Carbohydrate Research, 32 (1974) 37–46

THE SYNTHESIS OF PROTECTED GLYCOPEPTIDES CONTAINING THE AMINO ACID SEQUENCES 34–37 AND 34–38 OF BOVINE RIBONUCLEASE B*

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(Received June 11th, 1973; accepted August 16th, 1973)

ABSTRACT

2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-1-oyl-(L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**21**) and 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-1-oyl-(L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**22**), 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-1-oyl-(glycine ethyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine, and 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-1-oyl-(phenylalanine methyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine were synthesized by condensation of 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine with the appropriate protected amino acids and tri- and tetra-peptides. The amino acid sequences of **21** and **22** correspond to the protected amino acid sequences 34–37 and 34–38 of ribonuclease B that are adjacent to the carbohydrate–protein linkage.

INTRODUCTION

Plasma glycoproteins, including the immunoglobulins as well as numerous other glycoproteins possessing hormonal or enzymic activities, are characterized by a 2-acetamido-1-*N*-(aspart-4-oyl)-2-deoxy-D-glucosylamine linkage between their carbohydrate chain(s) and the protein backbone[2]. The carbohydrate chain includes a "core" region composed of an additional 2-acetamido-2-deoxy-β-D-glucopyranosyl residue and of α- (and in the few glycoproteins investigated up to now β-) D-mannopyranosyl residues[3]; the outer part is composed of 2-acetamido-2-deoxy-β-D-gluco-

*Amino sugars LXXXV. This is publication No. 612 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts. This work was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases (AM-03564), National Institutes of Health, U.S. Public Health Service. A preliminary communication has been presented[1].
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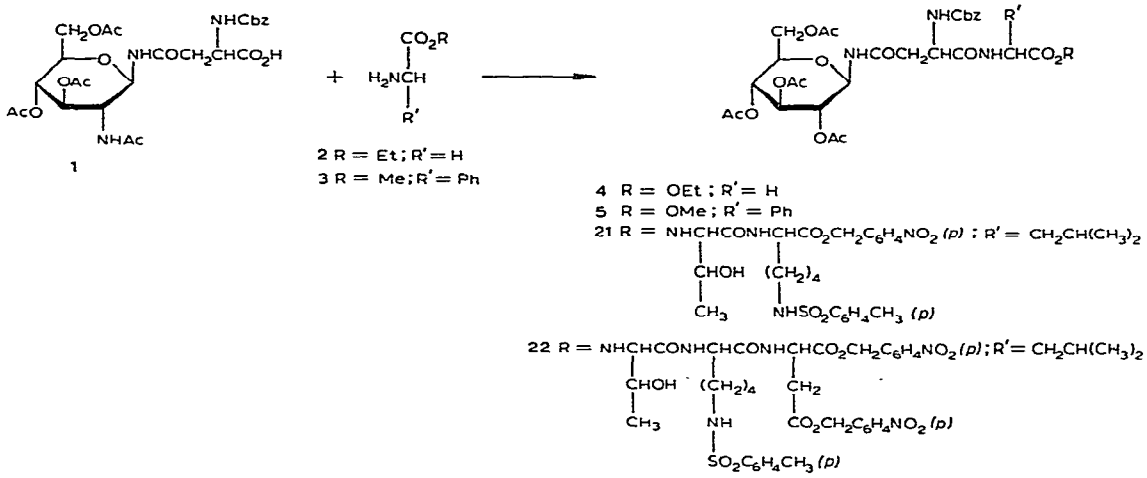
pyranosyl, β-D-galactopyranosyl, α-L-fucopyranosyl, and sialyl residues[2]. Although the biosynthesis of this outer part is well understood, very little is known of the synthesis of the "core" region and no enzymic systems have been isolated. On the other hand, it has been established that the structure Asn-X-Ser(or Thr) is always necessary for the attachment of a carbohydrate chain to the asparagine residue, and it has been suggested[4] that the nature of the residue X could direct the formation of the outer region. Finally, there is growing evidence that both the 2-acetamido-2-deoxy-D-glucose and D-mannose residues could be biochemically introduced into the glycopeptide molecule *via* activated isoprenoid sugar phosphates from the activated nucleotide sugars[5], a hypothesis difficult to prove with endogeneous acceptors and membrane-bound, particulate, enzymic systems.

