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Synthesis of pentasaccharide analogues of the *N*-glycan substrates of *N*-acetylglucosaminyltransferases III, IV and V using tetrasaccharide precursors and recombinant β-(1 → 2)-*N*-acetylglucosaminyltransferase II

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Abstract

Recombinant human UDP-GlcNAc:α-Man-(1 → 6)R β-(1 → 2)-*N*-acetylglucosaminyltransferase II (EC 2.4.1.143, GlcNAc-T II) was produced in the Sf9 insect cell/baculovirus expression system as a fusion protein with a $(His)_6$ tag and partially purified by affinity chromatography on a metal chelating column. The partially purified enzyme was used to catalyze the transfer of GlcNAc from UDP-GlcNAc to R-α-Man(1 → 6)(β-GlcNAc(1 → 2)α-Man(1 → 3))β-Man-*O*-octyl to form β-GlcNAc(1 → 2)R-α-Man(1 → 6)(β-GlcNAc(1 → 2)α-Man(1 → 3))β-Man-*O*-octyl where there is either no modification of the α-Man(1 → 6) residue (**7**), or where R is 3-deoxy (**8**), 4-deoxy (**9**) or 6-deoxy (**10**). The yields ranged from 64–80%. Products were characterized by ^1H and ^{13}C nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry. Compounds **7–10** are pentasaccharide analogues of the biantennary *N*-glycan substrates of *N*-acetylglucosaminyltransferases III, IV and V.

Keywords: Pentasaccharide analogues; *N*-Glycan substrates; *N*-Acetylglucosaminyltransferases; β-(1 → 2)-*N*-Acetylglucosaminyltransferase II

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1. Introduction

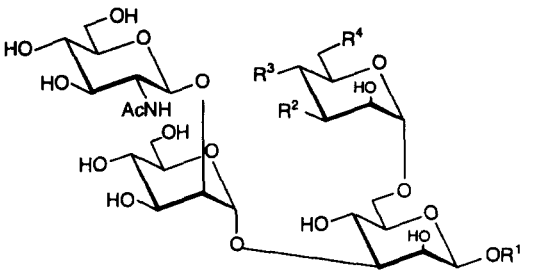
Highly branched complex asparagine-linked oligosaccharides (*N*-glycans) are important in many biological phenomena such as cell–cell interactions, embryonic development and the metastatic behaviour of cancer cells [1–8]. Recent experiments have shown that transgenic mouse embryos lacking a functional *N*-acetylglucosaminyltransferase I (GlcNAc-T I) gene are unable to make complex *N*-glycans and die at about 10 days after fertilization [9,10]. Six *N*-acetylglucosaminyltransferases (GlcNAc-T I to VI) control the synthesis of complex *N*-glycans by initiation of branches on the core structure [7,11,12]. It is therefore of interest to synthesize oligosaccharide analogues of the natural *N*-glycan substrates of these enzymes to obtain specific substrates for accurate enzyme assay, to study substrate specificity and to develop potential enzyme inhibitors.

With the increasing availability of recombinant glycosyltransferases [13–16], combined chemical-enzymatic synthesis of oligosaccharides is an attractive alternative to total chemical synthesis [17–27]. Glycosyltransferases usually give high yields and a high regio-specificity in glycosylation reactions [17]. Rabbit liver UDP-GlcNAc:α-Man-(1 → 6)R β-(1 → 2)-*N*-acetylglucosaminyltransferase II (EC 2.4.1.143, GlcNAc-T II) has been used by Hindsgaul's group [25,26] for the preparative synthesis of oligosaccharides. It is difficult to purify GlcNAc-T II from natural sources [28]. Recently, however, the genes encoding rat [29] and human [30] GlcNAc-T II have been cloned and recombinant forms of the active enzyme have become available. Since compound **6** (Fig. 1) is an excellent substrate for GlcNAc-transferases III [31], IV [32] and V [33], we have synthesized novel pentasaccharide analogues of compound **6** using synthetic modified tetrasaccharides as substrates for recombinant GlcNAc-T II. The stereo- and regiospecificities of the glycosidic linkages formed were determined by high resolution ^{1}H and ^{13}C NMR spectroscopy.

