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SYNTHESIS OF GLYCOPEPTIDE DERIVATIVES CONTAINING THE 2-ACETAMIDO-*N*-(L-ASPART-4-OYL)-2-DEOXY-β-D-GLUCOPYRANOSYLAMINE LINKAGE AND HAVING THE AMINO ACID SEQUENCES 32–34, 33–35, 33–37, AND 33–38 OF BOVINE RIBONUCLEASE*

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ABSTRACT

The synthesis is described of the glycotripeptide derivatives 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-seryl-L-nitroarginyl-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine, 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-seryl-L-nitroarginyl-L-aspart-1-oyl-(1-*p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine, and 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucine methyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine, and of the glycopentapeptide and glycohexapeptide derivatives 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysine-(*p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine and 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysyl-L-aspartic 1,4-di-*p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine.

INTRODUCTION

By use of synthetic dolichyl glycosyl phosphates and diphosphates as intermediates[1], our laboratory has undertaken the study of the biosynthesis of *N*-glycoproteins in calf pancreatic tissue. These glycoproteins contain the 2-acetamido-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine carbohydrate–protein linkage. In order to evaluate the role of the primary sequence of the peptide moiety, in addition to that of the known[2] "sequon" Asn–X–Ser(Thr), synthetic peptides reproducing the sequence around Asn-34 of beef ribonuclease have been selected, because the

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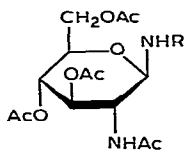
complete peptide sequence had previously been synthesized[3]. The attachment of one residue of 2-acetamido-2-deoxy-D-glucose to the asparagine residue of peptides of various lengths, followed by elongation of the peptide chain, constitutes a model synthesis for the preparation of similar glycopeptides containing the same sugar residue protected at O-3 and O-4 by benzyl groups. This residue may, in turn, be condensed with various oligosaccharides obtained by chemical synthesis, or isolated from natural sources, as shown by the recent synthesis of *N*-acetylchitobiose derivatives[4]. We now describe the synthesis of glycopeptides, derived from ribonuclease B, that contain the 2-acetamido-2-deoxy-D-glucose residue, and the sequences 33–37 and 33–38 surrounding the "sequon 34–36" Asn–Leu–Thr and glycotripeptides containing the sequences 32–34 and 33–35. All these sequences have amino acids on both sides of the L-asparagine residue linked to the carbohydrate component.

RESULTS AND DISCUSSION

In earlier syntheses of glycopeptides containing the sequence 2-acetamido-*N*-[L-aspart-1-oyl-(amino acid or polypeptide)-4-oyl]-2-deoxy-β-D-glucopyranosyl-



1 R = H

2 R = Cbz-Asp-OH

3 R = Cbz-L-Arg(NO2)-L-Asp-OH

4 R = Cbz-L-Arg(NO2)-L-Asp-OH

5 R = Cbz-L-Arg(NO2)-L-Asp-L-Leu-L-Thr-OMe

6 R = Cbz-L-Arg(NO2)-L-Asp-L-Leu-L-Thr-L-Lys(Ts)-ONBzl(*p*)

7 R = Cbz-L-Arg(NO2)-L-Asp-L-Leu-L-Thr-L-Lys(Ts)-L-Asp(ONBzl(*p*))-ONBzl(*p*)

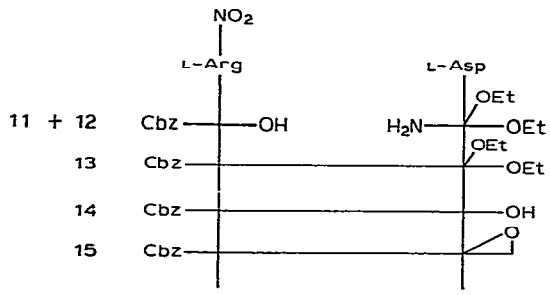
8 R = Cbz-L-Ser-L-Arg(NO2)-L-Asp-OH

9 R = Cbz-L-Ser-L-Arg(NO2)-L-Asp-ONBzl(*p*)

10 R = Cbz-L-Arg(NO2)-L-Asp-L-Leu-OMe

Ac = CH_3CO ; Cbz = $C_6H_5CH_2CO_2$; Me = CH_3 ; NBzl(*p*) = *p*-$NO_2C_6H_4CH_2$;
Ts = *p*-$CH_3C_6H_4SO_2$

amine[5,6], the peptide moiety was elongated by linking the carbonyl group of 2-acetamido-3,4,6-tri-*O*-acetyl-[*N*-(benzyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine[7] (**2**) with amino acids or polypeptide esters in the presence of *N*,*N'*-dicyclohexylcarbodiimide[8] (DCCI), *N*-ethyl-5-phenylisoxazolium 3'-sulfonate[9] (WRK), or 2-ethoxy-*N*-(ethoxycarbonyl)-1,2-dihydroquinoline[10] (EEDQ). An attempt to prepare 2-acetamido-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**3**) by linking the amino group of **2** with *N*-(benzyloxycarbonyl)-L-nitroarginine pentachlorophenyl ester[11] (**25**), after removal of the protective *N*-benzyloxycarbonyl group, was unsuccessful.