Synthetic glycopeptide acceptors could be of great help in order to test these various hypotheses and isolate the biosynthetic systems. Glycopeptides derived from beef ribonuclease B were selected because the complete amino acid sequence of ribonuclease is known[6] and has been synthesized[7,8], and the carbohydrate sequence adjacent to the carbohydrate–protein linkage in ribonuclease B is known[9] and has been, in part, synthesized[10]. In addition, the carbohydrate chain of ribonuclease B is composed only of the core section, and enzymic systems that synthesize isoprenoid alcohol D-mannopyranosyl phosphate and may be active in the biosynthesis of this carbohydrate chain have been isolated from pancreas in this laboratory[11].

As a model substance for the preparation of glycopeptides having the structure of part of the molecule of ribonuclease B, protected glycopeptides containing a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue and the amino acid sequences 34–37 (**21**) and 34–38 (**22**) of ribonuclease B have been synthesized.

RESULTS AND DISCUSSION

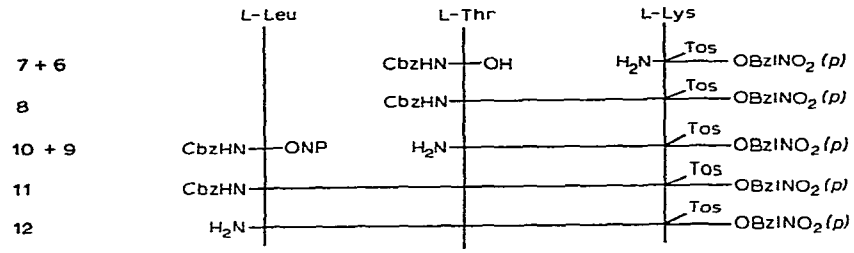
Many approaches are possible for the synthesis of glycopeptides. The pathway described in this paper, namely the attachment of a preformed oligopeptide to a glycosyl derivative of L-asparagine was selected because it could be applied to L-asparagine derivatives containing di- and tri-saccharides which, at the present time, are more cumbersome to synthesize than is the oligopeptide part; in addition, the unreacted carbohydrate moiety can be recovered. The direct condensation of a glycosylamine with the 4-carboxyl group of an L-aspartyl residue that is part of a peptide chain was not considered because of probable difficulties in recovering the unreacted glycosylamine from the glycopeptide and unreacted peptide mixture. As a first approach, attachment of a peptide to the 1-carboxyl group of a glycosyl–asparagine derivative was investigated; the carbohydrate moiety consisted of only one sugar residue and the peptide moiety of an amino acid and of a tri- and tetra-peptide, respectively, and the condensing agent was either *N,N'*-dicyclohexylcarbodiimide[12] (DCC) or *N*-ethyl-5-phenylisoxazolium 3'-sulfonate[13] (WRK). It was necessary to study the application of both condensing agents, as recent reports[14,15] have indicated that the WRK reagent gives less-satisfactory yields than the DCC reagent.



The condensation of the peptide moiety with 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine[16] (**1**) was at first investigated by coupling **1** with glycine ethyl ester (**2**) and L-phenylalanine methyl ester (**3**) as amino acid model substances, in the presence of DCC or WRK reagent to give **4** and **5**, respectively. In both condensations, the resulting compounds **4** and **5** could be obtained pure only after column chromatography when the DCC method was applied, whereas the WRK method gave crystalline compounds directly.