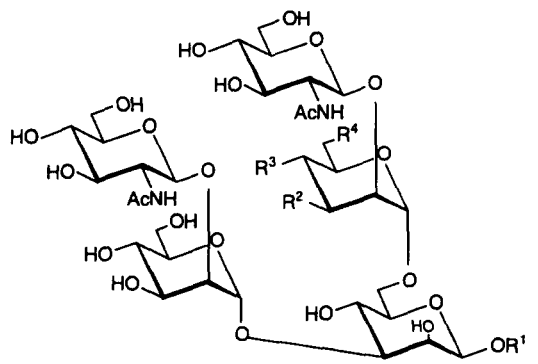
2. Results and discussion

Most glycosyltransferases show high specificity towards acceptor substrates in vivo [34]. However in vitro, at high substrate concentrations and in the absence of competing enzymes, the specificity is not absolute and deviations from the natural substrate are often tolerated (e.g., hydroxyl groups may be substituted by hydrogen, fluorine, *O*-methyl or larger groups). The tolerance for modifications is different for each enzyme and must be elucidated by substrate specificity studies [35–42].

GlcNAc-T II can convert **1** to **6** [43] and **2** to **7** [25], respectively (see Fig. 1 for formulae). The enzyme transfers a GlcNAc residue to the 2‴-position of the α-(1 → 6)-linked Man residue in α-Man‴(1 → 6)(β-GlcNAc″(1 → 2)α-Man′(1 → 3))β-Man-O-R. In previous substrate specificity studies we determined permissible substrate modifications for GlcNAc-T II [35]. Surprisingly, we found that substitutions of the 3‴-, 4‴- and 6‴-OH groups, near the site of enzyme action, by deoxy groups (compounds **3–5** [44]) or by *O*-methyl groups yielded good substrates. The V_{max} values for compounds **2–5** were, respectively, 8, 72, 11 and 14 μmol/min/mg [35].



1 R^1=-4GlcNAc-β-(1→4)GlcNAc-Asn-x; $R^2 = R^3 = R^4 = OH$
2 R^1= *n*-octyl; $R^2 = R^3 = R^4 = OH$
3 R^1= *n*-octyl; R^2 = H; $R^3 = R^4 = OH$
4 R^1= *n*-octyl; R^3 = H; $R^2 = R^4 = OH$
5 R^1= *n*-octyl; R^4 = H; $R^2 = R^3 = OH$



6 R^1=-4GlcNAc-β-(1→4)GlcNAc-Asn-x; $R^2 = R^3 = R^4 = OH$
7 R^1= *n*-octyl; $R^2 = R^3 = R^4 = OH$
8 R^1= *n*-octyl; R^2 = H; $R^3 = R^4 = OH$
9 R^1= *n*-octyl; R^3 = H; $R^2 = R^4 = OH$
10 R^1= *n*-octyl; R^4 = H; $R^2 = R^3 = OH$

Fig. 1.

In the present study, we have used recombinant GlcNAc-T II, expressed in Sf9 insect cells, to convert tetrasaccharide **2** and the deoxy analogues **3–5** to the modified pentasaccharides **7–10** in yields of 64–80% (Table 1). Compounds **8–10** are novel whereas the chemical synthesis of **7** has been reported previously [45]. A compound similar to **7** with a methoxycarbonyloctyl instead of an octyl spacer [25] as well as a 4′, 6‴-dideoxy derivative [26] have been synthesized by Hindsgaul's group using a chemoenzymatic approach.

The recombinant GlcNAc-T II used in this work is a fusion protein of the entire human GlcNAc-T II protein (cytoplasmic tail, trans-membrane domain, stem region and

Table 1
Enzymatic glycosylation with recombinant GlcNAc-T II

Product	Substrate	Method [a]	Reaction time(d) [b]	Yield (%) [c]	Molecular composition	FAB-MS (m/z) [d]
7	**2**	A	4	80		
8	**3**	A	7	64	$C_{42}H_{74}N_2O_{25}$	1029.4458 (MNa^+, −2.0 mmu)
8	**3**	B	5	67		
9	**4**	A	7	74	$C_{42}H_{74}N_2O_{25}$	1029.4491 (MNa^+, +1.3 mmu)
10	**5**	A	7	69	$C_{42}H_{74}N_2O_{25}$	1029.4453 (MNa^+, −2.5 mmu)

