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Note

Enzymic synthesis of the sialylglycopeptide, α-D-Neu*p*Ac-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4*N*)-L-Asn

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Enzymic glycosylation seems to be an attractive possibility for modification of glycoproteins[1]. In a recent project, we have been engaged in sialylation of glycoproteins in order to increase their plasmatic lifetime[2]. In *N*-glycoproteins, oligosaccharide and polypeptide components are linked by the residue, 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine (**6**). Therefore, we selected the glycosylasparagine derivative **6** as a model compound in studies of enzymic glycosylation, and we report herein the enzymic synthesis of the sialyl glycopeptide **8** from the well-known[3] derivative **6** *via* the galactosylated intermediate **7** on a semipreparative scale (50 μmol). Two enzymic steps were involved, *i.e.*, D-galactosylation catalyzed by immobilized *N*-acetyllactosamine synthase (EC 2.4.1.90), and sialylation catalyzed by soluble CMP-*N*-acetylneuraminate: β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose α-(2→6)-sialyltransferase (EC 2.4.99.1). The preparation of several sialyloligosaccharides by enzymic synthesis has already been reported by different groups[4,5], but this is the first report of an enzyme-catalyzed synthesis of a glycopeptide. The structure of compounds **7** and **8** are not known to be present in any glycoproteins, but the glycosylasparagine derivatives **6, 7,** and **8** have been isolated from the urine of patients suffering from aspartylglucosaminuria[6–8].

Compound **6** was synthesized by published procedures with minor modifications. Thus, silver azide, which was formerly used[9], was advantageously replaced by tetrabutylammonium azide for the conversion of chloride **1** into azide **2** (70% yield). The ^{1}H-n.m.r. spectrum of **6** was identical with the one reported by Vliegenthart *et al.*[10] for compound **6** isolated from natural sources. However, we observed, for the H-1 resonance of the β-D-Glc*p*NAc residue, a broad signal instead of the expected doublet. This anomaly was previously reported by Brisson and Carver[11] for *N,N'*-

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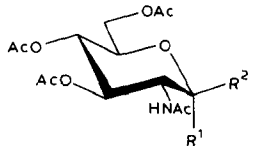
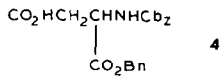


1 R^1 = Cl, R^2 = H
2 R^1 = H, R^2 = N_3
3 R^1 = H, R^2 = NH_2



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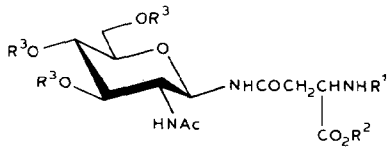
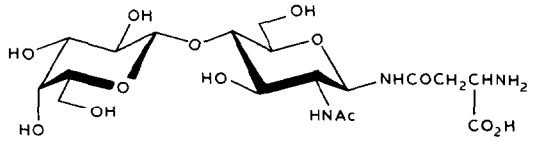


5 R^1 = Cbz, R^2 = Bn, R^3 = Ac
6 R^1 = R^2 = R^3 = H



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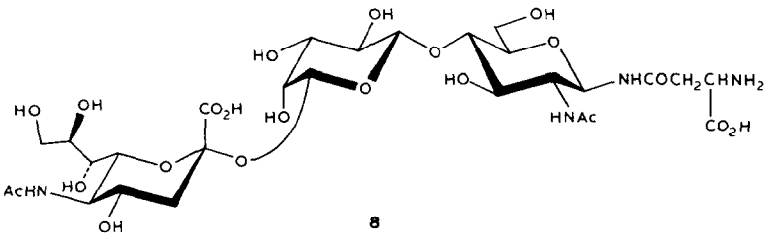

8

diacetylchitobiose. The same shape of the H-1 signal was also observed for compounds **7** and **8**.

Glycosylasparagine **6** could be galactosylated by use of the immobilized multienzyme system that regenerates UDP-α-D-galactose *in situ*, already described by us for oligosaccharides[12]. Owing to the poor affinity constant (K_m 15mM) of substrate **6** for β-D-galactosyltransferase, the enzymic galactosylation of **6** was achieved at high concentration of substrate (20mM). *N*-Acetyllactosaminylasparagine **7** was isolated as a pure compound in 26% yield after two gel filtrations. The same immobilized enzymes were utilized for three successive preparations of **7**. The observed optical rotation of **7** was an intermediate value between the one reported for

