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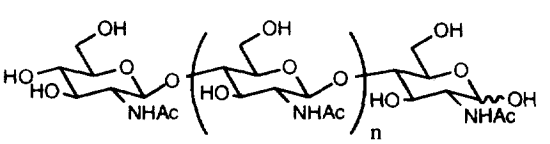
Glycosidase-catalysed Oligosaccharide Synthesis: Preparation of the *N*-Acetylchitooligosaccharides Penta-*N*-acetylchitopentaose and Hexa-*N*-acetylchitohexaose using the β-*N*-Acetylhexosaminidase of *Aspergillus oryzae*.

Suddham Singh , Richard Gallagher, Peter J. Derrick and David H.G. Crout*

Department of Chemistry, University of Warwick, Coventry CV4 7AL (U.K.)

Abstract: Using a crude β-*N*-acetylhexosaminidase from *Aspergillus oryzae* both tri-*N*-acetylchitotriose (GlcNAc)3 (**1**, n=1) and tetra-*N*-acetylchitotetraose (GlcNAc)4 (**1**, n=2) act respectively as both glycosyl donor and glycosyl acceptor to give product mixtures containing significant quantities of the corresponding penta- and hexasaccharides [**1** (n=3) and **1** (n=4), respectively] which are readily isolated and purified by charcoal-Celite chromatography.

Recently, there has been much interest in the synthesis of oligosaccharides by enzymatic methods using either the biosynthetic glycosyl transferases or glycosidases.[1-3] Glycosidase-catalysed reactions have the advantage that they require less complex substrates than glycosyl transferases. They operate by cleaving the glycosidic bond in a glycoside or oligosaccharide to give an enzyme bound intermediate which can be intercepted by water (hydrolysis) or by another hydroxylic species to generate a new glycosidic bond. When the intercepting species is a carbohydrate, another oligosaccharide is formed without the need for protection-deprotection sequences and with complete control of the configuration at the anomeric centre as determined by the specificity of the biocatalyst. We report here the use of the β-*N*-acetylhexosaminidase of *Aspergillus oryzae* to catalyse the formation of the chitooligosaccharides penta-*N*-acetylchitopentaose **1** (n=3) and hexa-*N*-acetylchitohexaose **1** (n=4) from the corresponding β1-4 linked tri- and tetrasaccharides, tri-*N*-acetylchitotriose (**1**, n=1) and tetra-*N*-acetylchitotetraose (**1**, n=2). Oligosaccharides consisting of β1-4 linked *N*-acetyl-D-glucosamine units (chitooligosaccharides) have been used as lysozyme substrates,[4-6] for biological studies of lectins,[7-10] as antitumour agents[11] and for their immunopotentiating effect.[12-13]



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Commercially available chitooligosaccharides are obtained by the hydrolysis of chitin or chitosan, either chemically[14-16] or enzymatically[17] Enzymatic methods of synthesis have also have been used. Thus hexa-*N*-acetylchitohexaose and di-*N*-acetylchitobiose have been obtained from tetra-*N*-acetylchitotetraose *via* glycosyl transfer catalysed by a chitinase from *Trichoderma reesei*. Chain elongation from di-*N*-acetylchitobiose is

catalysed by lysozyme in a medium of high ionic strength to give an oligomer mixture containing significant amounts of hexamer and heptamer.[18]

In the present synthesis, an enzyme is used which for preparative purposes can be obtained as an ammonium sulfate fraction from the inexpensive and readily available β-galactosidase from *A. oryzae*.[19] The tri-*N*-acetylchitotriose and the tetra-*N*-acetylchitotetraose used as starting materials were prepared from di-*N*-acetylchitobiose in a reaction catalysed by same enzyme.[20] The disaccharide acts as a glycosyl donor, transferring an *N*-acetylglucosamine unit on to itself to give the β1-4-linked trisaccharide. This in turn acts as acceptor of glycosyl transfer to give the β1-4 linked tetrasaccharide:

$$(GlcNAc)_2 \xrightarrow[\text{from } A.\ oryzae]{\beta\text{-}N\text{-Acetylhexosaminidase}} GlcNAc + (GlcNAc)_2 + (GlcNAc)_3 + (GlcNAc)_4$$

The products are readily separated by charcoal-Celite chromatography using water containing varying amounts of ethanol as eluent.[20]