L-Leu-L-Thr-R

16 R = OMe

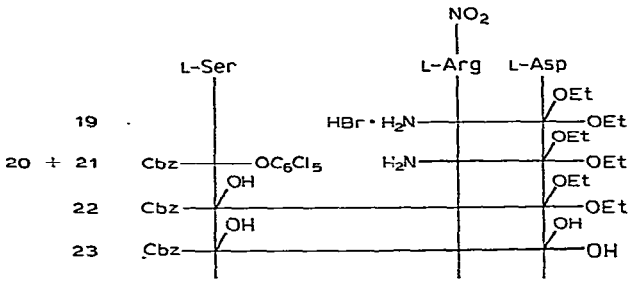
17 R = Ts
L-Lys-ONBzl(p)

18 R = Ts ONBzl(p)
L-Lys-L-Asp-ONBzl(p)

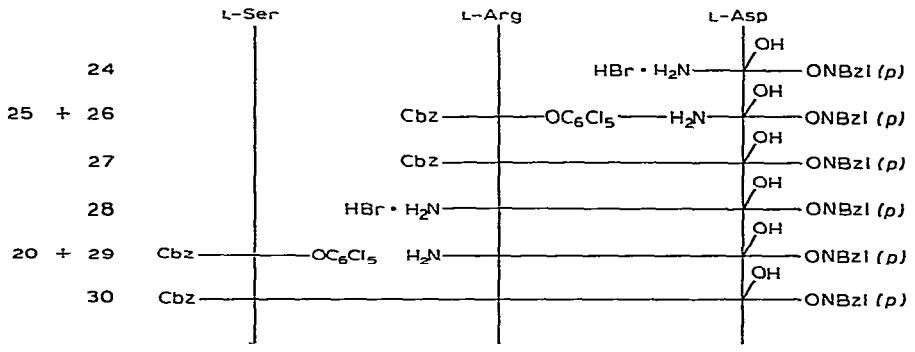
Scheme 1

In the second approach, *N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspartic anhydride (**15**), prepared according to Scheme 1, was condensed with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine[12] (**1**) to give both **3** and the L-aspart-1-oyl analog **4**. The usefulness of **3** as an intermediate for the synthesis of glycopeptides corresponding to the amino acid sequence 32–38 of bovine pancreatic ribonuclease was examined, and glycopeptides corresponding to sequences 33–36 (**5**), 33–37 (**6**), 33–38 (**7**), and 32–34 (**8**) were prepared. Methyl and *p*-nitrobenzyl ester groups were selected for the protection of the C-terminals, because (*a*) the methyl ester derivative can be readily converted into a reactive hydrazide[13], which is an intermediate for elongation of the peptide chain at the C-terminus, and (*b*) the *p*-nitrobenzyl group is stable under acidic conditions[14], and thus allows for elongation of the peptide chains at the N-terminus.

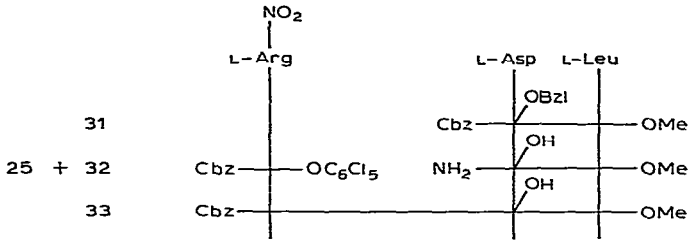
The elongation at the C-terminus of **3** to give crystalline **5** was at first investigated by coupling **9** in the presence of EEDQ with L-leucyl-L-threonine methyl ester (**16**),



Scheme 2



Scheme 3



Scheme 4

obtained from *N*-(benzyloxycarbonyl)-L-leucyl-L-threonine methyl ester[15]. Coupling of **3** with L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester (**17**) under the same conditions gave a poor yield, and EEDQ was replaced by WRK, which was also used for the preparation of the glycohexapeptide **7** from **3** plus L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysyl-L-aspartic 1,4-di-*p*-nitrobenzyl ester (**18**).

