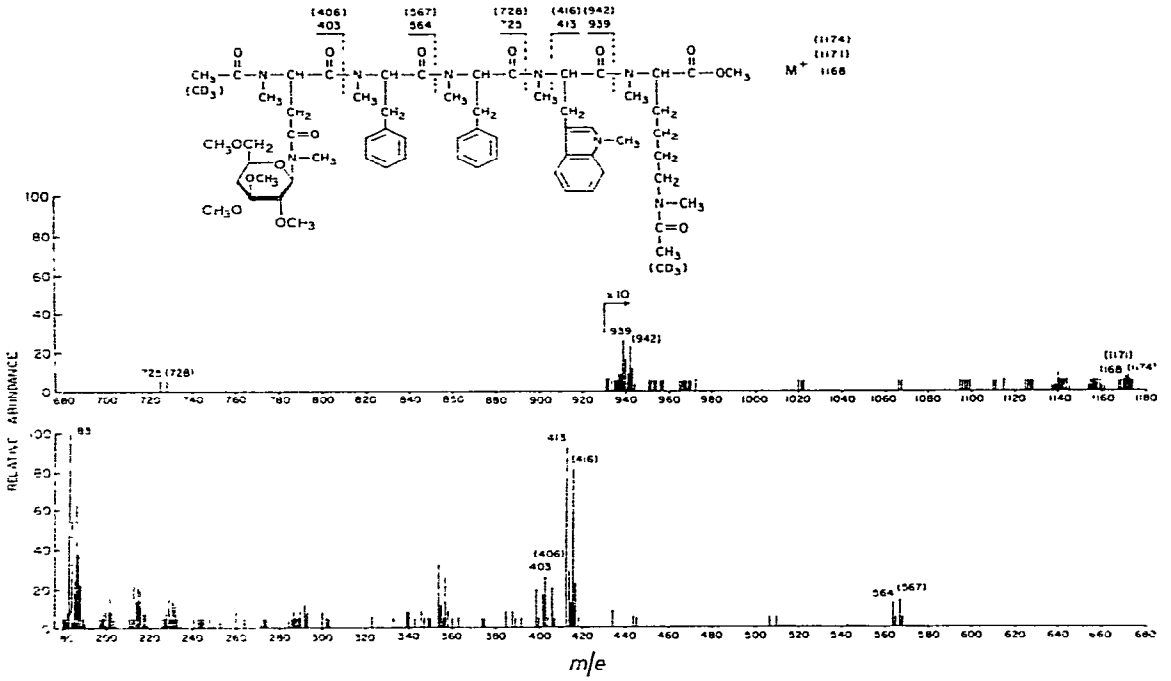


Fig. 1. Mass spectrum of glycopeptide 8 after *N*-acetylation and permethylation.