chemically synthesized[3] compound **7** and the one reported[7] for the natural compound **7**. Compound **7** exhibited a ^{1}H-n.m.r. spectrum identical with the one described in the literature[10]. *N*-Acetyllactosaminylasparagine **7** was subsequently incubated at 20mM concentration in aqueous buffer with soluble α-(2→6)-sialyltransferase and a stoichiometric amount of CMP-Neu5Ac[13], to give, in 38% yield after one gel filtration, sialyl-*N*-acetyllactosaminylasparagine (**8**) as a pure compound, characterized by n.m.r. spectroscopy. The ^{1}H-n.m.r. spectrum of **8** clearly exhibited signals for both anomeric protons and for the H-3*a* and H-3*e* protons of the Neu*p*5Ac residue. Their chemical shifts corresponded to the values reported by Vliegenthart *et al.*[10] for compound **8** isolated from the urine of a patient suffering from aspartylglucosaminuria. The proposed assignments for the ^{13}C-n.m.r. spectrum showed good agreement with the data reported in the literature[4,14,15].

The yield of sialylated compound **8** was about the same as the one reported in the literature for the enzymic sialylation of *N*-acetyllactosamine[5]; but it must be emphasized that, in our case, five times less enzymic activity was used for sialylating the same quantity of substrate. Immobilization of sialyltransferases should allow to scale up the level of sialyl-oligosaccharides and -glycopeptides synthesis to the millimole.

EXPERIMENTAL

General methods. — Melting points are uncorrected. Optical rotations were measured with a Roussel–Jouan electronic, digital micropolarimeter. ^{1}H-N.m.r. spectra were recorded at 250 MHz with a Bruker AM-250 spectrometer, the chemical shifts are given relative to the signal of tetramethylsilane as internal standard for solutions in $CDCl_3$, and as external reference (0.2% solution in $CDCl_3$) for solutions in D_2O. ^{13}C-N.m.r. spectra were recorded at 62.9 MHz with a Bruker AM-250 spectrometer; 1,4-dioxane was used as the internal standard (δ 67.40 from the signal of tetramethylsilane).

Dithiothreitol, UDP-α-D-glucose, α-D-glucopyranosyl phosphate, NAD^+, pyruvate kinase (EC 2.7.1.40), UDP-glucose pyrophosphorylase (EC 2.7.7.9), inorganic pyrophosphatase (EC 3.6.1.1), and UDP-galactose 4-epimerase (EC 5.1.3.2) were purchased from Sigma Chemical Co. *N*-Acetyllactosamine synthase was prepared from cow colostrum and partially purified by affinity chromatography on UDP-hexanolamine–Sepharose as described by Barker *et al.*[16] (170 units obtained from 2 L). α-(2→6)-Sialyltransferase (rat liver) was purchased from Boehringer Mannheim Corp. Enzymes of the galactosylation cycle were immobilized on Ultrogel A4 (4% Agarose) previously activated with cyanogen bromide according to Augé *et al.*[12]. CMP-Neu5Ac was synthesized from CMP with four immobilized enzymes according to our published procedure[13], and purified on DEAE-Sephadex A-25 (elution with a gradient of 0 to 0.75M triethylammonium hydrogencarbonate, pH 7.8).

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl azide (**2**). — A solution of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride (**1**) (19 g, 49 mmol) in dry toluene (570 mL) was heated at 80° for 1 h in the presence of

tetrabutylammonium azide[17] (98 mmol). The solution was evaporated and the residue was chromatographed on a silica gel column in 47:2:1 (v/v) ethyl acetate–hexane–acetone. Pure compound **2** crystallized from ethyl acetate–ether (12.8 g, 70%), m.p. 168–169°, $[\alpha]_D^{20}$ −44.5° (*c* 1.1, chloroform), $\nu_{max}^{CH_2Cl_2}$ 2120 cm^{-1} (N_3); ^{1}H-n.m.r. ($CDCl_3$): δ 5.82 (d, 1 H, $J_{2,NH}$ 8.5 Hz, NH), 5.28 (t, 1 H, $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 5.12 (t, 1 H, $J_{4,5}$ 9.5 Hz, H-4), 4.78 (d, 1 H, $J_{1,2}$ 9.5 Hz, H-1), 4.28 (dd, 1 H, $J_{5,6b}$ 5, $J_{6a,6b}$ 12.5 Hz, H-6b), 4.16 (dd, 1 H, $J_{5,6a}$ 2.5 Hz, H-6a), 3.93 (dt, 1 H, H-2), 3.8 (o, 1 H, H-5), 2.1, 2.04, and 1.97 (3 s, 3 H, 6 H, 3 H, 3 OAc and NAc); lit.[9] m.p. 170–171°, $[\alpha]_D^{23}$ −46.3° (*c* 1.1, chloroform).