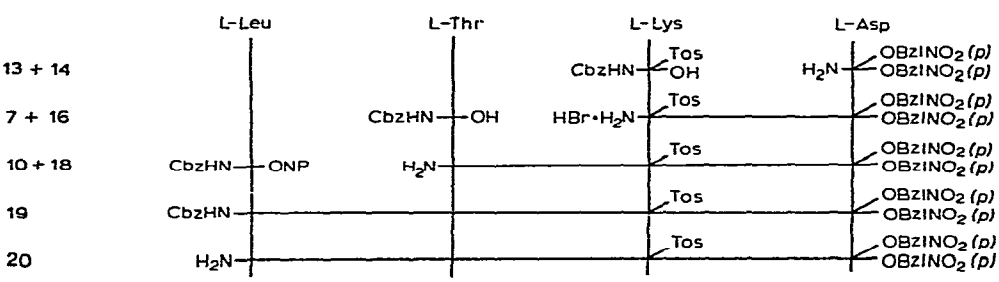
In order to study the efficiency of the condensing agent for larger peptide moieties, **1** was coupled with the protected amino acid sequence 34–37 (**12**) of beef ribonuclease in the presence of DCC and with the protected amino acid sequence 35–38 (**20**) in the presence of the WRK reagent. Condensation of an oligopeptide to the glycosyl–asparagine derivative was preferred to the sequential addition of amino acids to this derivative because the former procedure requires only one recovery of the unreacted carbohydrate–amino acid moiety. Thus, it is possible to devise a synthesis of the oligopeptide moiety based on the syntheses previously described[7,8], and especially to use the solid-support method for the synthesis of oligopeptides of molecular weight higher than the ones described in the present study. In the synthesis of the protected tripeptide **12**, N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester[17] (**6**) was coupled with *N*-benzyloxycarbonyl-L-threonine (**7**) in the presence of DCC to give crystalline *N*-benzyloxycarbonyl-L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester (**8**), which was treated with hydrogen bromide in acetic acid and then triethylamine. The resulting dipeptide having a free amino group (**9**) was condensed with the *p*-nitrophenyl ester of *N*-benzyloxycarbonyl-L-leucine[18] (**10**) to give *N*-benzyloxycarbonyl-L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester (**11**), which, on treatment with hydrogen bromide in acetic acid, afforded the tripeptide ester having a free amino group (**12**). Condensation of the latter compound with the L-asparagine derivative (**1**) in the presence of DCC resulted in the protected glycopeptide, 2-acetamido-3,4,6-tri-*O*-

acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-1-oyl-(L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**21**) having the amino acid sequence 34–37 of ribonuclease B.



NP = $C_6H_4NO_2$ (*p*); BzlNO$_2$ (*p*) = $CH_2C_6H_4NO_2$ (*p*); Cbz = $O_2CCH_2C_6H_5$

The synthesis of the protected tetrapeptide **20** was accomplished by condensation of N^{α}-benzyloxycarbonyl-N^{ε}-tosyl-L-lysine (**13**) with L-aspartic *p*-nitrobenzyl diester[19] (**14**) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) to give N^{α}-benzyloxycarbonyl-N^{ε}-tosyl-L-lysyl-L-aspartic *p*-nitrobenzyl diester (**15**), followed by treatment with hydrogen bromide in acetic acid. The resulting hydrobromide salt (**16**) was condensed with *N*-benzyolxycarbonyl-L-threonine (**7**) in the presence of DCC to give *N*-benzyloxycarbonyl-L-threonyl-N^{ε}-tosyl-L-lysyl-L-aspartic *p*-nitrobenzyl diester (**17**). Removal of the N^{α}-benzyloxycarbonyl group with hydrogen bromide in acetic acid, followed by treatment with triethylamine gave the protected tripeptide (**18**), which was condensed with the *p*-nitrobenzyl ester of *N*-benzyloxycarbonyl-L-leucine[18] (**10**) to give the tetrapeptide N^{α}-benzyloxycarbonyl-L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysyl-L-aspartic *p*-nitrobenzyl diester (**19**). Treatment of **19** with hydrogen bromide in acetic acid, followed by addition of triethylamine gave the protected tetrapeptide unit, L-leucyl-L-threonyl-N^{ε}-tosyl-L-lysyl-L-aspartic *p*-nitrobenzyl diester (**20**), which was coupled with **1** in the presence of WRK to give 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-aspart-1-oyl-(L-leucyl-L-threonyl-N^{ε}-tosyl-L-



NP = $C_6H_4NO_2$ (*p*); BzlNO$_2$ (*p*) = $CH_2C_6H_4NO_2$ (*p*); Cbz = $O_2CCH_2C_6H_5$

lysyl-L-aspartic *p*-nitrobenzyl diester)-4-oyl]-2-deoxy-β-D-glucopyranosylamine (**22**) having the amino acid sequence 34–38 of ribonuclease B.

The yields of the preparation of the glycosyltetrapeptide (**21**) in the presence of DCC and of the glycosylpentapeptide **22** in the presence of WRK were 7% and 12%, respectively, indicating a greatly improved yield when the WRK reagent was used instead of DCC and, thus, confirming the results of the condensation with single amino acids.