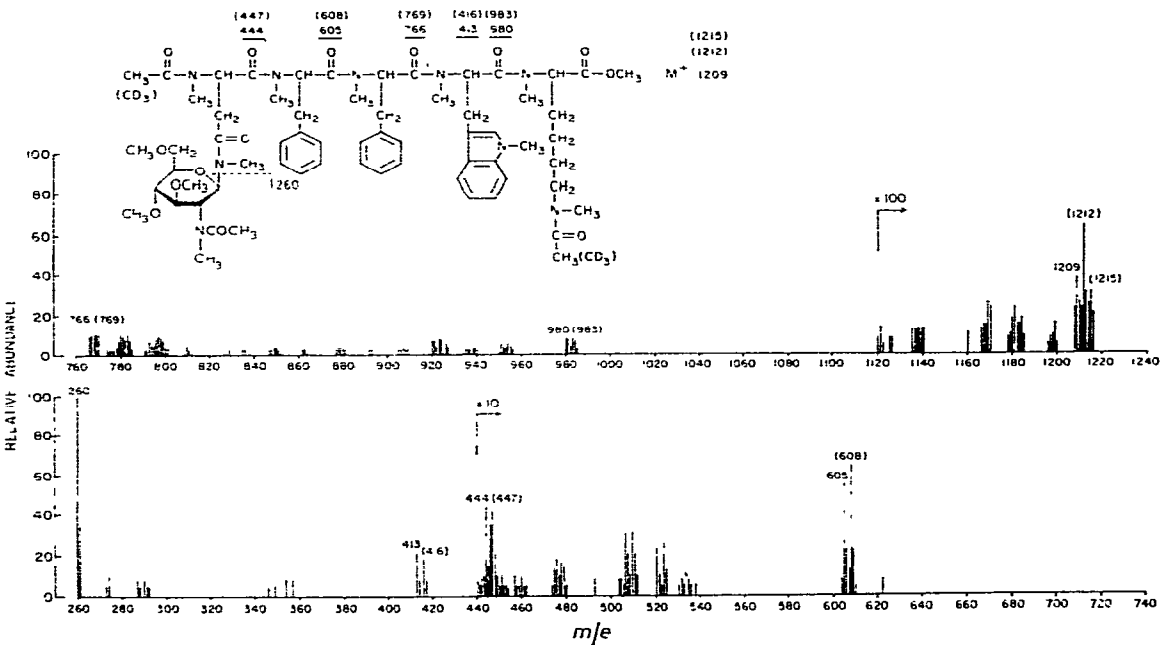


Fig. 2. Mass spectrum of glycopeptide 9 after *N*-acetylation and permethylation.

G-25 fine. These compounds were obtained in good yields, based upon the starting protected amino acid resin, and were found homogeneous by t.l.c. and l.c. The amino acid analyses were in good agreement with the theoretical values, including the 2-acetamido-2-deoxy-D-glucose content (see Table I).

As the structure of peptide **7** was already determined by mass spectrometry when the sequence of somatostatin was elucidated[21], it was expected that the sequence of the two glycopeptides **8** and **9** could be directly elucidated by mass spectrometry. Thus, **8** and **9** were acetylated with an equimixture of acetic anhydride and [$^2H_6$]-acetic anhydride and permethylated with methylsulfinylmethyl sodium and methyl iodide as described by Ling *et al.*[22] for the α- and γ-endorphins. In general, the mass spectra of *N*-acetylated and permethylated peptides exhibit peaks that correspond to the cleavage of the amide bond, with charge retention on the carbonyl portion of the molecule. Thus, the sequence of the two glycosylated peptides was readily deduced from the series of doublet peaks in the mass spectra (see Fig. 1 and 2). Trypsin digestion of the two corresponding glycosomatostatins[23] (Glc-Asn-5)-SS and (GlcNAc-Asn-5)-SS gave the pentapeptide fragment comprising residues 5 to 9 which, in t.l.c. (in two solvent systems) and l.c., showed the same R_F values and retention times as those of **8** and **9**, respectively.

EXPERIMENTAL

General methods. — Melting points were determined with a Hoover capillary melting-point apparatus and are uncorrected. Optical rotations were determined with a Perkin–Elmer Model 141 polarimeter. N.m.r. spectra were recorded with a JEOL JNM-PS-100 spectrometer, tetramethylsilane being the internal reference. Mass spectra were recorded with a Varian Mat CH-5, single-focusing, mass spectrometer equipped with a direct-inlet system, at an ionization current of 300 μA, an energy of 70 eV, and an acceleration voltage of 3 kV. Ascending t.l.c. on silica gel was performed on precoated silica gel 60 plates (Merck, 0.25 mm thick) for Boc aminoacids derivatives, and on Eastman chromatogram sheet No 13191 for peptides: the spots were detected by iodine vapor or the ninhydrin reagent. The solvent systems were: (*A*) 4:1:5 (upper phase) 1-butanol–acetic acid–water; (*B*) 5:3:11 (upper phase) 1-butanol–pyridine–0.1% acetic acid; and (*C*) 1:1 (upper phase) 1-butanol–0.1M acetic acid (all v/v). High-pressure, liquid chromatography (l.c.) was performed with a Waters Associates Model 204 liquid-chromatography system, equipped with two M-6000 A pumps, a 660 solvent programmer, a Schoeffel variable u.v. detector, a Spectra-Physics "Minigrator", and a Linear instrument 456 recorder; separation was accomplished on a 25 × 0.45-cm column, filled with a Zorbax ODS, in isocratic mode with the indicated percent composition of acetonitrile in buffer *D* or buffer *E*, at a flow rate of 1.5 mL/min and 0.1 absorbance full-scale at 210 nm. Buffer *D* was 0.25M triethylammonium phosphate at pH 3.00 (ref. 23), and buffer *E* was 0.01M triethylammonium phosphate at pH 3.00; 10–20 μg of compound was injected. Trifluoroacetic acid and *N*,*N*-dimethylformamide were distilled before use. 1-Hydroxy-

benzotriazole was recrystallized from methanol. All other solvents were reagent grade and not further purified.

Amino acid derivatives used for the synthesis were of the L configuration and were purchased from Bachem, Inc., Torrance, CA 90505. The α-amino group was protected exclusively with the *tert*-butyloxycarbonyl group (Boc). Glycosylamines **1** and **2** were synthesized from corresponding azides and bromides according to previously described methods[8–14]: melting points and rotations were in agreement with the literature values.