The glycotripeptide **8**, which represents sequence 32–34 of bovine ribonuclease, was obtained by elongation of **3** at the N-terminus after unmasking the amino group of the L-nitroarginine residue of **3**, and the resulting free amino group was condensed with *N*-(benzyloxycarbonyl)-L-serine pentachlorophenyl ester[13] (**20**). An

attempt to prepare **8** by a route similar to that of **3**, by first assembling the tripeptide *N*-(benzyloxycarbonyl)-L-seryl-L-nitroarginyl-L-aspartic acid (**23**) according to Scheme 2, and then converting it into the anhydride and condensing this with **1**, was unsuccessful, because **22** could not be saponified to afford **23**. However, the tripeptide *N*-(benzyloxycarbonyl)-L-seryl-L-nitroarginyl-L-aspartic acid 1-*p*-nitrobenzyl ester (**30**) could be prepared according to Scheme 3, and condensed with **1** in the presence of EEDQ to give a protected derivative of **8**, namely, 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-seryl-L-nitroarginyl-L-aspart-1-oyl-(*p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**9**).

For the preparation of the glycotripeptide 2-acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucine methyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**10**), which is symmetrical to the protein–carbohydrate linkage and represents sequence 33–35, the tripeptide *N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspartyl-L-leucine methyl ester (**33**) (prepared according to Scheme 4) was condensed with **1** in the presence of EEDQ, as just described for the preparation of **9**.

EXPERIMENTAL

General. — Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points". Rotations were determined for solutions in 1-dm semimicrotubes with a Perkin–Elmer No. 141 polarimeter. The *N*,*N*-dimethylformamide used was Spectroreagent grade. I.r. spectra were recorded, for potassium bromide discs, with a Perkin–Elmer spectrophotometer Model 237. Evaporations were performed *in vacuo*, the bath temperature being kept below 45°. Column chromatography was performed on Silica gel Merck (70–235 mesh; E. Merck, Darmstadt, Germany) used without pretreatment; the ratio of the weight of substance to the weight of silica gel was 1:60; the volume of the fractions collected was 2 ml/g of the substance. The homogeneity of compounds was verified by ascending t.l.c. on precoated plates of Silica gel (Merck) with solvents (v/v) *A* (7:3 chloroform–methanol), *B* (9:1 chloroform–methanol), *C* (19:1 chloroform–ethanol), *D* (9:1 chloroform–ethanol), *E* (14:1 chloroform–ethanol), *F* (14:1 chloroform–methanol), *G* (1:1 chloroform–methanol), and *H* (19:1 chloroform–methanol), and detection by spraying the plates with 20% sulfuric acid and heating for a few min at 200°. Preparative-layer chromatography was performed on precoated PLC plates of Silica gel G (Merck). The microanalyses were performed by Dr. W. Manser, Zurich, Switzerland.

N-(*Benzyloxycarbonyl*)-L-*nitroarginyl*-L-*aspartic 1,4-diethyl ester* (**13**). — *a.* A solution of *N*-(benzyloxycarbonyl)-L-nitroarginine (**11**, 3.53 g; Pierce, Rockford, IL 61105) in 1,4-dioxane (100 ml) was treated with butylamine (2.4 ml) and cooled to 11°. Ethyl chloroformate (0.92 ml) was added, with stirring, and the mixture was kept for 15 min at 11°. Then, a solution of L-aspartic 1,4-diethyl ester [**12**; obtained by treatment of the hydrochloride[19] (2.25 g)with triethylamine (1.4 ml) in 1,4-dioxane

(10 ml)] was added, and the mixture was stirred for 45 min at room temperature. The solvents were removed *in vacuo*, the residue was extracted with ethyl acetate (400 ml), and the extract was successively washed with M hydrochloric acid, water, 1% sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated to give a crude material that was crystallized from 2-propanol (2.1 g, 38%), m.p. 121–122°, $[\alpha]_D^{21}$ —5.1° (*c* 1.2, acetone); lit.[20] m.p. 122–123°, $[\alpha]_D^{19}$ —3.8° (acetone); t.l.c. (*C*): R_F 0.47; ν_{max}^{KBr} 3250 (NH), 1750–1530 (peptide Amide I, NO_2), and 1625 cm^{-1} (benzyloxycarbonyl C=O).

b. A mixture of L-aspartic 1,4-diethyl ester hydrochloride[22] (1.36 g) in chloroform (24 ml) was treated with triethylamine (0.84 ml), and **11** (2.12 g) was added, followed by *N,N'*-dicyclohexylcarbodiimide (1.30 g). A clear solution was obtained by stirring for a few min at room temperature and this was stirred overnight. The *N,N*-dicyclohexylurea was filtered off and the filtrate was washed successively with M hydrochloric acid, water, and 1% sodium hydrogencarbonate solution, dried (sodium sulfate), and evaporated; crystallization of the residue from 2-propanol gave 1.2 g (41%) of **13**, m.p. 121–122°; the i.r. spectrum was identical with that of the material obtained by method *a*.