We have not considered the possibility of a rearrangement of the 2-acetamido-2-deoxy-D-glucopyranosylaminyl residue from the 4-carboxyl to the 1-carboxyl group of asparagine during the condensation of **1** with the amino acids **2** and **3** or with the peptides **12** and **20**, which would result in derivatives having the glycosyl residue linked to the 1-carboxyl group and the amino acid or peptide residues linked to the 4-carboxyl group of the asparagine molecule for the following reasons: (*a*) No rearrangement has been observed during the coupling of various mono-, di-, and tri-saccharide glycosylamines with 1-benzyl *N*-benzyloxycarbonyl L-aspartate in the presence of DCC[10,20,21]; (*b*) this rearrangement was observed only under strong alkaline conditions[22–24], not used in the present work.

EXPERIMENTAL

General methods. — Melting points were determined with a Mettler FP-2 apparatus and correspond to "corrected melting points". Rotations were determined for solutions in 1-dm, semimicro tubes with a Perkin–Elmer No. 141 polarimeter. The *N,N*-dimethylformamide used was Spectro-reagent 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 (70–325 mesh, E. Merck, Darmstadt, Germany), used without pretreatment; the ratio of the weight of substance to the weight of silica gel was 1:50; the volume of the fractions collected was 3–4 ml per g of the substance; and the ratio of diameter of the column to its length was 1:18. The homogeneity of compounds was verified by ascending t.l.c. on precoated plates of Silica Gel (Merck); the spots were detected by spraying with 20% sulfuric acid and heating at 200° for a few min. The microanalyses were performed by Dr. W. Manser, Zurich, Switzerland. The amino acid composition of hydrolyzates of **21** and **22** were determined (A) with a Beckman Spinco Model 117 amino acid analyzer and (B) by g.l.c. of the *N*-trifluoroacetyl butyl esters with a Perkin–Elmer Model 900 gas chromatograph on a column of Tabsorb (Regis Chemical Co., Chicago, Ill. 60610) programmed for a rise of 4° per min from 75° to 225°. The peptides were hydrolyzed by heating with constant boiling-point hydrochloric acid (*ca.* 5.8M) for 24 h at 108°, followed by evaporation of the solution in a high vacuum in the presence of sodium hydroxide pellets. The dry residue was heated with 3M hydrogen chloride in butanol (0.5 ml) for 1 h at 100°, followed by treatment with a 25% solution of trifluoroacetic anhydride in dichloromethane (0.1 ml) for 1 h at 100°. The results are reported in

molecular proportions relative to the asparagine residue. Discrepancies between the found and calculated results for basic amino acid residues have been previously reported[25] when carbohydrate residues are present. In addition, analysis of crystalline N^{ε}-tosyl-L-lysine by method B showed the recovery of only 70% of L-lysine.

N-*Benzyloxycarbonyl*-L-*threonyl*-N^{ε}-*tosyl*-L-*lysine* p-*nitrobenzyl ester* (**8**). — Triethylamine (0.4 ml) was added to a mixture of *N*-benzyloxycarbonyl-L-threonine (**7**, 0.77 g, Cyclo Chemical Company, Los Angeles, Calif. 90001) and N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester (**6**) benzenesulfonate[17] (1.7 g) in 5:1 dichloromethane–*N*,*N*-dimethylformamide (30 ml) at 0°. *N*,*N'*-Dicyclohexylcarbodiimide (0.06 g) was added and, after 3 h at 0°, the reaction mixture was kept overnight at room temperature. The insoluble *N*,*N'*-dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The syrup was dissolved in ethyl acetate (200 ml), and the solution washed successively with 1% sodium hydrogen carbonate, water, M hydrochloric acid, and water, dried (magnesium sulfate), and evaporated. The residue was triturated with ether, and the resulting product (1.8 g, 93%) crystallized from hot ethyl acetate, m.p. 138.5–139°, $[\alpha]_D^{20}$ −10.5° (*c* 1.0, chloroform); i.r. data: ν_{max}^{KBr} 3320 (OH), 3250 (NH), 1725 (benzyloxycarbonyl group CO), and 1700–1600 cm^{-1} (peptide amide I); R_F 0.25 in 9:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{32}H_{38}N_4O_{10}S$: C, 57.24; H, 5.71; N, 8.35; S, 4.77. Found: C, 57.33; H, 5.78; N, 8.32; S, 4.92.