2,3,4,6-Tetra-O-*acetyl-1*-N-[*1-benzyl* N-(tert-*butyloxycarbonyl*)-L-*4-aspart-4-oyl*]-D-*glucopyranosylamine* (**3**). — To a solution of 1-benzyl *N*-(*tert*-butyloxycarbonyl)-L-aspartate {0.834 g, 2.58 mmol, m.p. 95–98°, $[\alpha]_D^{23}$ −20° (*c* 1, methanol) one peak in l.c. (iso., 34% acetonitrile, 17.5 min, ≥99%)} and 1-hydroxybenzotriazole (0.593 g, 4.39 mmol) in 1:1 (v/v) *N,N*-dimethylformamide–dichloromethane (5 mL) was added, at 0°, 1.1 equiv. of M dicyclohexylcarbodiimide solution in 1:9 (v/v) *N,N*-dimethylformamide–dichloromethane. After 5 min, the solution was filtered into a solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamine (**1**) (0.806 g, 2.35 mmol) in dichloromethane (5 mL). The mixture was stirred for 2 h at room temperature. After the usual processing involving washing with weak acid and base, the solvent was evaporated, and the remaining syrup was chromatographed on a column of silica gel (100 g) with 3:2 (v/v) chloroform–ethyl acetate to yield **3** (1.23 g, 82%), m.p. 169–171°, $[\alpha]_D^{23}$ +21° (*c* 1, chloroform); recrystallization from dichloromethane–ether raised the m.p. to 170–172°, $[\alpha]_D^{23}$ +22° (*c* 1, chloroform); ^{1}H-n.m.r. {100 MHz, [^{2}H]chloroform}: 7.23 (s, 5 H, $C_6H_5CH_2$), 6.38 (d, 1 H, $J_{NH\text{-}1,1}$ 9 Hz, N*H*CO), 5.63 (d, 1 H, $J_{NH,H'\text{-}2}$ 9 Hz, N*H*tBoc), 5.19 (t, 1 H, *J* 9 Hz), 5.10 (t, 1 H, *J* 9 Hz, *H*-1 of Glc), 5.05 (s, 2, $C_6H_5CH_2$), 4.83 (t, 1 H, *J* 9 Hz), 4.77 (t, 1 H, *J* 9 Hz), 4.48 (m, 1 H, *H'*-2 of Asp), 4.20 and 3.92 (2 H, J_{AB} 14, *J* 4 Hz, *J* 2 Hz, H_2-6 of Glc), 3.68 (m, 1 H, *H*-5 of Glc), 2.70 (m, 2 H, *H'*-3 of Asp), 2.02 (s, 3 H, CH_3CO), 1.99 (s, 6 H, CH_3CO), 1.85 (s, 3 H, CH_3CO), and 1.40 (s, 9 H, *tert*-butyl of Boc); m.s.: *m/e* 652 (M^+) (0.5), 596 (5), 552 (4), 517 (8), 461 (10), 417 (27), 331 (18), 271 (13), 169 (62), and 91 (100).

Anal. Calc. for $C_{30}H_{40}N_2O_{14}$: C, 55.21; H, 6.13; N, 4.29. Found: C, 55.06; H, 6.36; N, 4.46.

2-Acetamido-3,4,6-tri-O-*acetyl-1*-N-[*1-benzyl* N-(tert-*butyloxycarbonyl*)-L-*aspart-4-oyl*]-*2-deoxy*-D-*glucopyranosylamine* (**4**). — The procedure was the same as that used for **3**. 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine (**2**) (0.9 g) gave a syrup that was chromatographed on a column of silica gel (100 g) with ethyl acetate to yield 1.226 g (72%) of **4**, m.p. 157–158°, $[\alpha]_D^{23}$ +6.5° (*c* 1, chloroform); m.s.: *m/e* 651 (M^-) (0.3), 595 (1.5), 551 (0.8), 516 (7), 460 (14), 416 (9), 330 (33), 270 (24), 227 (67), 210 (64), 150 (83), and 91 (100).

Anal. Calc. for $C_{30}H_{41}N_3O_{13}$: C, 55.30; H, 6.30; N, 6.45. Found: C, 55.45; H, 6.39; N, 6.37.