N-(*Benzyloxycarbonyl*)-L-*nitroarginyl*-L-*aspartic acid* (**14**). — A solution of **13** (0.4 g) in 1:1 (v/v) methanol–water (8 ml) was treated with sodium hydroxide (0.1 g), and stirred for 1 h at room temperature to give a clear solution. After acidification with M hydrochloric acid, the methanol was evaporated off *in vacuo*, and **14** was extracted from the residual, aqueous solution with ethyl acetate. The extract was washed with water, dried (sodium sulfate), and evaporated, to give crystalline **14** (0.15 g), m.p. 118–119°, lit.[20] m.p. 97–101°; t.l.c. (*G*): R_F 0.14. Compound **14** was used without further purification for the preparation of **15**.

2-Acetamido-3,4,6-tri-O-acetyl-N-[N-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**3**). — *a*. A solution of *N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspartic anhydride (**15**) [obtained from **14** (1 g) and acetic anhydride under conditions similar to those described by Yamamoto[16]] in *N,N*-dimethylformamide (5 ml) was added to a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine[12] (**1**; obtained from 1.0 g of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl azide[17]) in ethyl acetate (50 ml). The mixture was kept overnight at room temperature, the solvents were evaporated, ethyl acetate (200 ml) was added, and the solution was successively washed with M hydrochloric acid and water, dried (sodium sulfate), and evaporated; the residue was taken up in 1% sodium hydrogencarbonate, and the suspension filtered. The filtrate was acidified with concentrated hydrochloric acid, to give a syrup which was extracted with ethyl acetate (200 ml). The extract was washed with water, dried (sodium sulfate), and evaporated; the residue (1.2 g, 64%) obtained showed in t.l.c. (*A*) three spots, R_F 0.2, 0.6, and 1.0 (corresponding to sugar compounds devoid of aspartoyl residues). This crude product was dissolved in warm acetonitrile (30 ml), and cooling gave the slowest-moving product (**3**; 0.2 g, 12%), m.p. 175–176° (dec.) (shrank at 173°), $[\alpha]_D^{20}$ +15.5° (*c* 1.0, methanol); ν_{max}^{KBr} 3300 (NH), 1750 (Ac), 1655

(benzyloxycarbonyl C=O), and 1650–1530 cm^{-1} (peptide Amide I, NO_2). The mother liquor was kept (for further isolation of **3** and **4** by column chromatography).

Anal. Calc. for $C_{32}H_{44}N_8O_{16}$: C, 48.23; H, 5.57; N, 14.07; O, 32.13. Found: C, 48.11; H, 5.57; N, 14.12; O, 32.10.

b. 2-Acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine[7] (**2**) was converted into the hydrobromide by dissolving it in glacial acetic acid (1.5 ml) and treating the solution with 30% hydrogen bromide in acetic acid (1.5 ml) for 1 h at room temperature. The hydrobromide was precipitated by anhydrous ether, rapidly filtered off, and dissolved in 0.8M sodium hydroxide (0.5 ml). The solution was added to a mixture of *N*-(benzyloxycarbonyl)-L-nitroarginine (71 mg), *tert*-butylamine (28 μl) in dry 1,4-dioxane (1 ml), and ethyl chloroformate (25 μl), and the mixture was kept for 10 min at 10°. After 2 h at room temperature, M hydrochloric acid was added, and the syrup so obtained was extracted with ethyl acetate. The extract was washed with water, dried (sodium sulfate), and evaporated; the residue gave crystals of **3** from ethanol (20 mg, 8%), m.p. 171–173° (dec.); the i.r. spectrum and mobility in t.l.c. were identical with those of the material obtained by method *a*.

*2-Acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl]-2-deoxy-β-D-glucopyranosylamine* (**4**). — The mother liquor from the preparation of **3** was evaporated, to give 1 g of a mixture of **3**, **4**, and a sugar compound not linked to an aspartoyl residue. A solution of this mixture in chloroform was applied to a column of silica gel, and successive elution with a linear gradient (1:0 to 9:1, v/v) of chloroform–methanol gave first 2-acetamido-*N*-acetyl-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine (0.22 g); needles after recrystallization from ethanol, m.p. 239–240° (dec.); lit.[18] m.p. 236–237°.