N-*Benzyloxycarbonyl*-L-*leucyl*-L-*threonyl*-N^{ε}-*tosyl*-L-*lysine* p-*nitrobenzyl ester* (**11**). — A solution of **8** (0.67 g) in glacial acetic acid (4 ml) was treated with 30% hydrogen bromide in acetic acid (4 ml) for 1 h at room temperature. Anhydrous ether was added to precipitate the hydrobromide of L-threonyl-N^{ε}-tosyl-L-lysine *p*-nitrobenzyl ester (**9**), which was rapidly filtered off and immediately dissolved in the minimum volume of *N*,*N*-dimethylformamide (1 ml). Triethylamine (0.14 ml), *N*-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[18] (**10**, 0.38 g), and chloroform (8 ml) were added, and the solution was stirred for 24 h at room temperature. After evaporation, the syrup was washed with ether, and the residue dissolved in ethyl acetate. The solution was washed successively with 1% sodium hydrogen carbonate, water, M hydrochloric acid, and water, and dried (sodium sulfate). Evaporation and crystallization from methanol afforded 0.35 g (44%), m.p. (sint. 75°) 153–154°, $[\alpha]_D^{20}$ −33° (*c* 1.2, chloroform); i.r. data: ν_{max}^{KBr} 3300 (OH, NH), 1740 (benzyloxycarbonyl group CO), and 1610 and 1620 cm^{-1} (peptide amide I); R_F 0.23 in 9:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{38}H_{48}N_5O_{11}S$: C, 58.31; H, 6.18; N, 8.95; S, 4.10. Found: C, 58.19; H, 6.24; N, 8.77; S, 4.20.

N^{α}-*Benzyloxycarbonyl*-N^{ε}-*tosyl*-L-*lysyl*-(L-*aspartic* p-*nitrobenzyl 1,4-diester*) (**15**). — N^{α}-Benzyloxycarbonyl-N^{ε}-tosyl-L-lysine (**19**, 1.24 g) and 1,4-di(*p*-nitrobenzyl) L-aspartate (**14**) benzenesulfonate[19] (1.6 g) in acetonitrile (50 ml) were treated with triethylamine (0.4 ml) and the solution was cooled to 0°. *N*,*N'*-Dicyclohexylcarbodiimide (0.62 g) was added, and the resulting mixture stirred for 4 h at 0° and at room temperature overnight. The solvent was evaporated and the residue dissolved in ethyl

acetate. After filtration from the *N,N'*-dicyclohexylurea, the filtrate was successively washed with M hydrochloric acid, water, 1% sodium hydrogen carbonate solution, water, and dried (sodium sulfate). After evaporation, the residue was crystallized from ethyl acetate (2.2 g, 77%), m.p. 144–145° (sint. at 141°), $[\alpha]_D^{20}$ +9.1° (*c* 1.4, chloroform); i.r. data: ν_{max}^{KBr} 3300 (NH), 1690 (benzyloxycarbonyl group C=O), and 1525–1725 cm^{-1} (peptide amide I); R_F 0.31 in 9:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{39}H_{41}N_5O_{13}S$: C, 57.14; H, 5.04; N, 8.53; S, 3.92. Found: C, 57.27; H, 5.15; N, 8.55; S, 3.97.

N$^{\varepsilon}$-*Tosyl*-L-*lysyl*-(L-*aspartic* p-*nitrobenzyl 1,4-diester*) *hydrobromide* (**16**). — A solution of **15** (1.0 g) in acetic acid (4 ml) was treated with 30% hydrogen bromide in acetic acid (4 ml). After 1 h, ether was added and the precipitated salt (0.9 g, 96%) was collected and recrystallized from methanol–ether (0.75 g, 80%), m.p. 105–106°; $[\alpha]_D^{20}$ +2.8° (*c* 1.1, *N,N*-dimethylformamide); i.r. data: ν_{max}^{KBr} 3275, 3375 (NH), 2950 (NH_2^+), and 1525–1730 cm^{-1} (peptide amide I).

Anal. Calc. for $C_{31}H_{35}N_5O_{11}S \cdot HBr$: C, 48.55; H, 4.73; N, 9.13; S, 4.18. Found: C, 48.48; H, 4.78; N, 8.98; S, 4.27.