2-Acetamido-N-(L-*aspart-4-oyl*)-*2-deoxy*-β-D-*glucopyranosylamine* (**6**). — An equimolecular mixture of **3** (ref. 18) (0.54 g, 1.56 mmol) and 1-benzyl *N*-benzyloxycarbonyl-L-aspartate (**4**) {0.56 g, m.p. 82–83°, $[\alpha]_D^{20}$ −10° (*c* 2.5, acetone)} was stirred with *N,N'*-dicyclohexylcarbodiimide (0.4 g, 2 mmol) in dry dichloromethane and treated according to the reported procedure[3] to afford **5** (0.68 g, 61%), m.p. 225°, $[\alpha]_D^{20}$ +7° (*c* 1, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 7.38 (m, 10 H, arom.), 7.18 (d, 1 H, *J* 8 Hz, NH), 6.06 (d, 1 H, *J* 8 Hz, NH), 5.82 (d, 1 H, *J* 8 Hz, NH), 5.09 (m, 7 H, H-1,3,4, 2 PhCH_2O), 4.66 (m, 1 H, H-2), 4.30 (dd, 1 H, $J_{5,6b}$ 4.5, $J_{6a,6b}$ 12 Hz, H-6b), 4.16 (m, 2 H, H-6a, H-α), 3.72 (m, 1 H, H-5), 2.89 (dd, 1 H, $J_{\alpha,\beta'}$ 4.5, $J_{\beta,\beta'}$ 16.5 Hz, H-β'), 2.70 (dd, 1 H, $J_{\alpha,\beta}$ 4.5 Hz, H-β), 2.08, 2.06, 2.05, and 1.83 (4 s, 3 H each, 3 OAc and NAc).

Alkaline treatment of **5** (0.605 g, 0.85 mmol), followed by catalytic hydrogenation performed as previously described[3], gave **6** (0.168 mg, 56%), m.p. 220° (from aqueous ethanol), $[\alpha]_D^{18}$ +18.6° (*c* 0.945, water); ^{1}H-n.m.r. (D_2O): δ 5.05 (br, 1 H, H-1), 3.94 (dd, 1 H, $J_{\alpha,\beta'}$ 4, $J_{\alpha,\beta}$ 6.5 Hz, H-α), 3.85 (dd, 1 H, $J_{5,6a}$ 2, $J_{6a,6b}$ 12.5 Hz, H-6a), 3.78 (t, 1 H, $J_{1,2} = J_{2,3}$ 10 Hz, H-2), 3.70 (dd, 1 H, $J_{5,6b}$ 5 Hz, H-6b), 3.57 (t, 1 H, $J_{3,4}$ 10 Hz, H-3), 3.48 (m, 1 H, H-5), 3.43 (t, 1 H, $J_{4,5}$ 10 Hz, H-4), 2.94 (dd, 1 H, $J_{\beta,\beta'}$ 17 Hz, H-β'), 2.81 (dd, 1 H, H-β), and 2.0 (s, 3 H, NAc); lit.[19] m.p. 219–221°, $[\alpha]_D^{26}$ +24° (*c* 1.0, water); lit.[7] $[\alpha]_D^{20}$ +23°.

O-β-D-*Galactopyranosyl-(1→4)-2-acetamido-1*-N-(L-*aspart-4-oyl*)-*2-deoxy*-β-D-*glucopyranosylamine* (**7**). — Compound **6** (168 mg, 0.5 mmol), α-D-glucopyranosyl phosphate (200 mg, 0.55 mmol), phosphoenolpyruvate (114 mg, 0.55 mmol), and UDP-α-D-glucose (7.5 mg, 0.012 mmol) were added to a solution of the following cofactors in 0.1M Tris buffer (pH 8, 0.5 mL): NAD^+ (19 mg, 0.025 mmol), $MnCl_2$ (10 mg, 0.050 mmol), $MgCl_2$ (20 mg, 0.1 mmol), KCl (130 mg, 1.75 mmol), dithiothreitol (38 mg, 0.25 mmol), and NaN_3 (2.5 mg). To this solution, adjusted to pH 8.0, was added the suspension of the following immobilized enzymes in the same buffer (20 mL): β-D-galactosyltransferase (3.8), pyruvate kinase (11.8), UDP-D-glucose pyrophosphorylase (2.2), inorganic pyrophosphatase (13), and UDP-D-galactose 4-epimerase (2.5 units). The reaction was allowed to proceed at 30° under N_2 with gentle shaking for 4 days. The gel was removed by filtration, washed with twice-distilled water, and the filtrate was freeze-dried. The residue was dissolved in a minimum volume of water and applied to a column (2.6 × 70 cm) of Bio-Gel P-2 (200–400 mesh), equilibrated and eluted with water. The fractions containing compound **7**