The efficiency of this process makes it possible to generate the tri- and tetrasaccharides in quantities large enough to study their behaviour as substrates for the β-*N*-acetylhexosaminidase of *A. oryzae*. Thus incubation of the tetrasaccharide with the β-*N*-acetylhexosaminidase gave a mixture containing the di-, tri, tetra-, penta-, and hexasaccharides (all exclusively β1-4 linked). These products were readily separated from each other by charcoal-Celite chromatography.

$$(GlcNAc)_4 \xrightarrow[\text{from } A.\ oryzae]{\beta\text{-}N\text{-Acetylhexosaminidase}} \begin{array}{l}(GlcNAc)_2 + (GlcNAc)_3 + (GlcNAc)_4 \\ + (GlcNAc)_5 + (GlcNAc)_6\end{array}$$

When the trisaccharide was used as substrate, a similar mixture was obtained except that it contained appreciable amounts of monomer:

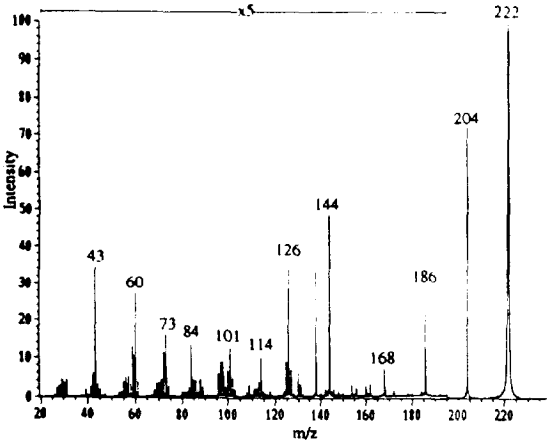
$$(GlcNAc)_3 \xrightarrow[\text{from } A.\ oryzae]{\beta\text{-N-Acetylhexosaminidase}} \begin{array}{l}GlcNAc + (GlcNAc)_2 + (GlcNAc)_3 + \\ (GlcNAc)_4 + (GlcNAc)_5 + (GlcNAc)_6\end{array}$$

The system is kinetically complex because each compound present can act as both glycosyl donor and acceptor. The product mixture evolves towards a quasi equilibrium and is intercepted at a point at which the concentrations of the desired oligomers reaches a maximum as determined by HPLC Since the evolution of the mixture is quite slow, its composition is readily monitored.

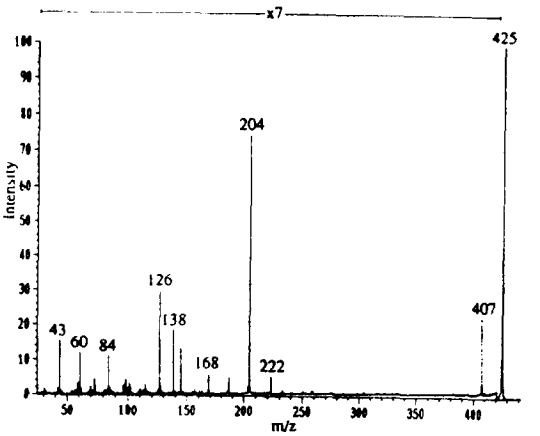
The structures of the products were studied by tandem mass spectrometry using collision induced dissociation (CID), a process whereby a small portion of the kinetic energy of the mass-selected incident ion is transformed into vibronic energy upon collision with helium atoms. Its purpose is to promote unimolecular decompositions.

The mass spectra obtained for *N*-acetylglucosamine and for the oligosaccharides (**1**, n=0-4) are shown in Fig. 1. The molecular ion in all cases is the protonated species. We have adopted the nomenclature proposed by Domon and Costello[21] for describing fragmentation processes in oligosaccharides (Fig. 2). The main daughter ions observed are from B and Y type fragmentations (Fig.1) which clearly show steps of 203 amu

