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Carbohydrate Research 259 (1994) 93–101

CARBOHYDRATE RESEARCH

Synthesis of tetrasaccharide analogues of the *N*-glycan substrate of β-(1 → 2)-*N*-acetylglucosaminyltransferase II using trisaccharide precursors and recombinant β-(1 → 2)-*N*-acetylglucosaminyltransferase I

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(Received August 13th, 1993; accepted in revised form January 12th, 1994)

Abstract

Recombinant rabbit UDP-GlcNAc: α-Man-(1 → 3R) β-(1 → 2)-*N*-acetylglucosaminyltransferase I (EC 2.4.1.101, GlcNAc-T I) produced in the Sf9 insect cell/baculovirus expression system has been used to convert compounds of the form 3-R-α-Man(1 → 6)(α-Man(1 → 3))β-Man-*O*-octyl to 3-R-α-Man(1 → 6)(β-GlcNAc(1 → 2)α-Man(1 → 3))β-Man-*O*-octyl where R is OH (**14**), *O*-methyl (**17**), *O*-pentyl (**18**), *O*-(4,4-azo)pentyl (**19**), *O*-(5-iodoacetamido)pentyl (**20**) and *O*-(5-amino)pentyl (**21**); 2-deoxy-α-Man(1 → 6)(β-GlcNAc(1 → 2)α-Man(1 → 3))β-Man-*O*-octyl (**16**), 4-*O*-methyl-α-Man(1 → 6)(β-GlcNAc(1 → 2)α-Man(1 → 3))β-Man-*O*-octyl (**22**), 6-*O*-methyl-α-Man(1 → 6)(β-GlcNAc(1 → 2)α-Man(1 → 3))β-Man-*O*-octyl (**23**) and α-Man(1 → 6)[β-GlcNAc(1 → 2)(4-*O*-methyl)α-Man(1 → 3)]β-Man-*O*-octyl (**15**) were also synthesized by this procedure. The yields ranged from 80 to 99%. Products were characterized by high resolution ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry. Compounds **14**, **15**, **17**, **22**, and **23** are excellent substrates for UDP-GlcNAc: α-Man(1 → 6R) β-(1 → 2)-*N*-acetylglucosaminyltransferase II and the other compounds are inhibitors of this enzyme.

1. Introduction

Highly branched complex asparagine-linked oligosaccharides (*N*-glycans) have been implicated in many biological phenomena such as cell–cell interactions and

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SSDI 0008-6215(94)00022-8

the metastatic behaviour of cancer cells [1–7]. This has stimulated studies on the six *N*-acetylglucosaminyltransferases (GlcNAc-T I to VI) responsible for the initiation of *N*-glycan branches [6,8,9]. It is of interest to synthesize oligosaccharide analogues of the natural *N*-glycan substrates of the GlcNAc-transferases as substrates for specific enzyme assays and specificity studies and as potential enzyme inhibitors.

Combined chemical–enzymatic synthesis of oligosaccharides is an attractive alternative to total chemical synthesis [10–24]. Enzymatic glycosylation often permits regio- and stereo-selective formation of glycosidic bonds without the use of protective groups. On the other hand, chemical synthesis offers greater flexibility, especially for the synthesis of oligosaccharide analogues of the natural *N*-glycans.

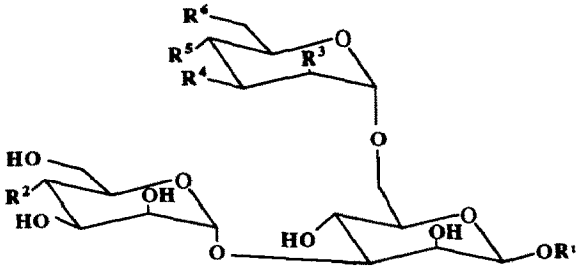
Two approaches are available for enzymatic glycosylation, i.e., glycosyltransferases can be used to transfer monosaccharides from sugar-nucleotide donors to oligosaccharide acceptors, or, alternatively, glycosidases can be used for oligosaccharide synthesis by reversal of the catabolic reaction [18,22,25]. Glycosyltransferases are normally preferred because they give higher yields and a greater regio-specificity than glycosidases.