2,3,4,6-Tetra-O-*acetyl-1*-N-[N-(tert-*butyloxycarbonyl*)-L-*aspart-4-oyl*]-D-*glucopyranosylamine* (**5**). — Compound **3** (0.978 g) was hydrogenated in 10:1 (v/v)

ethanol–water (220 mL) in the presence of 10% palladium-on-charcoal (0.15 g) for 5 h at room temperature and atmospheric pressure. After filtration through a Celite column and evaporation of the solvent, a white, amorphous powder was obtained (0.735 g, 87%), $[\alpha]_D^{23}$ +31° (*c* 1, chloroform); t.l.c.: R_F 0.62 (*A*), 0.66 (*B*), and 0.46 (*C*); l.c. (iso., 32% acetonitrile in buffer *E*): 6.8 min, min. purity 97.5%.

Anal. Calc. for $C_{23}H_{34}N_2O_{14}$: C, 49.11; H, 6.05; N, 4.98. Found: C, 49.25; H, 6.08; N, 5.03.

*2-Acetamido-3,4,6-tri-O-acetyl-*N-[N-(tert-*butyloxycarbonyl*)-L-*aspart-4-oyl*]-*2-deoxy*-D-*glucopyranosylamine* (**6**). — The procedure was the same as that used for **5**. Compound **4** (1.2 g) gave, after hydrogenation, 0.92 g of the free acid **6** (90%) as a white amorphous powder, m.p. 193–195° $[\alpha]_D^{23}$ +23.4° (*c* 1, chloroform); t.l.c.: R_F 0.60 (*A*) 0.62 (*B*), and 0.35 (*C*); l.c. (iso., 24% acetonitrile in buffer *E*): 11.4 min, min. purity 98%.

Anal. Calc. for $C_{23}H_{35}N_3O_{13}$: C, 49.20; H, 6.24; N, 4.99. Found: C, 49.07; H, 6.17; N, 5.05.

L-*Asparaginyl*-L-*phenylalanyl*-L-*phenylalanyl*-L-*tryptophanyl*-L-*lysine* (**7**), *1*-N-[L-*aspart-1-oyl*-(L-*phenylalanyl*-L-*phenylalanyl*-L-*tryptophanyl*-L-*lysine*)-*4-oyl*]-*β*-D-*glucupyranosylamine* (**8**), *and 2-acetamido-1*-N-[L-*aspart-1-oyl*-(L-*phenylalanyl*-L-*phenylalanyl*-L-*tryptophanyl*-L-*lysine*)-*4-oyl*]-*β*-D-*glucopyranosylamine* (**9**). — Coupling of 2-*N*-(*tert*-butyloxycarbonyl)-6-*N*-(2-chlorobenzyloxycarbonyl)-L-lysine to the (chloromethyl)resin (0.9 mequiv./g, Lab Systems, Inc., San Mateo, CA 94401) was performed by the Monahan and Gilon procedure[17]. A ratio of 0.6 mequiv. of 2-*N*-(*tert*-butyloxycarbonyl)-6-*N*-(2-chlorobenzyloxycarbonyl)-L-lysine to 0.54 mequiv. of potassium *tert*-butoxide per g of resin was used, which resulted in a substitution of 0.30 mmol of lysine per gram of resin. (Boc-lysyl) resin (1.5 g, 0.45 mmol of lysine) was treated in a four-cycle procedure according to the following scheme: (a) dichloromethane wash: (b) 1:9:10 (v/v) ethane-1,2-dithiol–trifluoroacetic acid–dichloromethane, 20 min; (c) dichloromethane wash (3 ×); (d) 1:9 (v/v) triethylamine–dichloromethane neutralization: (e) dichloromethane wash; (f) 1:9 (v/v) triethylamine–dichloromethane; (g) dichloromethane wash: (h) methanol wash (2 ×); (i) dichloromethane wash (2 ×); (j) Boc-amino acid (1.5 mmol) plus 1 equiv. of 2M dicyclohexylcarbodiimide in dichloromethane, 2 h, except for Boc-asparagine, **5**, and **6**; Boc-Asn was coupled as its *p*-nitrophenyl ester in *N*,*N*-dimethylformamide overnight, and **5** and **6** were coupled with 1 equiv. of dicyclohexylcarbodiimide and 1.5 equiv. of 1-hydroxybenzotriazole in 1:1 (v/v) *N*,*N*-dimethylformamide–dichloromethane overnight; (k) methanol wash (3 ×); and (l) dichloromethane wash (3 ×). Coupling efficiency was monitored with the Kaiser test[25]. The protected peptide-resins were treated with 1.5 mL of methoxybenzene and 10 mL of hydrogen fluoride per g of peptide-resin for 0.5 h at −20° and for 0.3 h at 0°. The resins were first washed (3 ×) with 1:1 (v/v) diethyl ether–chloroform, and then the peptide was eluted with 1:9 (v/v) acetic acid–water. Lyophilization of the extracts gave 320, 310, and 352 mg of the crude peptides **7**, **8**, and **9**, respectively. The two glycopeptides **8** and **9** were *O*-deacetylated with a saturated solution of ammonia in methanol (15 mL per 300 mg

of peptide) for 4 h at room temperature. After evaporation of the solvent *in vacuo* at 40°, the residues were diluted with water and lyophilized. The crude peptides were purified by partition chromatography[20] on Sephadex *G*-25 fine with the solvent system *A* to yield 144, 123, and 188 mg of **7**, **8**, and **9**, respectively. The properties and amino acid contents are reported in Table I.