[a] A: GlcNAc-T II purified on Ni-NTA column, 45–90 ng enzyme/mL; B: GlcNAc-T II immobilised on Ni-NTA resin, ~100 μg enzyme/mL.
[b] Days at 28°C.
[c] Isolated yields, 2–7 mg
[d] In glycerol matrix unless otherwise stated

catalytic domain), a $(His)_6$ sequence upstream of the amino-terminal end and an enterokinase cleavage site [46]. The fusion protein was partially purified by metal chelating affinity chromatography on a nickel-nitrilo-tri-acetic acid (Ni-NTA) resin [47]. This preparation was stable at 4°C for several months. Alkaline phosphatase was added to the enzyme incubations to degrade the product UDP [48] which is an inhibitor of GlcNAc-T II [43]. Only two equivalents of commercially available UDP-GlcNAc per mole of acceptor substrate were needed to achieve complete conversion of the substrates as observed by thin layer chromatography (TLC). GlcNAc-T II requires a divalent cation like manganese or cobalt for activity [43] but unlike GlcNAc-T I which gives highest rates of incorporation with cobalt [36], GlcNAc-T II was more active with manganese. Under the conditions of incubation used in this study, almost complete conversion of oligosaccharide acceptor to product was detected by TLC after 4–7 days and yields of purified products ranged from 64 to 80%. Traces of trisaccharide acceptor were detected by TLC after purification on the SepPak cartridges and were removed by gel filtration on a Bio-Gel P-2 column. No impurities were detected in the final products by TLC and NMR analyses.

We also tested recombinant GlcNAc-T II bound to the Ni-NTA resin as an enzyme source for these reactions. The potential advantages of such an immobilized "solid-state" enzyme preparation are speed of enzyme purification, ease of separation of enzyme from reactants by centrifugation and re-utilization of the enzyme. We found that the resin-bound GlcNAc-T II was enzymatically active and that only traces of enzyme activity were solubilized from the beads during the reaction. The immobilized enzyme converted compound **3** to compound **8** (Table 1, method B) with a yield similar to that obtained with the partially purified soluble enzyme (Table 1, method A). However, approximately 2000-fold more enzyme was required to achieve this yield with the immobilized enzyme (Table 1). Prolonged reaction times (8–14 days) did not lead to lower yields in the case of the partially purified soluble enzyme, but with the Ni-NTA-bound enzyme, product is slowly degraded to a compound with the same mobility as substrate on TLC indicating that an *N*-acetylhexosaminidase may also be bound to the Ni-NTA resin. Furthermore, the bound GlcNAc-T II activity is not stable for more than a few days at 28°C so that

the enzyme beads could not be re-utilized. The partially purified soluble enzyme preparation is therefore a preferable reagent since small amounts of enzyme (~ 100 ng/3 mg acceptor) can be used and there is less degradation of product.

The structures of **7–10** were confirmed by ^{1}H and ^{13}C NMR and FAB-MS (Table 1). The ^{1}H NMR data for **7** obtained by chemo-enzymatic synthesis were identical to the data previously reported for **7** obtained by total chemical synthesis [45]. The new glycosidic linkages introduced into **8–10** by enzymatic incorporation of the GlcNAc'''' residue are in β-configuration since the signals for H-1'''' are doublets with $J_{H1'''',H2''''} = 8.5$ Hz. The coupled $^{1}H-^{13}C$ NMR spectrum (HMBC) shows the direct C-1–H-1 coupling constants in the expected range for all α-glycosidic linkages ($^{1}J_{C\text{-}1,H\text{-}1} = 168–170$ Hz) and β-glycosidic linkages ($^{1}J_{C\text{-}1,H\text{-}1} = 155–165$ Hz) [49–51]. Long-range couplings across glycosidic bonds were observed in the HMBC experiment between C-1''''/H-2''' and H-1''''/C-2''' of **8–10**, which proves the 1''''–2''' regio-specificity of the newly formed glycosidic linkages. FAB-MS of **7–10** gave a fragment with $m/z = 366$ indicative of HexNAc-Hex, and **8–10** gave an additional fragment with $m/z = 350$ indicative of HexNAc-(deoxy)Hex-.