UDP-GlcNAc: α-Man(1 → 3R) β-(1 → 2)-*N*-acetylglucosaminyltransferase I (EC 2.4.1.101, GlcNAc-T I), isolated from rabbit liver and from human milk, has been used by Hindsgaul's group [12,17] for the preparative synthesis of oligosaccharides. It is difficult to purify GlcNAc-T I from natural sources [26,27]. We and others have recently cloned the genes encoding rabbit, human and mouse GlcNAc-T I [28–32]. Expression of recombinant rabbit GlcNAc-T I in Sf9 insect cells using a baculovirus vector [33] has made large amounts of enzyme available for oligosaccharide synthesis. We describe here the synthesis of novel tetrasaccharide analogues of the *N*-glycan substrate of GlcNAc-T II using synthetic modified trisaccharide acceptor substrates and recombinant GlcNAc-T I. A preliminary abstract reporting part of this work has appeared [34].

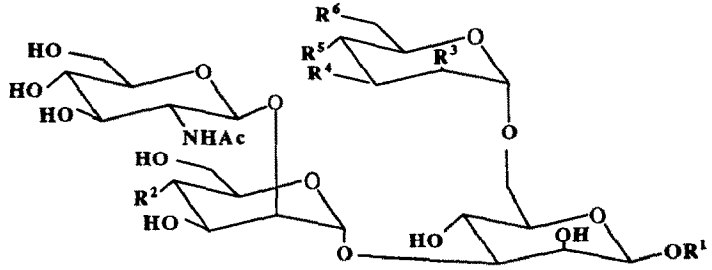
2. Results and discussion

Although the substrate specificity of most glycosyltransferases is very high in vivo [35], the specificity for donor and acceptor is not absolute under in vitro conditions (e.g., high substrate concentrations, no competing enzymes). Small deviations from the natural substrate such as the substitution of an OH-group by a hydrogen, fluorine or methoxy group are often tolerated by the enzymes. The synthetic carbohydrate chemist can take advantage of this promiscuous behaviour of the glycosyltransferases to synthesize novel compounds. The tolerance for substrate modifications is different for each enzyme and must be elucidated by specificity studies [12,19,27,36–39].

GlcNAc-T I converts **1** to **13** [26,40] and **2** to **14** [27,41] respectively (see Scheme 1 for formulae). Previous substrate specificity studies with synthetic analogues of compound **2** [27] revealed that compounds like **4–11** [42] (Paulsen and Springer, unpublished data) (Scheme 1), with modifications in the α-(1 → 6)-linked mannose,



1: R^1 = 1-4GlcNAcβ1-4GlcNAc-Asn-x, $R^2 = R^3 = R^4 = R^5 = R^6$ = OH
2: R^1 = octyl, $R^2 = R^3 = R^4 = R^5 = R^6$ = OH
3: R^1 = octyl, R^2 = *O*-methyl, $R^3 = R^4 = R^5 = R^6$ = OH
4: R^1 = octyl, R^3 = deoxy, $R^2 = R^4 = R^5 = R^6$ = OH
5: R^1 = octyl, R^4 = *O*-methyl, $R^2 = R^3 = R^5 = R^6$ = OH
6: R^1 = octyl, R^4 = *O*-pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
7: R^1 = octyl, R^4 = *O*-(4,4-azo)pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
8: R^1 = octyl, R^4 = *O*-(5-iodoacetamido)pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
9: R^1 = octyl, R^4 = *O*-(5-amino)pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
10: R^1 = octyl, R^5 = *O*-methyl, $R^2 = R^3 = R^4 = R^6$ = OH
11: R^1 = octyl, R^6 = *O*-methyl, $R^2 = R^3 = R^4 = R^5$ = OH