N-*Benzyloxycarbonyl*-L-*threonyl*-N$^{\varepsilon}$-*tosyl*-L-*lysyl*-(L-*aspartic* p-*nitrobenzyl 1,4-diester*) (**17**). — Solutions of *N*-benzyloxycarbonylthreonine (**7**, 0.126 g, Cyclo Chemical Co.) in dichloromethane (10 ml) and **16** (0.383 g) in *N,N*-dimethylformamide (2 ml) and triethylamine (0.07 ml) were cooled to 0°, and mixed. *N,N'*-Dicyclohexylcarbodiimide (0.10 g) was added and the mixture stirred for 8 h at 0° and overnight at room temperature. The dichloromethane was evaporated and the *N,N'*-dicyclohexylurea was filtered off. Upon dilution of the filtrate with water, the tripeptide was precipitated. After filtration, it was dissolved in ethyl acetate and the solution washed with M hydrochloric acid, water, 1% sodium hydrogen carbonate solution, and water, and then dried (sodium sulfate). After evaporation, crystallization from methanol gave 0.35 g (76%), m.p. 84–85°, $[\alpha]_D^{22}$ −4.2° (*c* 1.0, chloroform); i.r. data: ν_{max}^{KBr} 3290 (NH); 1675 (benzyloxycarbonyl CO), and 1525–1725 cm^{-1} (peptide amide I); R_F 0.18 in 9:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{43}H_{48}N_6O_{15}S$: C, 56.03; H, 5.25; N, 9.12. Found: C, 56.06; H, 5.21; N, 9.06.

N-*Benzyloxycarbonyl*-L-*leucyl*-L-*threonyl*-N$^{\varepsilon}$-*tosyl*-L-*lysyl*-(L-*aspartic* p-*nitrobenzyl 2,4-diester*) (**19**). — Compound **17** (0.49 g) was dissolved in acetic acid (2 ml) and 30% hydrogen bromide in acetic acid (2 ml) was added. After 1 h at room temperature, ether was added to precipitate the hydrobromide of **18**, which was washed with ether by decantation. It was dissolved in *N,N*-dimethylformamide (2 ml) and triethylamine (0.07 ml) was added to give free **18**. After addition of *N*-benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester[18] (**10**, 0.191 g) in chloroform (5 ml), the pale-yellow solution was kept for 24 h at room temperature. The chloroform was evaporated, ethyl acetate (5 ml) added, and the solution was successively washed with 1% sodium hydrogen carbonate solution, water, M hydrochloric acid, water, and then dried (magnesium sulfate). After evaporation of the solvent, the syrupy residue was crystallized from methanol (0.3 g, 58%), m.p. 108–109°, $[\alpha]_D^{22}$ −20° (*c* 1.4, chloro-

form); i.r. data: ν_{max}^{KBr} 3300 (NH), 1680 (benzyloxycarbonyl CO), and 1525–1730 cm^{-1} (peptide amide I); R_F 0.20 in 9:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{49}H_{59}N_7O_{16}S \cdot H_2O$: C, 55.95; H, 5.84; N, 9.32; S, 3.04. Found: C, 55.80; H, 5.69; N, 9.33; S, 3.55.

*2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[N-(benzyloxycarbonyl)-*L*-aspart-1-oyl-(glycine ethyl ester)-4-oyl]-2-deoxy-β-*D*-glucopyranosylamine* (**4**). — *A.* To a solution of 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-aspart-4-oyl]-2-deoxy-β-D-glucopyranosylamine[16] (**1**, 0.59 g) in dichloromethane (35 ml) was added a solution of glycine ethyl ester (**2**) [prepared by treatment of a solution of glycine ethyl ester hydrochloride (0.14 g) in *N,N*-dimethylformamide (2 ml) and triethylamine (0.14 ml)]. The suspension was stirred at 0° till a clear solution was obtained, *N,N'*-dicyclohexylcarbodiimide (0.21 g) was added, and the mixture stirred for 24 h at room temperature. The suspension was filtered, and the filtrate evaporated. The residue was extracted with chloroform (100 ml), and the solution washed successively with M hydrochloric acid, 1% sodium hydrogen carbonate, and water, and dried (magnesium sulfate). After evaporation, the crystalline residue (0.25 g) was dissolved in *N,N*-dimethylformamide. The *N,N'*-dicyclohexylurea crystallized at room temperature. After filtration, the mother liquor was refrigerated, and gave a product, m.p. 252–254°, which was further purified from the remaining *N,N'*-dicyclohexylurea by column chromatography on silica gel. Chloroform eluted *N,N'*-dicyclohexylurea (R_F 0.63 in 14:1, v/v, chloroform–methanol) and 7:1 (v/v) chloroform–methanol gave crystalline **4** (0.085 g, 12.5%), which was recrystallized from hot acetonitrile, m.p. 253–255°, $[\alpha]_D^{20}$ +8.9° (*c* 1.1, *N,N*-dimethylformamide; i.r. data: ν_{max}^{KBr} 3300 (NH), 1725 (OAc), 1690 (benzyloxycarbonyl group CO), and 1540–1640 cm^{-1} (peptide amide I); R_F 0.43, in 14:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{30}H_{40}N_4O_{14}$: C, 52.94; H, 5.92; N, 8.23; O, 32.91. Found: C, 52.94; H, 5.86; N, 8.08; O, 32.80.