In conclusion, we demonstrate the use of recombinant GlcNAc-T II for the synthesis of novel modified *N*-glycans and prove that the enzyme attaches a GlcNAc residue in β-(1''''–2''')-linkage to the modified acceptors **3–5** to produce compounds **8–10**. These compounds are intended as reagents for the study of the substrate requirements of GlcNAc-T III, IV and V and may also enable the development of inhibitors for these enzymes. The genes for GlcNAc-T III [52,53] and V [54,55] have recently been cloned and active recombinant enzymes have been obtained.

3. Experimental

General methods.—Alkaline phosphatase (calf intestine) was analytical grade (grade I) and was purchased from Boehringer Mannheim GmbH. TLC was performed on Silica Gel F_{254} (Merck) with detection by UV absorption and/or by charring with EtOH–H_2O–H_2SO_4 14:4:1 (v/v). Bio-Gel P-2 (200–400 mesh, Bio-Rad Laboratories) was used for gel filtration. ^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR spectra were recorded at 125.8 MHz, using a Varian Unity Plus 500 spectrometer. Assignments of ^{1}H and ^{13}C resonances were based on 2D-experiments ($^{1}H-^{1}H$ COSY, $^{1}H-^{1}H$ TOCSY, $^{1}H-^{13}C$ HMQC, $^{1}H-^{13}C$ HMBC) and polarisation-transfer experiments (APT). HMBC experiments were optimized for the detection of long-range couplings (5–7 Hz). High-resolution fast atom bombardment-mass spectrometry (FAB-MS) was performed in the positive-ion mode on a ZAB-SE mass spectrometer (VG Analytical) with polyethyleneglycol as internal standard, xenon as bombarding atom (8 keV, 1.2 mA dark anode current), and accelerated voltage scan in a narrow mass range. Compounds **2** [20,56] and **3, 4** and **5** [44,57] were synthesized as previously described.

Preparation of recombinant GlcNAc-T II.—The recombinant GlcNAc-T II used in this work is a fusion protein of the entire human GlcNAc-T II protein (cytoplasmic tail, trans-membrane domain, stem region and catalytic domain), a $(His)_6$ sequence upstream of the amino-terminal end and an enterokinase cleavage site. The details of this enzyme

preparation will be published elsewhere [46]. Briefly, Sf9 insect cells (2×10^8) were infected with recombinant baculovirus at a multiplicity of infection of 2 plaque forming units/cell. The cells were cultured at 28°C, harvested at 4 days post-infection and homogenised in 9 mL of 2% Triton X-100 in 0.2 M NaCl in the presence of protease inhibitors. The homogenate was centrifuged at 40,000 rpm at 4°C for 1 h and enzyme was partially purified from the supernatant by metal chelating affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA, Qiagen) resin [47], according to the instructions of the manufacturer. GlcNAc-T II was utilized for enzyme incubations either while still bound to the Ni-NTA resin or after elution from the resin with 200 mM imidazole (overall yield was 20%). Pressure dialysis was used to concentrate the soluble enzyme and to remove imidazole.

Preparative enzymatic glycosylation with recombinant GlcNAc-T II.—Method A. Compounds **7–10** were synthesized following the general procedure described below. The incubation was carried out in a total volume of 0.75–4.3 mL containing oligosaccharide acceptor (2.0–5.0 mM), MES [2-(*N*-morpholino)ethanesulfonate] buffer (50 mM, pH 6.8), UDP-GlcNAc (5–20 mM), $MnCl_2$ (17 mM), Triton X-100 (0.004–0.010%), bovine serum albumin (1 mg/mL) and alkaline phosphatase (50 mU/mL). Soluble GlcNAc-T II was added last at a concentration of 4.7 mU/mL (compound **2**), or 1.35 mU/mL (compound **3**), or 2.6 mU/mL (compounds **4** and **5**) and the solution was mixed by inversion. The reaction was kept at 28°C in the dark, without stirring, and was monitored by TLC ($CHCl_3$–MeOH–H_2O, 10:8:1, v/v/v). After 4–7 days of incubation, oligosaccharide acceptor was either not detected or detected only in traces by TLC. The reaction mixture was applied to a C_{18} SepPak cartridge (Waters Associates) pre-equilibrated with 5 mL MeOH followed by 30 mL H_2O. The cartridge was washed with H_2O (10 mL) and with CH_2Cl_2 (10 mL); washing with CH_2Cl_2 removes Triton but not **7–10** since these compounds are not soluble in this solvent. Products were eluted with MeOH (8 mL) and further purified by gel filtration on a Bio-Gel P-2 column with water as eluent to remove traces of acceptor and other impurities. The yields are shown in Table 1.