13: R^1 = 1-4GlcNAcβ1-4GlcNAc-Asn-x, $R^2 = R^3 = R^4 = R^5 = R^6$ = OH
14: R^1 = octyl, $R^2 = R^3 = R^4 = R^5 = R^6$ = OH
15: R^1 = octyl, R^2 = *O*-methyl, $R^3 = R^4 = R^5 = R^6$ = OH
16: R^1 = octyl, R^3 = deoxy, $R^2 = R^4 = R^5 = R^6$ = OH
17: R^1 = octyl, R^4 = *O*-methyl, $R^2 = R^3 = R^5 = R^6$ = OH
18: R^1 = octyl, R^4 = *O*-pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
19: R^1 = octyl, R^4 = *O*-(4,4-azo)pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
20: R^1 = octyl, R^4 = *O*-(5-iodoacetamido)pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
21: R^1 = octyl, R^4 = *O*-(5-amino)pentyl, $R^2 = R^3 = R^5 = R^6$ = OH
22: R^1 = octyl, R^5 = *O*-methyl, $R^2 = R^3 = R^4 = R^6$ = OH
23: R^1 = octyl, R^6 = *O*-methyl, $R^2 = R^3 = R^4 = R^5$ = OH
24: R^1 = 1-4GlcNAcβ1-4GlcNAc-Asn-x, R^3 = O-GlcNAcβ1-, $R^2 = R^4 = R^5 = R^6$ = OH

Scheme 1.

Table 1
Enzymatic glycosylation with recombinant GlcNAc-T I

Product	Substrate	Reaction time (days) [a]	Yield (%)	$[\alpha]D$ [b] (°)	Molecular composition	FABMS (m/z) [c]
14	**2**	5	99			
15	**3**	5	81	n.d. [d]	$C_{35}H_{63}NO_{21}$	856.3801 (MNa^+, +1.1 mmu)
16	**4**	6	88	n.d.	$C_{34}H_{61}NO_{20}$	804.3865 (MH^+, −2.3 mmu)
17	**5**	5	99	n.d.	$C_{35}H_{63}NO_{21}$	834.3989 (MH^+, +1.8 mmu)
18	**6**	2	94	n.d.	$C_{39}H_{71}NO_{21}$	890.4597 (MH^+, +0.4 mmu)
19	**7**	4	80	+11.6	$C_{39}H_{69}N_3O_{21}$	916.4562 (MH^+, +6.0 mmu) 918.4676 (MH_3^+, +1.8 mmu) [e]
20	**8**	4	82	+12.6	$C_{41}H_{73}IN_2O_{22}$	1075.4724 (M − I + TG + Na^+, +0.6 mmu) 969.4686 (M − I + H + Na^+, +5.5 mmu) [f]
21	**9**	5	80	n.d.	$C_{39}H_{72}N_2O_{21}$	905.4706 (MH^+, +1.0 mmu)
22	**10**	6	80	n.d.	$C_{35}H_{63}NO_{21}$	834.3943 (MH^+, −2.8 mmu)
23	**11**	6	85	n.d.	$C_{35}H_{63}NO_{21}$	834.3946 (MH^+, −2.5 mmu)

[a] Days at room temperature.
[b] c 0.5, MeOH.
[c] In glycerol matrix unless otherwise stated.
[d] Not determined because of a small amount of compound.
[e] MH^+ was detected in a thioglycerol matrix; in glycerol only the reduced mass peak MH_3^+ was detected.
[f] The iodine containing mass peaks of **20** and of **8** could not be detected, probably due to reduction of the CH_2I group to CH_3 in the matrix (in both glycerol and thioglycerol matrix) and substitution of the iodine by thioglycerol (in thioglycerol matrix). TG = thioglycerol.

are excellent substrates for GlcNAc-T I. The 4-*O*-methyl-α-Man(1 → 3) analogue (**3**) was also found to be a good substrate (Reck, unpublished data). In the present study, we have used recombinant GlcNAc-T I, expressed in Sf9 insect cells [33], to synthesize compounds **14–23** in yields of 80–99% (Table 1). Compounds **15** and **17–23** are novel whereas the chemical syntheses of **14** and **16** have been reported previously [43,44].