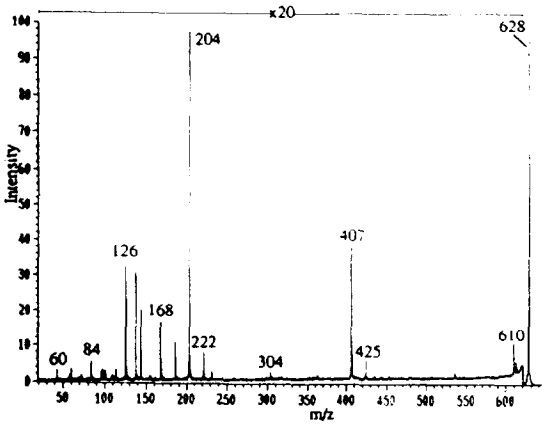
N-acetylglucosamine



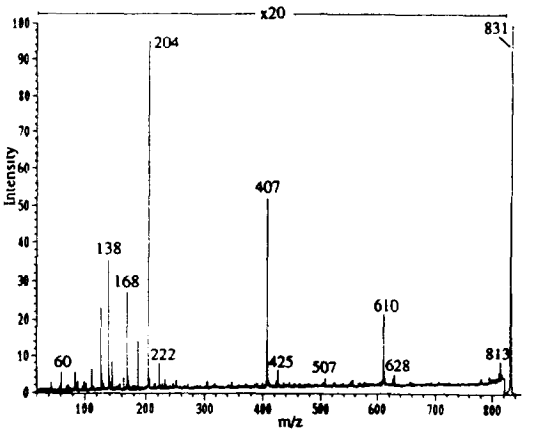
Di-*N*-acetylchitobiose (**1**, n = 0)



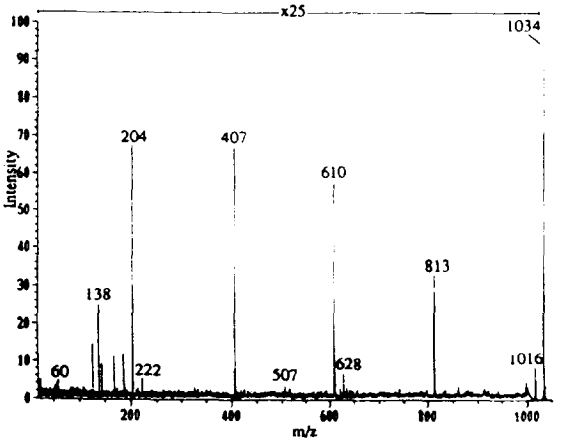
Tri-*N*-acetylchitotriose (**1**, n = 1)



Tetra-*N*-acetylchitotetraose (**1**, n = 2)



Penta-*N*-acetylchitopentose (**1**, n = 3)



Hexa-*N*-acetylchitohexose (**1**, n = 4)

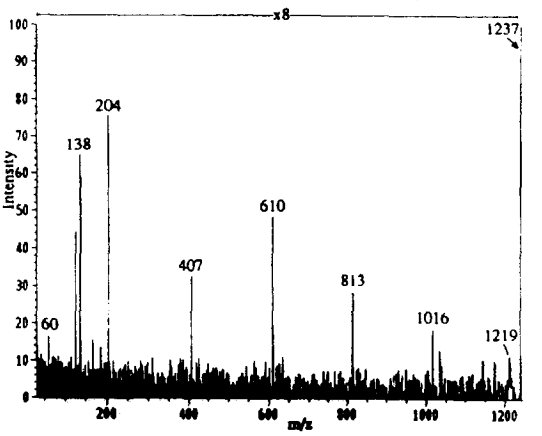


Table 1. Listing of mass spectral data for *N*-acetylglucosamine and oligosaccharides **1** (n=0-4).

	Oligosaccharide Sample (n)					
m/z	Mono	Di (0)	Tri (1)	Tetra (2)	Penta (3)	Hexa (4)
1237						$[MH]^+$
1219						B_6
1034					$[MH]^+$	-
1016					B_5	B_5
831				$[MH]^+$	-	-
813				B_4	B_4	B_4
628			$[MH]^+$	-	-	-
610			B_3	B_3	B_3	B_3
507			-	$^{0,2}X_2$	$^{0,2}X_2$	
425		$[MH]^+$	Y_2	Y_2	-	-
407		B_2	B_2	B_2	B_2	B_2
304		$^{0,2}X_1$	$^{0,2}X_1$	$^{0,2}X_1$	-	-
222	$[MH]^+$	Y_1	Y_1	Y_1	Y_1	-
204	B_1	B_1	B_1	B_1	B_1	B_1
186	B_1-H_2O	B_1-H_2O	B_1-H_2O	B_1-H_2O	B_1-H_2O	
168	B_1-2(H_2O)	B_1-2(H_2O)	B_1-2(H_2O)	B_1-2(H_2O)	B_1-2(H_2O)	B_1-2(H_2O)
144	+	+	+	+	+	-
138	+	+	+	+	+	+
126	+	+	+	+	+	+
114	+	+	+	+	-	-
101	$^{0,2}X_0$	$^{0,2}X_0$	-	-	-	-
84	+	+	+	+	-	-
73	+	+	+	+	-	-
60	+	+	+	+	+	+
43	+	+	+	+	+	-
29	HCO^+	HCO^+	-	-	-	-