Anal. Calc. for $C_{16}H_{24}N_2O_9$: C, 49.43; H, 6.27; N, 7.25; O, 37.08. Found: C, 49.50; H, 6.20; N, 7.14; O, 37.33.

Further elution gave **4** (0.35 g, 21%), which crystallized from ethanol, m.p. 187–189°, $[\alpha]_D^{20}$ −9.5° (*c* 0.8, methanol) ; t.l.c. (*A*): R_F 0.6; ν_{max}^{KBr} 3300 (NH), 1740 (OAc), 1650 (benzyloxycarbonyl C=O), 1650–1550 (peptide Amide I), and 1525 cm^{-1} (NO_2).

Anal. Calc. for $C_{32}H_{14}N_8O_{16}$: C, 48.23; H, 5.57; N, 14.07; O, 32.13. Found: C, 48.18; H, 5.52; N, 14.00; O, 31.92.

After complete elution of **4**, compound **3** (0.25 g, 15%) was eluted; it contained a contaminant that tailed to the origin in t.l.c.; the overall yield of **3** was 27%.

*2-Acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucyl-L-threonine methyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine* (**5**). — To a solution of *N*-(benzyloxycarbonyl)-L-leucyl-L-threonine methyl ester[15] (**16**; 50 mg) in glacial acetic acid (1 ml) was added 30% hydrogen bromide in acetic acid (1 ml), and the mixture was kept for 1 h at room temperature. The acids were removed by evaporation *in vacuo*, and the residue was washed with anhydrous ether, and dried in a vacuum desiccator in the presence of sodium hydroxide pellets, to give L-leucyl-L-threonine methyl ester, hydrobromide salt. Treatment with

triethylamine (18 µl) in acetonitrile (2 ml) gave L-leucyl-L-threonine methyl ester. The mixture was evaporated to remove acetonitrile and the excess of triethylamine. To the residue were added **3** (100 mg) in 1:1 (v/v) benzene–ethanol (10 ml) and EEDQ (32 mg). The mixture was stirred overnight, and evaporated *in vacuo*, and the residue was diluted with water and extracted with ethyl acetate (50 ml). The extract was successively washed with M hydrochloric acid, water, 1% sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated *in vacuo*. The crude product crystallized from absolute ethanol, to yield **5** (40 mg, 31%), m.p. 224–225° (dec.; softening at 201°), $[\alpha]_D^{21}$ −10.4°(*c* 0.4, methanol); t.l.c. (*B*): R_F 0.4; ν_{max}^{KBr} 3375–3275 (NH), 1740 (OAc), 1660 (benzyloxycarbonyl C=O), 1660–1540 (peptide Amide I), and 1530 cm^{-1} (NO_2).

Anal. Calc. for $C_{43}H_{64}N_{10}O_{19}$: C, 50.39; H, 6.29; N, 13.66; O, 29.66. Found: C, 50.30; H, 6.24; N, 13.51; O, 29.75.

*2-Acetamido-3,4,6-tri-O-acetyl-*N*-[*N*-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucyl-L-threonyl-*N*$^{\varepsilon}$-tosyl-L-lysine p-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine* (**6**). — *a*. To a solution of *N*-(benzyloxycarbonyl)-L-leucyl-L-threonyl-*N*$^{\varepsilon}$-tosyl-L-lysine *p*-nitrobenzyl ester[5] (**17**, 100 mg) in glacial acetic acid (2 ml) was added 30% hydrogen bromide in acetic acid (2 ml). The mixture was kept for 1 h at room temperature, and the hydrobromide was obtained by evaporating off the acids *in vacuo*, washing with anhydrous ether, and drying in a vacuum desiccator in the presence of sodium hydroxide pellets. The hydrobromide was dissolved in acetonitrile (2 ml), and the solution was treated with triethylamine (18 µl), and evaporated *in vacuo*. To the residue were added **3** (100 mg) dissolved in 1:1 (v/v) benzene–ethanol (15 ml) and EEDQ (32 mg), the mixture was stirred overnight, and the solvents were evaporated *in vacuo*. The residue was dissolved in ethyl acetate, and processed as described for **5**. The residue (100 mg) showed two spots in t.l.c. (*C*): R_F 0.49 and R_F 0.44, and was chromatographed on p.l.c. plates (20 × 20 cm) (*C*). After being dried in air, the plates were re-eluted with the same solvent-mixture, and compounds were detected with 20% sulfuric acid. The band containing **6** (R_F 0.49) was removed from the plates, and stirred overnight with ethyl acetate. Filtration of the suspension through Celite, evaporation of the filtrate, and crystallization of the residue from ethanol gave **6** (35 mg, 19%), m.p. 217–219°, $[\alpha]_D^{21}$ −13.2° (*c* 0.6, methanol); t.l.c. (*D*): R_F 0.31: ν_{max}^{KBr} 3300 (NH), 1740 (OAc), 1660 (benzyloxycarbonyl C=O), and 1630–1525 cm^{-1} (peptide Amide, NO_2).