Method B. The enzyme incubation contained in a total volume of 1.0 mL compound **3** (3.1 mM), MES [2-(*N*-morpholino)ethanesulfonate] buffer (100 mM, pH 6.8), UDP-GlcNAc (15 mM), $MnCl_2$ (15 mM), Triton X-100 (0.05%) and bovine serum albumin (1.0 mg/mL). Recombinant GlcNAc-T II bound to Ni-NTA resin (0.4 mL, ~ 140 μg enzyme) was added to the incubation mixture. The mixture was kept in suspension by gentle inversion at room temperature in the dark. After 5 days of incubation, acceptor was detected only in trace amounts by TLC ($CHCl_3$–MeOH–H_2O, 10:8:1, v/v/v). The reaction mixture was filtered through glass wool to remove resin-immobilized enzyme and the product was purified as described above for Method A. The yield of purified product was 67% (Table 1).^{1}H and ^{13}C NMR parameters for **8–10** are as follows (only partial data are given).

Compound **8**.—^{1}H NMR (500 MHz, D_2O, HDO = 4.8 ppm): δ 5.15 (d, 1 H, $J_{1',2'}$ 1.0 Hz, H-1'), 4.78 (bs, 1 H, H-1‴), 4.69 (bs, 1 H, H-1), 4.63 (d, 1 H, $J_{1'''',2''''}$ 8.5 Hz, H-1''''), 4.57 (d, 1 H, $J_{1'',2''}$ 8.5 Hz, H-1''), 4.21 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, H-2'), 4.15 (d, 1 H, $J_{2,3}$ 2.5 Hz, H-2), 4.06 (m, 1 H, H-2‴), 4.02 (dd, 1 H, $J_{5,6a}$ 5.5 Hz, $J_{6a,6b}$ 11.5 Hz, H-6a), 2.19 (m, 1 H, H-3*eq*‴), 2.07 (2 × s, 6 H, CH_3CO), 1.89 (m, 1 H, H-3*ax*‴),

1.65–1.56 (m, 2 H, CH_2CH_2O), 1.40–1.24 (m, 10 H, CH_2 octyl), 0.88 (t, 3 H, CH_3 octyl).^{13}C NMR (125.8 MHz, D_2O, CH_3 octyl = 13.35), coupling constants from HMBC: δ 174.70 (2 × C=O), 100.02 (d, $J_{C\text{-}1'''',H\text{-}1''''}$ 165 Hz, C-1''''), 99.71 (d, $J_{C\text{-}1,H\text{-}1}$ 158 Hz, C-1), 99.56 (d, $J_{C\text{-}1'',H\text{-}1''}$ 160 Hz, C-1''), 99.27 (d, $J_{C\text{-}1',H\text{-}1'}$ 170 Hz, C-1'), 95.54 (d, $J_{C\text{-}1''',H\text{-}1'''}$ 170 Hz, C-1'''), 76.43 (C-2'), 74.34 (C-2'''), 70.22 (C-2), 70.12 (CH_2O octyl), 65.47 (C-6), 32.43 (C-3'''), 31.02, 28.62, 28.39, 28.32, 25.02, 21.94 (6 × CH_2 octyl), 22.24 (2 × CH_3 CO), 13.35 (CH_3 octyl).