were identified by t.l.c. on celulose (4:1 phenol–water, w/v; ninhydrin spray), pooled, and freeze-dried. Compound **7** was still contaminated by substrate **6**. An additional purification on a column (2.6 × 50 cm) of Bio-Gel P-2 (minus 400 mesh) in water afforded pure **7** (65 mg, 26%), $[\alpha]_D^{18}$ +8.9° (*c* 1.12, water); ^{1}H-n.m.r. (D_2O): δ 5.10 (br, 1 H, H-1), 4.48 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 3.93 (d, 1 H, $J_{3',4'}$ 3 Hz, H-4'), 3.67 (dd, 1 H, $J_{2',3'}$ 10 Hz, H-3'), 3.54 (dd, 1 H, H-2'), 2.96 (dd, 1 H, $J_{\alpha,\beta'}$ 4, $J_{\beta,\beta'}$ 17 Hz, H-β'), 2.85 (dd, 1 H, $J_{\alpha,\beta}$ 7 Hz, H-β), and 2.02 (s, 3 H, NAc); lit.[3] $[\alpha]_D^{20}$ +6° (*c* 1.1, methanol); lit.[7] $[\alpha]_D^{20}$ +12° (*c* 1.12, water).

O-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-*2-nonulopyranosylonic acid)-(2→6)*-O-β-D-*galactopyranosyl-(1→4)-2-acetamido-1*-N-(L-*aspart-4-oyl)-2-deoxy-β*-D-*glucopyranosylamine* (**8**). — Compound **7** (65 mg, 0.130 mmol) and CMP-Neu5Ac ditriethylammonium salt (108 mg, 0.130 mmol) were dissolved in 0.05M sodium cacodylate (pH 6.0, 6.5 mL) containing 0.05M NaCl, and incubated for 24 h at 37° in a plastic tube with α-(2→6)-sialyltransferase (500 munits) and bovine serum albumin (6.5 mg). The mixture was freeze-dried, the residue taken up in water (2 mL), and the solution applied to a column (2.5 × 6 cm) of Bio-Gel P-2 (200–400 mesh) equilibrated and eluted with water. The fractions containing pure sialylglycopeptide **8**, as evidenced by t.l.c. on cellulose (4:1 phenol–water, w/v; ninhydrin spray) were pooled and freeze-dried to afford **8** as a white powder (40 mg, 38%), $[\alpha]_D^{20}$ −7.4° (*c* 0.815, water); ^{1}H-n.m.r. (D_2O): δ 5.13 (br, 1 H, H-1), 4.43 (d, 1 H, $J_{1',2'}$ 8 Hz, H-1'), 2.84 (m, 2 H, H-β,β'), 2.65 (dd, 1 H, $J_{3''a,3''e}$ 12.5, $J_{3''e,4''}$ 5 Hz, H-3''*e*), 2.02 (s, 3 H, NAc), 2.00 (s, 3 H, NAc), and 1.70 (t, 1 H, $J_{3''a,4''}$ 12.5 Hz, H-3''*a*); ^{13}C-n.m.r. (D_2O): δ 22.47, 22.57 ($NHCOCH_3$), 35.39 (C-β Asn), 40.51 (C-3''), 51.33 (C-α Asn), 52.29 (C-5''), 53.95 (C-2), 60.53 (C-6), 63.10 (C-9''), 63.83 (C-6'), 68.60 (C-4'',7''), 68.83 (C-4'), 71.16 (C-2'), 72.16 (C-8''), 72.75 (C-6''), 72.99 (C-3'), 73.31 (C-3), 74.13 (C-5'), 76.61 (C-5), 78.73 (C-1), 80.60 (C-4), 100.63 (C-2''), 103.92 (C-1'), 173.06, 174.05 (C-1'', C-γ Asn), and 175.45 (C=O).

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