Anal. Calc. for $C_{62}H_{84}N_{13}O_{24}S \cdot H_2O$: C, 51.52; H, 6.00; N, 12.60; S, 2.22. Found: C, 51.35; H, 5.95; N, 12.42; S, 2.21.

b. To a solution of 2-ethyl-5-phenylisoxazolium 3′-sulfonate (32 mg) in acetonitrile at 0° were added **3** (100 mg) and 4-methylmorpholine (13 µl) in acetonitrile (10 ml). The mixture was stirred, and the bath was removed; after 65 min, all of the compounds had dissolved, and to the solution was added L-leucyl-L-threonyl-*N*$^{\varepsilon}$-tosyl-L-lysine *p*-nitrobenzyl ester hydrobromide [obtained from **17** (100 mg) as just described] dissolved in acetonitrile and treated with 4-methylmorpholine (13 µl). The mixture was stirred for 24 h at room temperature, and the solvents were removed

in vacuo. The residue was dissolved in chloroform, and the solution was successively washed with M hydrochloric acid, water, 1% sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated *in vacuo*. The residue (95 mg) was chromatographed on p.l.c. plates (*C*). The band containing **6** was removed from the plates, and processed as just described. Crystallization from ethanol gave **6** (50 mg, 28%), m.p. 219–220°; the i.r. spectrum and mobility in t.l.c. were identical with those of the material obtained by method *a*.

2-Acetamido-3,4,6-tri-O-acetyl-N-[N-(benzyloxycarbonyl)-L-nitroarginyl-L-aspart-1-oyl-(L-leucyl-L-threonyl-N$^{\varepsilon}$-tosyl-L-lysyl-L-aspartic 1,4-di-p-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**7**). — To a solution of 2-ethyl-5-phenylisoxazolium 3′-sulfonate (32 mg) in acetonitrile (10 ml) at 0° were added **3** (100 mg) and 4-methylmorpholine (13 μl) in acetonitrile (10 ml). The suspension was treated as described for the preparation of **6**, and a solution of *N*-(benzyloxycarbonyl)-L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysyl-L-aspartic di-*p*-nitrobenzyl ester (**18**, 135 mg) (transformed into the hydrobromide salt as described earlier[5]) in acetonitrile (5 ml) and 4-methylmorpholine (13 μl) was added. The mixture was treated as described for the preparation of **2** (method *b*), and the residue (35 mg) was chromatographed on p.l.c. plates (*D*). The band containing **7** (R_F 0.3) was removed from the plates, and processed as described for **6** (method *b*). Crystallization from ethanol gave 20 mg (10%) of **7**, m.p. 214–215° (dec.), $[\alpha]_D^{21}$ −14° (*c* 0.22, methanol); ν_{max}^{KBr} 3400–3250 (NH), 1740 (OAc), 1625 (benzyloxycarbonyl C=O), and 1625–1525 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{73}H_{95}N_{15}O_{29}S \cdot 2H_2O$: C, 51.13; H, 5.82; N, 12.25; S, 1.87. Found: C, 51.01; H, 5.71; N, 12.39; S, 1.49.

2-Acetamido-3,4,6-tri-O-acetyl-N-[N-(benzyloxycarbonyl)-L-seryl-L-nitroarginyl-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**8**). — A solution of **3** (0.2 g) in acetic acid (2 ml) was treated with 30% hydrogen bromide in acetic acid (2 ml) for 1 h at room temperature. After removal of the acids *in vacuo*, the hydrobromide was washed with anhydrous ether, and dried in a vacuum desiccator in the presence of sodium hydroxide pellets. It was then dissolved in *N*,*N*-dimethylformamide (5 ml), and triethylamine (36 μl) was added, followed by *N*-(benzyloxycarbonyl)-L-serine pentachlorophenyl ester[11] (0.1 g). The mixture was stirred overnight at room temperature, the *N*,*N*-dimethylformamide was evaporated, and the residue was dissolved by treatment with 1% sodium hydrogencarbonate and ethyl acetate. After clarification by filtration, the aqueous layer was separated, acidified with concentrated hydrochloric acid, and extracted with ethyl acetate. The extract was washed with water, dried (sodium sulfate), and evaporated, to give an amorphous powder (35 mg, 16%). Attempts to crystallize **8** were unsuccessful. The analytical sample was purified by dissolving **8** in methanol, and precipitating with anhydrous ether; m.p. 106–107° (softening at 97°), $[\alpha]_D^{21}$ −14.6° (*c* 1.4, methanol); t.l.c. (*A*): R_F 0.11; ν_{max}^{KBr} 3400–3300 (NH), 1725 (OAc), 1650 (benzyloxycarbonyl C=O), and 1625–1525 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{35}H_{49}N_9O_{18}$: C, 47.56; H, 5.59; N, 14.26; O, 32.58. Found: C, 47.50; H, 5.64; N, 14.02; O, 32.40.