Key: + Peak present in mass spectrum; - Peak absent in mass spectrum.

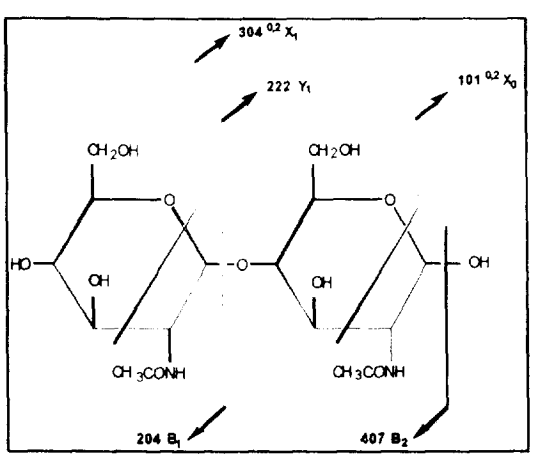


Fig. 1. Oligosaccharide fragmentation nomenclature.

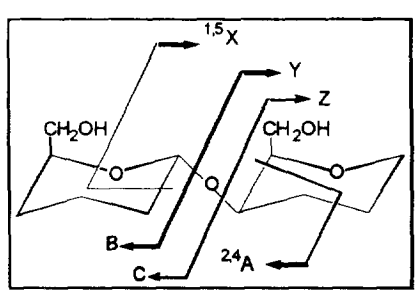


Fig. 2. Major fragment ions observed in di-*N*-acetylchitobiose (**1**, n=0).

corresponding to losses of *N*-acetylglucosamine groups. In the case of the hexa-*N*-acetyl chitohexaose for example, daughter ion masses of 204, 407, 610, 813, 1016 and 1219 amu correspond to subsequent B_1, B_2, B_3, B_4, B_5 and B_6 fragment losses. Similar patterns are shown in all the samples. From the pentasaccharide down to the di-*N*-acetylchitobiose, the Y_i ion series is also observed. Penta-*N*-acetylchitopentaose shows ions at 222, 425, and 628 amu corresponding to Y_1 Y_2 and Y_3 respectively but no ions are observed corresponding to Y_4. Ring cleavages of the type $^{0,2}X_i$ are also observed. In all the spectra several low mass fragments are detected. These correspond to water losses from the *N*-acetylglucosamine ring and from loss of the *N*-acetyl substituent.

Experimental

General.— ^{1}H-NMR spectra were determined at 400 MHz using a Bruker WH 400 spectrometer. ^{13}C NMR spectra were determined at 100.62 MHz using the same instrument. Optical rotations were determined using an AA-1000 polarimeter (Optical Activity Ltd). with a 2 dm cell. Optical rotations are given in units of 10^{-1} deg cm^2 g^{-1}. β-Galactosidase from *Aspergillus oryzae* was obtained from the Sigma Chemical Company. Celite 535 was obtained from Fluka and activated charcoal (Darco G-60, 100 mesh) was obtained from the Aldrich Chemical Company. HPLC analyses were carried out using a Gilson HPLC instrument with a Hypersil 5 APS (aminopropyl silica) column (20 x 4.6 mm) with UV detection at 210 nm and MeCN-H_2O (75:25) as eluent at a flow rate of 1.5 mL min^{-1}. High-energy Collision Induced Dissociation (CID) spectra were obtained on a Kratos Concept II HH four-sector tandem mass spectrometer (Kratos Analytical Ltd, Manchester, UK) of EBEB geometry equipped with a scanning-array detection system.[22] The ions were produced using a caesium ion liquid secondary ion mass spectrometry ion source (Cs^+ LSIMS) which bombards the sample with a 12 keV Cs^+ ion beam producing protonated molecular ions.[23] The ion source was operated at an accelerating potential of 8 kV with the MS-1 resolution set to 1200 (10% valley). By selecting only the ^{12}C monoisotope $(M+H)^+$ ion any matrix-associated chemical noise peaks, which occur at every mass, are eliminated in the MS/MS experiment. The collision cell was floated to 4 kV potential to give 4 keV energy collisions for all the CID experiments with the helium collision gas pressure set such that the selected precursor ion peak was decreased to 30 % of its original intensity. This degree of attenuation corresponds to an average of one collision with helium for a given ion.[24] The CID mass spectra were acquired by means of a linked scan of the electric and magnetic fields such that the ratio B/E is constant. Each mass spectrum is the product of 10 accumulated spectra covering a mass range of 10 to 2000 amu at a scan speed of 10 sec/dec. Glycerol was obtained from Fisons plc, Loughborough, UK and thioglycerol from Aldrich Chemical Co.Ltd, Gillingham, Dorset, UK. All the oligosaccharide samples were dissolved in water and a 2 µl aliquot was placed on the probe tip and dried down. An equal volume of glycerol/thioglycerol (1:1, v/v) was added as the matrix.