The recombinant GlcNAc-T I used in this work was a highly active partially purified enzyme preparation obtained from the insect cell culture medium by a single ion-exchange purification step. The enzyme was concentrated to allow use of small reaction volumes. Substrate conversion was monitored by TLC.

Complete conversion of the acceptor to the product was detected with the relatively stable compounds **14**, **17**, and **18** and only a simple desalting procedure was necessary to provide pure product. The mild conditions of pH and temperature used for the enzyme reaction permitted the high-yield conversion of the unstable compound **8** to the unstable product **20**. For compounds **15**, **16**, **19**, and **21–23** only traces of substrate could be detected after reaction for 6 days. The commercially available nucleotide-sugar donor UDP-GlcNAc was present in a four-fold excess over acceptor (not optimized) to overcome inhibition by the product UDP which is a competitive inhibitor of GlcNAc-T I [26]. GlcNAc-T I

Table 2
Selected ^{1}H and ^{13}C NMR parameters for **14–23** [a]

Compound	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
H-1 ($J_{1,2}$)	4.74 (bs)	4.68 (bs)	4.69 (bs)	4.74 (bs)	4.73 (bs)	4.74 (bs)	4.74 (bs)	4.74 (bs)	4.66 (bs)	4.67 (bs)
H-1′ ($J_{1',2'}$)	5.19 (bs)	5.13 (1.7)	5.15 (bs)	5.20 (1.6)	5.17 (bs)	5.19 (bs)	5.21 (1.6)	5.20 (1.6)	5.12 (bs)	5.12 (1.5)
H-1″ ($J_{1'',2''}$)	4.62 (8.4)	4.57 (8.4)	4.57 (8.4)	4.62 (8.4)	4.57 (8.4)	4.62 (8.5)	4.63 (8.4)	4.63 (8.4)	4.54 (8.4)	4.54 (8.4)
H-1‴ ($J_{1''',2'''}$)	4.98 (1.5)	4.93 (1.5)	5.08 (m)	5.02 (1.8)	4.96 (bs)	4.99 (bs)	5.01 (1.7)	5.01 (1.5)	4.88 (1.5)	4.88 (1.5)
H-2 ($J_{2,3}$)	4.19 (2.9)	4.13 (3.1)	4.15 (2.9)	4.20 (2.8)	4.13 (m)	4.19 (m)	4.20 (m)	4.20 (2.7)	4.12 (2.5)	4.12 (3.0)
H-2′ ($J_{2',3'}$)	4.25 (3.2)	4.20 (3.4)	4.21 (3.3)	4.26 (3.3)	4.20 (m)	4.25 (m)	4.26 (3.3)	4.26 (3.2)	4.18 (3.0)	4.19 (3.0)
H-2‴ ($J_{2''',3'''}$)	4.06 (3.4)	4.01 (3.3)	2.21 (ddd, H-2‴$_{ax}$) 1.74 (ddd, H-2‴$_{eq}$)	4.30 (3.3)	4.15 (m)	4.22 (m)	4.24 (m)	4.24 (2.9)	3.97 (3.5)	3.98 (3.5)
OCOCH_3	2.12	2.08	2.07	2.12	2.09	2.12	2.13	2.12	2.04	2.05
CH_2CH_3(t)	0.93	0.88	0.88	0.92	0.92, 0.89	0.92	0.94	0.93	0.86	0.87
Other H		3.54 (s) (CH_3O)		3.52 (s) (CH_3O)		1.08 (s) (CH_3CN_2)	3.81 (s) (CH_2I) 3.28 (t) (CH_2NHR)	3.08 (t) (CH_2N)	3.54 (s) (CH_3O)	3.41 (s) (CH_3O)
CH_2I							−3.3			

[a] In D_2O, HDO 4.80 ppm. Chemical shifts (δ) in ppm, coupling constants (J) in Hz. Apostrophe superscripts: β-linked Man, no apostrophe; α3-linked Man′; β2-linked GlcNAc″; α6-linked Man‴.

requires a divalent cation like manganese or cobalt for activity [27,45]. We chose cobalt instead of manganese because we found in previous studies that its addition resulted in 50% higher enzyme activities [27]. Under these conditions product inhibition, which has been found to be a severe problem in the use of some other glycosyltransferases [25], is not a problem with GlcNAc-T I.