N-(*Benzyloxycarbonyl*)-L-*seryl*-L-*nitroarginyl*-L-*aspartic 1,4-diethyl ester* (**22**). — Compound **13** (0.24 g) was converted into the hydrobromide (**19**) by dissolving **13** in glacial acetic acid (1.5 ml), treating the solution with 30% hydrogen bromide in acetic acid (1.5 ml) for 1 h at room temperature, removing the acids *in vacuo*, washing with anhydrous ether, and drying in a vacuum desiccator in the presence of sodium hydroxide pellets. The residue was dissolved in *N,N*-dimethylformamide (5 ml), and triethylamine (27 µl) and *N*-(benzyloxycarbonyl)-L-serine pentachlorophenyl ester[11] (80 mg) were added. The mixture was stirred overnight, the *N,N*-dimethylformamide was removed *in vacuo*, and the residue was dissolved in ethyl acetate (50 ml). The solution was successively washed with 5% citric acid solution, water, 1% sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated *in vacuo*. The residue was crystallized from ethanol–ether, to give **22** (35 mg, 30%), m.p. 71–73°, $[\alpha]_D^{21}$ −24.3° (*c* 0.81, *N,N*-dimethylformamide); t.l.c. (*H*): R_F 0.14; ν_{max}^{KBr} 3300 (NH), 1650 (benzyloxycarbonyl C=O), and 1725–1525 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{25}H_{37}N_7O_{11} \cdot 0.5C_2H_5OH$: C, 49.21; H, 6.35; N, 15.45; O, 28.99. Found: C, 49.42; H, 6.42; N, 15.12; O, 29.26.

N-(*Benzyloxycarbonyl*)-L-*nitroarginyl*-L-*aspartic 1*-p-*nitrobenzyl ester* (**27**). — To a solution of L-aspartic 1-*p*-nitrobenzyl ester hydrobromide[21] (**24**, 88 mg) in *N,N*-dimethylformamide (2 ml) were added triethylamine (35 µl) and *N*-(benzyloxycarbonyl)-L-nitroarginyl pentachlorophenyl ester[11] (**25**; 0.15 g) in *N,N*-dimethylformamide (15 ml). The mixture was stirred overnight at room temperature, *N,N*-dimethylformamide was evaporated off, and the residue was dissolved in ethyl acetate. The solution was successively washed with M hydrochloric acid and water, dried (sodium sulfate), and evaporated, to give a syrup that crystallized from warm ethanol–water on cooling (50 mg, 33%), m.p. 124° (dec., shrank at 117–118°), $[\alpha]_D^{20}$ −14.4° (*c* 0.61, *N,N*-dimethylformamide); t.l.c. (*G*): R_F 0.7; ν_{max}^{KBr} 3500–3300 (NH), 1640 (benzyloxycarbonyl C=O), and 1630–1510 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{25}H_{29}N_7O_{11} \cdot H_2O$: C, 48.30; H, 5.02; N, 15.77. Found: C, 48.53; H, 4.73; N, 15.43.

N-(*Benzyloxycarbonyl*)-L-*seryl*-L-*nitroarginyl*-L-*aspartic 1*-p-*nitrobenzyl ester* (**30**). — A solution of L-nitroarginyl-L-aspartic 1-*p*-nitrobenzyl ester (**29**) was prepared by treating a solution of **27** (0.1 g) in glacial acetic acid (1.5 ml) with 30% hydrogen bromide in acetic acid (1.5 ml), keeping the mixture for 1 h at room temperature, evaporating off the acids *in vacuo*, washing with anhydrous ether, drying in a desiccator in the presence of sodium hydroxide pellets, and adding *N,N*-dimethylformamide (1 ml) and triethylamine (24 µl). To the resulting solution was added *N*-(benzyloxycarbonyl)-L-nitroarginyl pentachlorophenyl ester[11] (**25**, 80 mg), and the mixture was stirred overnight. The *N,N*-dimethylformamide was evaporated off and the residue was processed as described for the preparation of **22**, to give an oil that crystallized from ethanol–ether (60 mg, 53%), m.p. 137–138° (shrank at 125°),

$[\alpha]_D^{20}$ −12.3° (*c* 0.76, *N,N*-dimethylformamide); t.l.c. (*A*): R_F 0.16; ν_{max}^{KBr} 3400–3275 (NH), 1660 (benzyloxycarbonyl C=O), and 1550–1490 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{28}H_{34}N_8O_{13}$: C, 48.70; H, 4.96; N, 16.23. Found: C, 48.61; H, 5.00; N, 16.17.