Enzyme preparation.— The β-*N*-acetylhexosaminidase was obtained from the crude β-galactosidase from *Aspergillus oryzae* (Sigma grade IX, 50 g) as previously described.[19,20]

*Preparation of penta-N-acetylchitopentaose (**1**, n=3) and hexa-N-acetylchitohexaose (**1**, n=4):*

*From tetra-N-acetylchitotetraose (**2**, n=2):* To a solution of tetra-*N*-acetylchitotetraose (175 mg) in phosphate buffer (pH-6.5, 0.04 M, 0.8 ml) was added β-*N*-acetylhexosaminidase from *Aspergillus oryzae* (0.5 ml, 0.094 U/mg protein, 149 mg protein/ml). The mixture was incubated at 30 °C for 54 h. The reaction was stopped by heating in a boiling water bath for 5 min. The reaction mixture was applied to a carbon - Celite (1:1) column and the column was eluted with ethanol-water (10:90, 300 ml) to give di-*N*-acetychitobiose (8 mg), ethanol-water (15:85, 500 ml) to give tri-*N*-acetylchitotriose (28 mg), ethanol-water (20:80, 650 ml) to give tetra-*N*-acetylchitotetraose (71 mg), ethanol-water (25:75, 350 ml), to give penta-*N*-acetylchitopentaose (35 mg) and ethanol-water (30:70, 200 ml) to give hexa-*N*-acetylchitohexaose (12 mg).

Penta-*N*-acetylchitopentaose (**1**, n=3): $[\alpha]_D^{27}$ = -9.43° (c 0.49 H_2O) , Lit.[15] $[\alpha]_D^{20}$ = -9.1° + 0.2 (c 1, H_2O). ^{1}H-NMR (δ, D_2O): 1.98, 2.00 and 2.01 (all s, 15 H, 5 x CH_3), 3.38 - 3.88 (m, 30 H, 30 OH), 4.52 (d, J = 8.00 Hz, H-1, internal residues), 4.53 (d, J = 8.36 Hz, H-1, internal residues); 4.64 (d, J = 8.16 Hz, 0.45 H, H-1-β); 5.10 (d, J = 2.63 Hz, 0.55 H, H-1-α). ^{13}C-NMR data (δ, D_2O) are given in Tables 2 and 3.

Table 2. ^{13}C N.m.r Data for Pentamer **1** (n=3) (α-anomer).

Residue or group	C-1	C-2	C-3	C-4	C-5	C-6	Me	
Reducing end	91.07	54.27	69.86	80.23	70.63	60.75	22.51	175.11
Internal residues	101.88	55.65	72.68[a] 72.74[b]	79.51[c] 79.74[d]	75.15	60.56	22.74	175.25
Non-reducing end	102.09	56.22	74.05	70.32	76.53	61.15	22.74	175.25

[a]Residues 2 and 3; [b] residue 4; [c] residues 3 and 4; [d] residue 2.

Table 3. ^{13}C N.m.r Data for Pentamer **1** (n=3) (β-anomer).

Residue or group	C-1	C-2	C-3	C-4	C-5	C-6	Me	
Reducing end	95.45	56.73	73.10	79.74	75.23	60.56	22.81	175.37

Signals for the internal residues and non-reducing terminal residue were as for the α-anomer (Table 2).