B. To a solution of *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (0.125g) in acetonitrile (20 ml) at 0° was added **1** (0.298 g) and *N*-methylmorpholine (0.05 ml) in acetonitrile (40 ml). The reaction mixture was stirred for 65 min at room temperature until the suspension had clarified. A solution of glycine ethyl ester (**2**) hydrochloride (0.07 g, Aldrich Chemical Co.) in acetonitrile (12 ml) and *N*-methylmorpholine (0.05 ml) was added, and stirring was continued for 24 h. The acetonitrile was evaporated, dichloromethane (100 ml) added, and the solution washed successively with 1% hydrogen carbonate, water, M hydrochloric acid, and water, and dried (magnesium sulfate). After evaporation, crystallization of the residue from acetonitrile gave **4** (0.082 g, 24%), m.p. 253–254°; the i.r. spectrum was identical with that of the material obtained by method *A*.

*2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[N-(benzyloxycarbonyl)-*L*-aspart-1-oyl-(phenylalanine methyl ester)-4-oyl]-2-deoxy-β-*D*-glucopyranosylamine* (**5**). — *A.* L-Phenylalanine methyl ester (**3**) hydrochloride (0.108 g, Aldrich Chemical Co.) was condensed with **1** (0.298 g) in dichloromethane in the presence of *N,N'*-dicyclohexylcarbodiimide (0.103 g) after treatment with triethylamine (0.07 ml), essentially as described for the

preparation of **4** by method *A*. The resulting crude, white solid (0.35 g) was purified by column chromatography on silica gel. Chloroform eluted *N,N'*-dicyclohexylurea (R_F 0.63 in 9:1, v/v, chloroform–ethanol) and 14:1, v/v, chloroform–ethanol **5**, which was recrystallized from acetonitrile to give 0.05 g (13%), m.p. 271–272°, $[\alpha]_D^{20}$ +7.5° (*c* 1.0, *N,N*-dimethylformamide); i.r. data: ν_{max}^{KBr} 3300 (NH), 1735 (OAc), 1690 (benzyloxycarbonyl group CO), and 1520–1640 cm^{-1} (peptide amide I); R_F 0.48 in 14:1 (v/v) chloroform–methanol.

Anal. Calc. for $C_{36}H_{44}N_4O_{14}$: C, 57.15; H, 5.86; N, 7.40; O, 29.60. Found: C, 57.26; H, 5.88; N, 7.17; O, 29.22.

B. Compound **5** was prepared from **1** (0.298 g) by the same procedure *B* as described for **4**, starting from L-phenylalanine methyl ester (**3**) hydrochloride (0.108 g) instead of **2**. The crude product (0.15 g) was recrystallized from acetonitrile (0.13 g, 34%), m.p. 274–276°; the i.r. spectrum was identical with that of the product prepared by method *A*.