Compound **9**.—^{1}H NMR (500 MHz, D_2O, HDO = 4.8 ppm): δ 5.15 (d, 1 H, $J_{1',2'}$ 1.6 Hz, H-1'), 4.98 (bs, 1 H, H-1'''), 4.68 (bs, 1 H, H-1), 4.63 (d, 1 H, $J_{1'''',2''''}$ 8.4 Hz, H-1''''), 4.57 (d, 1 H, $J_{1'',2''}$ 8.4 Hz, H-1''), 4.21 (dd, 1 H, $J_{2',3'}$ 3.2 Hz, H-2'), 4.14 (d, 1 H, $J_{2,3}$ 3.1 Hz, H-2), 4.11 (ddd, 1 H, $J_{2''',3'''}$ 3.31 Hz, $J_{3''',4'''eq}$ 4.18 Hz, $J_{3''',4ax'''}$ 11.9 Hz, H-3'''), 3.99 (m, 1 H, H-2'''), 3.85, 3.69 (2 × m, 2 H, CH_2O octyl) 2.08, 2.07 (2 × s, 6 H, CH_3CO), 1.69 (m, 1 H, H-4*eq*'''), 1.66–1.58 (m, 2 H, CH_2CH_2O), 1.55 (ddd, $J_{gem} = J_{4ax''',5'''}$ 12.0 Hz, H-4*ax*''') 1.39–1.25 (m, 10 H, CH_2 octyl), 0.88 (t, 3 H, CH_3 octyl). ^{13}C NMR (125.8 MHz, D_2O, CH_3CN = 0.765), coupling constants from HMBC: δ 174.68 (2 × C = O), 99.71 (d, $J_{C\text{-}1,H\text{-}1}$ 155 Hz, C-1), 99.56 (d, $J_{C\text{-}1'',H\text{-}1''}$ 160 Hz, C-1''), 99.36 (d, $J_{C\text{-}1'''',H\text{-}1''''}$ 160 Hz, C-1''''), 99.23 (d, $J_{C\text{-}1',H\text{-}1'}$ 168 Hz, C-1'), 97.23 (d, $J_{C\text{-}1''',H\text{-}1'''}$ 169 Hz, C-1'''), 76.43 (C-2'), 74.23 (C-2'''), 70.21 (C-2), 70.14 (CH_2O octyl), 64.28 (C-3'''), 31.02, 28.63, 28.37, 28.32, 25.04, 21.95 (6 × CH_2 octyl), 29.57 (C-4'''), 22.24 (2 × CH_3CO), 13.34 (CH_3 octyl).

Compound **10**.—^{1}H NMR (500 MHz, D_2O, HDO = 4.8 ppm): δ 5.15 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.89 (d, 1 H, $J_{1''',2'''}$ 1.5 Hz, H-1'''), 4.69 (bs, 1 H, H-1), 4.59 (d, 1 H, $J_{1'''',2''''}$ 8.5 Hz, H-1''''), 4.58 (d, 1 H, $J_{1'',2''}$ 8.5 Hz, H-1''), 4.21 (dd, 1 H, $J_{2',3'}$ 3.3 Hz, H-2'), 4.17 (dd, 1 H, $J_{2''',3'''}$ 3.4 Hz, H-2'''), 4.15 (d, 1 H, $J_{2,3}$ 3.3 Hz, H-2), 3.98 (dd, 1 H, $J_{5,6a}$ 4.3 Hz, $J_{6a,6b}$ 11.3 Hz, H-6a), 2.08 (2 × s, 6 H, CH_3CO), 1.66–1.58 (m, 2 H, CH_2CH_2O), 1.39–1.26 (m, 10 H, CH_2), 1.29 (d, 3 H, $J_{5''',6'''}$ 6.5 Hz, H-6'''), 0.88 (t, 3 H, CH_3-octyl). ^{13}C NMR (125.8 MHz, D_2O, CH_3CN = 0.765), coupling constants from HMBC: δ 174.70 and 174.58 (2 × C=O), 99.74 (d, $J_{C\text{-}1,H\text{-}1}$ 159 Hz, C-1), 99.57 (d, $J_{C\text{-}1'',H\text{-}1''}$ 163 Hz, C-1''), 99.28 (C-1'), 99.13 (d, $J_{C\text{-}1'''',H\text{-}1''''}$ 161 Hz, C-1''''), 96.73 (d, $J_{C\text{-}1''',H\text{-}1'''}$ 169 Hz, C-1'''), 76.42 (C-2'), 75.85 (C-2'''), 70.24 (C-2), 70.10 (CH_2O octyl), 65.73 (C-6), 31.02, 28.59, 28.39, 28.32, 25.02, 21.96 (6 × CH_2 octyl), 22.24 (2 × CH_3CO), 16.79 (C-6'''), 13.35 (CH_3 octyl).

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