Hexa-*N*-acetylchitohexaose (**1**, n=4): $[\alpha]_D^{27}$ = -11.75° (c 0.40, H_2O) , Lit.[15] $[\alpha]_D^{20}$ = -11.4 ± 0.4° (c 0.8, H_2O). ^{1}H-NMR (δ, D_2O) (for the α-anomer): 1.97 and 1.99 (all s, 18 H, 6 x CH_3), 3.37 - 3.90 (m, 36 H, 36 OH), 4.50 (d, J = 8.08 Hz, H-1, internal residues); 4.51 (d, J = 8.28 Hz, H-1 internal residue); 4.62 (bd, J = 7.72 Hz, 0.42 H, H-1-β); 5.12 (bs, 0.58 H, H-1-α). ^{13}C-NMR data (δ, D_2O) are given in Tables 4 and 5.

Table 4. ^{13}C N.m.r Data for Hexamer **1** (n=4) (α-anomer).

Residue or group	C-1	C-2	C-3	C-4	C-5	C-6	Me	C=O
Reducing end	91.07	54.25	69.85	80.23	70.63	60.56	22.50	175.10
Internal residues	101.88	55.67	72.68[a] 72.74[b]	79.51[c] 79.74[d]	75.14	60.56	22.73	175.27
Non-reducing end	102.10	56.20	74.06	70.31	76.53	61.15	22.73	175.27

[a]Residues 2-3; [b] residue 5; [c] residues 3-5; [d] residue 2.

Table 5. ^{13}C N.m.r Data for Hexamer **1** (n=4) (β-anomer).

Residue or group	C-1	C-2	C-3	C-4	C-5	C-6	Me	C=O
Reducing end	95.45	56.73	73.09	79.74	75.15	60.56	22.73	175.27

Signals for internal residue and all other non-reducing termal carbon atoms were as for the α-anomer (Table 4).

*From Tri-N-acetylchitotriose (**1**, n = 1):* To a solution of tri-*N*-acetylchitotriose (500 mg) in phosphate buffer (pH-6.5, 0.04 M, 6 ml) was added β-*N*-acetylhexosaminidase from *Aspergillus oryzae* (0.5 ml, 0.094 U/mg protein, 149 mg protein/ml). The mixture was incubated at 30 °C for 52 h. The reaction was stopped by heating in boiling water bath for 5 min. The reaction mixture was then applied to a carbon-Celite column and column was eluted with ethanol-water (5:95, 400 ml) to give *N*-acetyglucosamine (71 mg), ethanol-water (10:90, 700 ml) to give di-*N*-acetychitobiose (120 mg), ethanol-water (15:85, 800 ml) to give tri-*N*-acetylchitotriose (132 mg), ethanol-water (20:80, 500 ml) to give tetra-*N*-acetylchitotetraose (89 mg), ethanol-water (25:75, 400 ml), to give penta-*N*-acetylchitopentaose (52 mg) and ethanol-water (30:70, 300 ml) to give hexa-*N*-acetylchitohexaose (20 mg).

*Tandem mass spectral data for chitooligosaccharides (**1**, n= 0-4).* *N*-acetylglucosamine; $C_8H_{15}NO_6$. Mol. Wt.= 221.1 amu. $(MH)^+$= 222.1; di-*N*-acetylchitobiose (**1**, n=0). $C_{16}H_{28}N_2O_{11}$. Mol. Wt.= 424.2 amu. $(MH)^+$= 425.18; Tri-*N*-acetylchitotriose (**1**, n=1). $C_{24}H_{41}N_3O_{16}$. Mol. Wt.= 627.3 amu. $(MH)^+$= 628.3; tetra-*N*-acetylchitotetraose (**1**, n=2). $C_{32}H_{54}N_4O_{21}$. Mol. Wt.= 830.3 amu. $(MH)^+$= 831.3; penta-*N*-acetylchitopentaose (**1**, n=3); $C_{40}H_{67}N_5O_{26}$. Mol. Wt.= 1033.4 amu; $(MH)^+$= 1034.4; hexa-*N*-acetylchitohexaose (**1**, n=4); $C_{48}H_{80}N_6O_{31}$. Mol. Wt.= 1236.5 amu. $(MH)^+$= 1237.49

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