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Synthesis, Evaluation, and Mechanism of *N*,*N*,*N*-Trimethyl-D-glucosamine-(1→4)-chitooligosaccharides as Selective Inhibitors of Glycosyl Hydrolase Family 20 β-*N*-Acetyl-D-hexosaminidases

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GH20 β-*N*-acetyl-D-hexosaminidases are enzymes involved in many vital processes. Inhibitors that specifically target GH20 enzymes in pests are of agricultural and economic importance. Structural comparison has revealed that the bacterial chitin-degrading β-*N*-acetyl-D-hexosaminidases each have an extra +1 subsite in the active site; this structural difference could be exploited for the development of selective inhibitors. *N*,*N*,*N*-trimethyl-D-glucosamine (TMG)-chitotriomycin, which contains three GlcNAc residues, is a natural selective inhibitor against bacterial and insect β-*N*-acetyl-D-hexosaminidases. However, our structural alignment analysis indicated that the two GlcNAc residues at the reducing end might be unnecessary. To prove this hypothesis, we designed and synthesized a series of TMG-chitotriomycin analogues containing one to four GlcNAc units. Inhibitory kinetics and molecular docking showed that TMG-$(GlcNAc)_2$, is as active as TMG-chitotriomycin [TMG-$(GlcNAc)_3$]. The selective inhibition mechanism of TMG-chitotriomycin was also explained.

Introduction

β-*N*-Acetyl-D-hexosaminidases (EC 3.2.1.52) catalyze the removal of β-linked *N*-acetyl-D-glucosamine (GlcNAc) or *N*-acetyl-D-galactosamine (GalNAc) from the nonreducing ends of a variety of oligosaccharides and glycoconjugates. These enzymes belong to glycosyl hydrolase families 3, 20, and 84 according to the CAZy database (http://www.cazy.org/). Glycosyl hydrolase family 20 (GH20) β-*N*-acetyl-D-hexosaminidases are widely distributed in mammals, microorganisms, plants, and insects. However, they differ from on another in physiological functions. Mammalian β-*N*-acetyl-D-hexosaminidases are located in lysosome and are responsible for the catabolism of the glycosyl components of proteins and lipids.[1] Human HexA is responsible for the removal of terminal nonreducing GalNAc from G_{M2} gangliosides. Dysfunction of HexA results in massive accumulation of G_{M2} gangliosides, which can result in Tay–Sachs and Sandhoff diseases.[1] Bacterial β-*N*-acetyl-D-hexosaminidases degrade chitin, the β-(1→4)-linked GlcNAc polymer, which is used as a source of nutrient by the bacteria.[2] Fungal and insect β-*N*-acetyl-D-hexosaminidases play a role in the recycling of chitin during growth and development.[3,4] Plant β-*N*-acetyl-D-hexosaminidases exhibit the highest activity in germinating seeds, suggesting the importance of these enzymes in the storage of glycoproteins.[5]

Although GH20 β-*N*-acetyl-D-hexosaminidases vary in physiological functions, they share the same substrate-assisted retaining catalytic mechanism.[6–12] In this mechanism, the 2-acetamido group of the nonreducing end sugar of the substrate acts as a nucleophile to attack C-1, leading to the formation of a bicyclic oxazolinium intermediate. The intermediate is then attacked by a water molecule activated by the catalytic glutamate. Inhibitors that mimic the oxazolinium intermediate have been designed on the basis of this mechanism. Currently known highly efficient inhibitors, with K_i values in the nM range, include 1,2-dideoxy-2′-methyl-α-D-glucopyranoso-[2,1-*d*]-Δ2′-thiazoline (NGT),[13] *N*-acetylglucosaminono-1,5-lactone *O*-(phenylcarbamoyl)oxime (PUGNAc),[14] and nagstatin.[15] However, these inhibitors are not specific, because their targets cover most of the known GH20 β-*N*-acetyl-D-hexosaminidases.