*2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N-[N-(*benzyloxycarbonyl*)-L-*aspart-1-oyl*-(L-*leucyl*-L-*threonyl*-N$^{\varepsilon}$-*tosyl*-L-*lysine* p-*nitrobenzyl ester*)-*4-oyl*]-*2-deoxy-β*-D-*glucopyranosylamine* (**21**). — To a solution of **11** (0.391 g) 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 resulting hydrobromide was precipitated with anhydrous ether and rapidly filtered off. Treatment with triethylamine (0.07 ml) in *N,N*-dimethylformamide (2 ml) gave the free amino acid peptide **12**, which was condensed with **1** (0.298 g) in dichloromethane (20 ml) in the presence of *N,N'*-dicyclohexylcarbodiimide (0.105 g) essentially under the same conditions as described for the preparation of **4**. The crude product (0.2 g) was purified by column chromatography on silica gel. Chloroform eluted *N,N'*-dicyclohexylurea and 19:1 (v/v) chloroform–ethanol gave **21**, which was recrystallized from 1:1 (v/v) methanol–chloroform (0.045 g, 7.5%), m.p. 248–250°, $[\alpha]_D^{20}$ −3.4° (*c* 0.3, *N,N*-dimethylformamide); t.l.c. (19:1, v/v, chloroform–ethanol): R_F 0.48, (9:1, v/v, chloroform–methanol): R_F 0.91; i.r. data: ν_{max}^{KBr} 3300 (NH), 1740 (OAc), 1700 (benzyloxycarbonyl group CO), and 1520–1660 cm^{-1} (peptide amide I).

Anal. Calc. for $C_{56}H_{73}N_8O_{21}S$: C, 54.86; H, 6.00; N, 9.13; S, 2.62. Found: C, 54.83; H, 6.07; N, 9.12; S, 2.76. Amino acids: Asp 1.00 (A, B); Lys 0.13 (A), 0.17, 0.29 (B); Leu 0.33 (A), 0.93, 0.92 (B); and Thr 0.88 (A), 1.06, 0.99 (B).

*2-Acetamido-3,4,6-tri-*O*-acetyl-1-*N-{N-(*benzyloxycarbonyl*)-L-*aspart-1-oyl*-[L-*leucyl*-L-*threonyl*-N$^{\varepsilon}$-*tosyl*-L-*lysyl*-(L-*aspartic* p-*nitrobenzyl 1,4-diester*]-*4-oyl*}-*2-deoxy-β*-D-*glucopyranosylamine* (**22**). — To a solution of *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (0.063 g) in acetonitrile (20 ml) at 0° was added **1** (0.15 g) and *N*-methylmorpholine (0.025 ml) in acetonitrile (20 ml). The reaction mixture was stirred at room temperature until the suspension had clarified (65 min). A solution of the hydrobromide of L-leucyl-L-threonyl-*N*$^{\varepsilon}$-tosyl-L-lysyl-(L-aspartic *p*-nitrobenzyl 2,4-diester) (**20**) was prepared by treatment of **19** (0.258 g) in acetic acid (1 ml) with 30% hydrogen bromide in acetic acid (1 ml) for 1 h at room temperature, followed by addition of ether and washing with this solvent by decantation. The precipitate was

dissolved in acetonitrile (10 ml) and treated with *N*-methylmorpholine (0.025 ml) to give **20**. This solution was added to the clear solution of **1** and stirring was continued for 24 h. The acetonitrile was evaporated and chloroform (75 ml) added. The solution was successively washed with a 1% sodium hydrogen carbonate solution, water, M hydrochloric acid, and water, and dried (sodium sulfate). After evaporation, crystallization of the residue from acetonitrile gave 37 mg (12%), m.p. 232–234° (dec) (sint. at 215°), $[\alpha]_D^{20}$ −6.7° (*c* 0.5, *N*,*N*-dimethylformamide); i.r. data: ν_{max}^{KBr} 3300 (NH), 1735 (OAc), 1650 (benzyloxycarbonyl group CO), and 1525–1690 cm^{-1} (peptide amide I); R_F 0.65 in 14:1 (v/v) chloroform–methanol, R_F 0.89 in 9:1 (v/v) chloroform–methanol. On evaporation of the mother liquor, additional material was obtained (5 mg).

Anal. Calc. for $C_{67}H_{84}N_{10}O_{26}S$: C, 54.47; H, 5.73; N, 9.46; S, 2.17. Found: C, 54.40; H, 5.72; N, 9.32; S, 2.24. Amino acids: Asp 2.00 (A, B); Lys 0.13 (A), 0.17, 0.31 (B); Leu 1.02 (A), 1.01, 0.99 (B); and Thr 1.00 (A), 1.19, 1.00 (B).

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