Derivatization of glycopeptides **8** *and* **9** *for mass spectrometric analysis.* — Trypsin digestion, *N*-acetylation, with acetic anhydride and [$^2H_6$]acetic anhydride, and permethylation were performed as described[22].

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REFERENCES

1 W. H. DAUGHADAY, in E. WILLIAMS (Ed.), *Textbook of Endocrinology*, Saunders, New York, 1974, pp. 31–54.
2 P. BRAZEAU, W. VALE, R. BURGUS, N. LING, M. BUTCHER, J. RIVIER, AND R. GUILLEMIN, *Science*, 179 (1973) 77–79.
3 J. RIVIER, M. BROWN, C. RIVIER, N. LING, AND W. VALE, *Proc. Eur. Peptide Symp., 14th*, (1976) 427–451.
4 D. SARANTAKIS, J. TEICHMAN, E. L. LIEN, AND R. L. FENICHEL, *Biochem. Biophys. Res. Commun.*, 73 (1976) 336–353.
5 R. D. MARSHALL AND A. NEUBERGER, in A. GOTTSCHALK (Ed.), *Glycoproteins*, 2nd edn., Elsevier, Amsterdam, 1972, pp. 453–470.
6 H. G. GARG AND R. W. JEANLOZ, *Carbohydr. Res.*, 70 (1979) 47–58 and references therein.
7 R. B. MERRIFIELD, *J. Am. Chem. Soc.*, 85 (1963) 2149–2154.
8 R. U. LEMIEUX, *Methods Carbohydr. Chem.*, 2 (1962) 221–222.
9 Y. INOUYE, K. ONODERA, S. KITAOKA, AND H. OCHIAI, *J. Am. Chem. Soc.*, 79 (1957) 4218–4222.
10 A. BERTHO AND D. AURES, *Justus Liebigs Ann. Chem.*, 592 (1955) 54–69.
11 F. MICHEEL AND H. WULFF, *Chem. Ber.*, 89 (1956) 1521–1530.
12 G. S. MARKS, R. D. MARSHALL, AND A. NEUBERGER, *Biochem. J.*, 87 (1963) 274–279.
13 A. BERTHO AND J. MAIER, *Justus Liebigs Ann. Chem.*, 498 (1932) 50–61.
14 C. H. BOLTON AND R. W. JEANLOZ, *J. Org. Chem.*, 28 (1963) 3228–3230.
15 W. KÖNIG AND R. GEIGER, *Chem. Ber.*, 103 (1970) 788–798.
16 N. LING, *Biochem. Biophys. Res. Commun.*, 74 (1977) 248–255.
17 M. W. MONAHAN AND G. GILON, *Biopolymers*, 12 (1973) 2513–2519.
18 S. SAKAKIBARA, Y. SHIMONISHI, M. OKADA, AND Y. KISHIDA, *Proc. Eur. Peptide Symp., 8th*, (1967) 44–49.
19 J. LENARD AND A. B. ROBINSON, *J. Am. Chem. Soc.*, 89 (1967) 181–182.
20 D. YAMASHIRO, *Nature (London)*, 201 (1964) 76–77.
21 N. LING, R. BURGUS, J. RIVIER, W. VALE, AND P. BRAZEAU, *Biochem. Biophys. Res. Commun.*, 50 (1973) 127–133.
22 N. LING, R. BURGUS, AND R. GUILLEMIN, *Proc. Natl. Acad. Sci. U.S.A.*, 73 (1976) 3942–3947.
23 S. LAVIELLE, N. LING, P. BRAZEAU, R. BENOIT, T. WASADA, D. HARRIS, R. UNGER, AND R. GUILLEMIN, *Biochem. Biophys. Res. Commun.*, 91 (1979) 614–622.
24 J. RIVIER, R. WOLBER, AND R. BURGUS, *Proc. Am. Peptide Symp., 5th*, (1977) 52–55.
25 E. KAISER, R. COLESCOTT, C. BOSSINGER, AND P. COOK, *Anal. Biochem.*, 34 (1970) 595–598.