It is always a challenge to design selective inhibitors against different GH20 β-*N*-acetyl-D-hexosaminidases, which can have different functions. We compared the crystal structures of three known β-*N*-acetyl-D-hexosaminidases—SmCHB (chitobiase from *Serratia marcescens*, PDB ID: 1QBB),[6] SpHex (β-*N*-acetyl-D-hexosaminidase from *Streptomyces plicatus*, PDB ID: 1HP5),[7] and HsHexB (β-*N*-acetyl-D-hexosaminidaseB from

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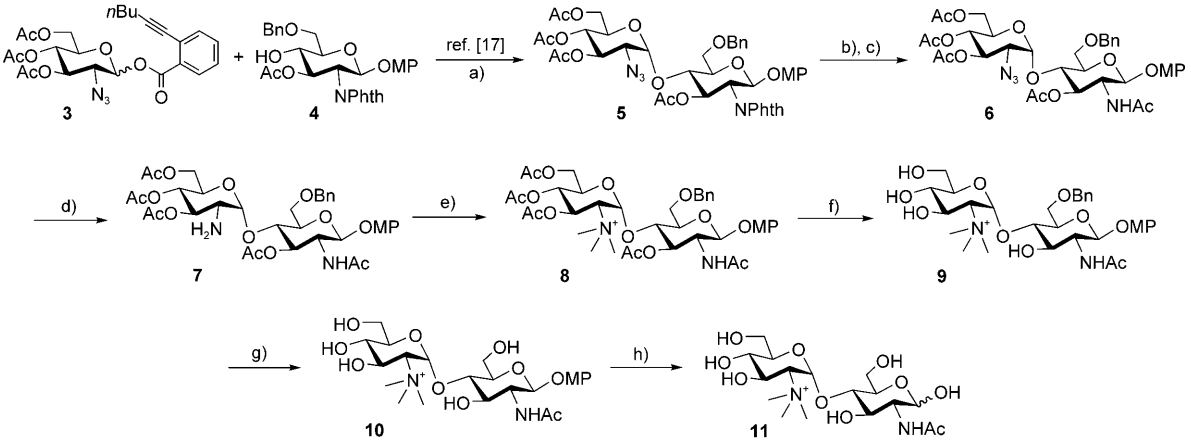


Scheme 1. Synthesis of the TMG-α-GlcNAc derivatives **10** and **11**. a) PPh_3AuOTf, 96%; b) $H_2NCH_2CH_2NH_2$, EtOH, reflux; c) Ac_2O, DMAP, pyridine, RT, 93%; d) propane-1,3-dithiol, Et_3N, pyridine, H_2O, RT, 92%; e) MeI, *i*Pr_2NEt, THF, RT, 80%; f) K_2CO_3, MeOH, CH_2Cl_2, RT, 94%; g) H_2, $Pd(OH)_2$/C, MeOH, CH_2Cl_2, aq. HCl, RT, 89%; h) $Ag(DPAH)_2$, CH_3CN, H_2O, RT, 63%.

Homo sapiens, PDB ID: 1NP0)[8,9]—and found that the bacterial enzymes each have an extra +1 subsite that consists of two conserved residues, valine and tryptophan, at the entrance of the active pocket. We postulated that this structural difference might thus provide important clues for the designing of species-specific drugs.