The structures of **14–23** were confirmed by ^{1}H and ^{13}C NMR and FABMS (Table 2). The ^{1}H NMR data for **14** and **16** were identical to the data previously reported for chemically synthesized **14** and **16** [43,44] (Paulsen and Springer, unpublished data). The signal for H-2′ of the α-Man(1 → 3) residue in **14–23** is shifted downfield by ca. 0.2 ppm (to 4.18–4.26 ppm) relative to the H-2′ signal of the respective precursors **2–11**, indicating that the 2′-O is glycosylated. The new glycosidic linkage is in a β configuration since the H-1″ signal for **14–23** is a doublet with $J_{1'',2''} = 8.4$ Hz at 4.54–4.63 ppm (Table 2). FABMS of **14** and **16–23** gave a fragment with $m/z = 366$ indicative of HexNAc-Hex-, whereas **15** gave a fragment with $m/z = 380$ indicative of HexNAc-(*O*-methyl)-Hex-.

Compounds **14–23** have been used (Reck et al., *Glycoconjugate J.*, in press) for substrate specificity studies and specific inhibition of UDP-GlcNAc: α-Man(1 → 6R) β-(1 → 2)-*N*-acetylglucosaminyltransferase II (GlcNAc-T II; EC 2.4.1.143) which converts **13** to **24** [6,8]. Compound **14** is a good substrate for assays of GlcNAc-T II activity. The photolabile diazirine **19** and the iodoacetamide derivative **20** show specific irreversible inhibition of GlcNAc-T II and may prove useful for identification of the active site of the enzyme. Compounds **16**, **18**, **19** (without irradiation), and **21** are reversible inhibitors with K_i values of 0.13, 2.4, 1.0, and 2.4 mM, respectively. Compounds **15**, **17**, **22**, and **23** are substrates for GlcNAc-T II.

3. Experimental

General methods.—Optical rotations were measured at 23°C (*c* 0.5, MeOH) using a Perkin–Elmer 241 Polarimeter. TLC was performed on Silica Gel F_{254} (Merck) with detection by UV absorption and/or by charring with 14:4:1 EtOH–H_2O–H_2SO_4. Bio-Gel P-2 (200–400 mesh, Bio-Rad Laboratories) was used for gel filtration chromatography. ^{1}H NMR spectra were recorded at 500 MHz (Bruker AM 500) and at 300 MHz (Bruker AM 300). ^{13}C NMR spectra were recorded at 75.5 MHz (Bruker AM 300). Only partial NMR data are reported. Assignments of ^{1}H resonances were based on 2D-COSY experiments. High resolution fast atom bombardment-mass spectrometry (FABMS) was performed in the positive-ion mode on a ZAB-SE mass spectrometer (VG analytical) with polyethyleneglycol as internal standard, Xe as the bombarding atom (8 kev, 1.2 mA dark anode current), and accelerated voltage scan in a narrow mass range.

Grace's insect medium, fetal calf serum (FCS), TC Yeastolate, TC lactalbumin hydrolysate, gentamycin sulfate, and amphotericin (Fungizone) were obtained from Gibco Laboratories, Grand Island, NY. UDP-hexanolamine-Sepharose was synthesized using UDP-hexanolamine (Sigma) and cyanogen bromide-activated Sepharose

(Pharmacia) according to the manufacturer's instructions. CM-Sephadex C-50 was obtained from Pharmacia.

Spodoptera frugiperda (Sf9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcMNPV) were obtained from the laboratory of Dr. M.D. Summers (Texas A&M University, College Station, TX). The recombinant baculovirus vMAG-GnTI was constructed as detailed elsewhere [33] (Sarkar *Glycoconjugate J.*, in press); vMAG-GnTI encodes a fusion protein between the signal sequence of myelin-associated glycoprotein (MAG) and the catalytic domain of GlcNAc-T I.