*2-Acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-*L*-seryl-*L*-nitroarginyl-*L*-aspart-(1-*p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-*D*-glucopyranosylamine* (**9**). — To a solution of **30** (0.138 g) in 1:1 (v/v) benzene–ethanol (15 ml) were added **1** (ref. 12; obtained from the catalytic hydrogenation of 0.1 g of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glycopyranosyl azide[17]) and EEDQ (50 mg). The mixture was stirred overnight at room temperature, the solvents were evaporated off, and the residue was dissolved in ethyl acetate. The solution was successively washed with M hydrochloric acid, water, 1% sodium hydrogencarbonate, and water, dried (sodium sulfate), and evaporated. The residue crystallized from ethyl acetate–ethanol (53 mg, 26%), m.p. 186–187°, $[\alpha]_D^{20}$ −8.1° (*c* 0.44, *N,N*-dimethylformamide); t.l.c. (*E*): R_F 0.08; ν_{max}^{KBr} 3350 (NH), 1720 (OAc), 1650 (benzyloxycarbonyl C=O), and 1650–1525 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{42}H_{54}N_{10}O_{20} \cdot H_2O$: C, 48.69; H, 5.45; N, 13.52. Found: C, 48.92; H, 5.45; N, 13.06.

N-(*Benzyloxycarbonyl*)-*L*-*nitroarginyl*-*L*-*aspartyl*-*L*-*leucine methyl ester* (**33**). — A solution of 4-benzyl-*N*-(benzyloxycarbonyl)-L-aspartyl-L-leucine methyl ester[22] (**31**, 0.242 g) in 19:1 (v/v) acetic acid–water (20 ml) was hydrogenated in the presence of 10% palladium-on-charcoal (20 mg) for 6 h at room temperature and 2.5 atm. The catalyst was filtered off, and the filtrate was evaporated to dryness. The residue (**32**) was dried in a vacuum desiccator in the presence of sodium hydroxide pellets (to remove the last traces of acetic acid), and dissolved in *N,N*-dimethylformamide (10 ml). To the solution was added *N*-(benzyloxycarbonyl)-L-nitroarginyl pentachlorophenyl ester[11] (**25**, 0.3 g), and the mixture was stirred overnight. The *N,N*-dimethylformamide was evaporated off, and the residue was processed as described for the preparation of **22**, to give **33**, which was crystallized from methanol–ether (0.125 g, 12%), m.p. 95–97° (softening at 91°), $[\alpha]_D^{20}$ +21.3° (*c* 0.55, *N,N*-dimethylformamide); t.l.c. (*A*): R_F 0.44; ν_{max}^{KBr} 3300 (NH), 1650 (benzyloxycarbonyl C=O), and 1650–1525 cm^{-1} (peptide Amide I, NO_2).

Anal. Calc. for $C_{25}H_{37}N_7O_{10}$: C, 50.41; H, 6.26; N, 16.46; O, 26.86. Found: C, 50.36; H, 6.26; N, 16.53; O, 26.85.

*2-Acetamido-3,4,6-tri-*O*-acetyl-*N*-[*N*-(benzyloxycarbonyl)-*L*-nitroarginyl-*L*-aspart-1-oyl-(*L*-leucine methyl ester)-4-oyl]-2-deoxy-β-*D*-glucopyranosylamine* (**10**). — To a solution of **33** (0.1 g) in 1:1 (v/v) benzene–ethanol (15 ml) were added **1** [obtained from the catalytic hydrogenation of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl azide[17] (0.1 g)] and EEDQ (50 mg). The mixture was processed as described for the preparation of **9**. The residue crystallized from ethanol, to give **10** (26 mg, 17%), m.p. 219–220° (dec.; shrank at 204°), $[\alpha]_D^{20}$ +15.5° (*c* 0.9, *N,N*-

dimethylformamide); t.l.c. (*F*): R_F 0.2; ν_{max}^{KBr} 3300 (NH), 1740 (OAc), 1700–1525 (peptide Amide I, NO_2), and 1650 cm^{-1} (benzyloxycarbonyl C=O).

Anal. Calc. for $C_{39}H_{57}N_9O_{17}$: C, 50.70; H, 6.22; N, 13.65. Found: C, 50.61; H, 6.19; N, 13.68.

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