Preparation of recombinant GlcNAc-T I.—Sf9 cells were grown at 28°C in Grace's insect medium supplemented with 10% FCS, TC Yeastolate, lactalbumin hydrolysate, 50 mg/mL gentamycin sulfate, and 2.5 mg/mL amphotericin (Fungizone) in spinner flasks (Bellco Glass Inc., Vineland, NJ) [46–49]. Sf9 cells at a density of $2.0–2.8 \times 10^8$ cells/L were infected with vMAG-GnTI virus (stock solution of 2×10^8 pfu/mL) at a multiplicity of infection of 2 pfu/cell. The cells were cultured in spinner flasks each containing 250 mL of medium at 28°C. Supernatant (1.0 L) from the vMAG-GnTI-infected insect cell cultures was collected at 4 days after addition of virus and was passed through a CM-Sephadex C-50 column equilibrated with 25 mM MES, pH 6.1, 0.1% Triton X-100, and 0.02% sodium azide (buffer A). The column was washed with buffer A (500 mL), buffer A containing 0.1 M NaCl (2.5 L), and enzyme activity was eluted with 1.0 M NaCl in buffer A. Fractions containing enzyme activity were pooled, dialyzed against buffer A and concentrated to 425 mL with polyethylene glycol 20000 (Carbowax, Fisher) [50]. On storage at 4°C for 7 days, enzyme activity precipitated probably due to the low salt concentration. About 10% of the enzyme activity could be re-dissolved with 1.0 M NaCl in buffer A to yield a solution with an enzyme activity of 0.4 μmol/min/mL. This solution was stable for several months at 4°C. In subsequent preparations, dialysis was avoided and the enzyme was concentrated by filtration under N_2 pressure using an Amicon concentration unit.

Preparative enzymatic glycosylation with recombinant GlcNAc-T I.—Compounds **15–23** were synthesized under identical conditions following the general procedure described below. The synthesis was scaled up five times for **14**. The incubation for **15–23** was carried out in a total volume of 1.8 mL containing acceptor (2.6 mM), MES [2-(*N*-morpholino)ethane sulfonate] buffer (0.12 M, pH 6.1), UDP-GlcNAc (11 mM), $CoCl_2$ (50 mM), and bovine serum albumin (2.25 mg). GlcNAc-T I (20 μL, 8 mU, where 1 unit is 1 μmol/min) was added and the solution was mixed by inversion. The reaction was kept at room temperature in the dark, without stirring, and was monitored by TLC (5:1 CH_3CN–H_2O). Acceptor was either not detectable or detectable only in traces by TLC after 2–6 days of incubation and the mixture was applied to a C_{18} SepPak cartridge (Waters Associates) pre-equilibrated with 5 mL MeOH followed by 30 mL H_2O [51]. The cartridge was washed with H_2O (10 mL) and the product eluted with MeOH (8 mL), to provide pure **14**, **17**, and **18**. Compounds **15**, **16**, **19**, **20**, **22**, and **23** were further purified by gel filtration on a Biogel P-2 column with H_2O as the solvent to remove traces of impurities. Compound **21** was purified by gel filtration on a Biogel P-2 column with

0.02 M NaCl in H_2O as the solvent followed by adsorption to and elution from a C_{18} SepPak cartridge to remove salt.

Acknowledgments

This research was supported by grants from the Medical Research Council of Canada (to H.S. and I.B.), by the Canadian Protein Engineering Network of Centres of Excellence (to H.S.), and by the Bundesministerium für Forschung und Technologie (to H.P.). F.R. is supported by a post-doctoral fellowship from the Deutsche Studienstiftung and the BASF Aktiengesellschaft. We thank Dr. A.A. Grey, Carbohydrate Research Centre, Toronto, NMR Centre, for recording NMR spectra and Dr. H. Pang, Carbohydrate Research Centre, Toronto, Mass Spectrometry Laboratory, for recording and interpreting FABMS spectra.

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