To validate the hypothesis, TMG-chitotriomycin[16] was chosen as the starting inhibitor, from which *N*,*N*,*N*-trimethyl-D-glucosamine (TMG) derivatives with one to four β-(1→4)-linked GlcNAc residues were designed and synthesized. TMG-chitotriomycin is a recently disclosed and efficient inhibitor for bacterial, fungal, and insect β-*N*-acetyl-D-hexosaminidases, but has no effect on the enzymes from plants and mammals,[16] a promising feature that might allow for the development of novel and eco-friendly fungicides as well as insecticides. It is also a pseudotetrasaccharide containing three β-(1→4)-linked GlcNAc units linked to TMG at the nonreducing end. Although the structure of TMG-chitotriomycin is known, its inhibition mechanism remains to be clarified. It has been speculated that the charged nitrogen atom in TMG is vital for its inhibitory activity,[16] but Kanzaki et al. have shown that TMG itself is inactive against β-*N*-acetyl-D-hexosaminidase from the insect *Spodoptera litura*.[16] This surprising result led them to presume that the $(GlcNAc)_3$ moiety is important for inhibition of β-*N*-acetyl-D-hexosaminidase. The question arising from this is why and how the sugar component affects the interaction between TMG-chitotriomycin and its target enzymes. On the other hand, TMG was originally postulated by Kanzaki et al.[16] to be α-(1→4)-linked to $(GlcNAc)_3$, but was later found by our group to be β-(1→4)-linked.[17] It is thus important to confirm if the α-conformation indeed has no activity toward β-*N*-acetyl-D-hexosaminidase. In this study, two series of both α- and β-(1→4)-linked TMG-chitotriomycin analogues with different number of GlcNAc units were synthesized and their inhibition of GH20 β-*N*-acetyl-D-hexosaminidases from different organisms was evaluated. Furthermore, an inhibition mechanism that partially supported our hypothesis was postulated in terms of the enzymes' structures.

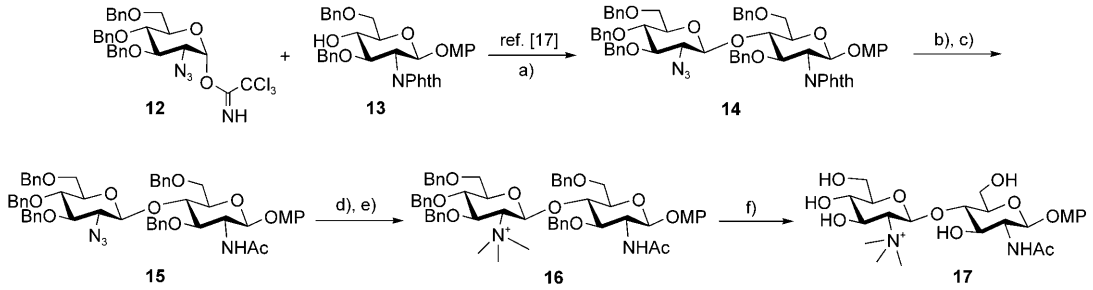
Results

Synthesis

The TMG-GlcNAc, TMG-chitobiose, and TMG-tetraose derivatives **10**/**11**, **17**, **22**/**23**, and **30**/**31** were readily synthesized by modifying previously reported synthetic approaches for TMG-chitotriomycin (**1**) and its α-TMG congener **2**,[17] as shown in Schemes 1–4, below.

The α-(1→4)-disaccharide **5** (Scheme 1) was readily prepared by coupling the glycosyl *ortho*-hexynylbenzoate **3** with the glucosamine-4-OH derivative **4** in the presence of PPh_3AuOTf as catalyst in Et_2O at −30 °C to room temperature.[17] Removal of the *N*-Phth group on **5** with ethylenediamine, followed by acetylation of the resulting amine, provided the acetamide **6** in 93% yield.[18] In the presence of propane-1,3-dithiol and Et_3N in aqueous pyridine,[19] the azido group on **6** was readily converted into an amine group in 92% yield. Use of other conditions, such as PPh_3/silica gel/THF/H_2O[20] and Zn/HOAc,[21] led to migration of the *O*-acetyl groups to the nascent NH_2 group. Treatment of the resulting amine derivative **7** with excess CH_3I in the presence of (*i*Pr)$_2$NEt readily yielded the ammonium compound **8**, in 80% yield after purification by column chromatography (Sephadex LH-20). Subsequently, compound **8** was subjected to basic conditions (K_2CO_3, MeOH/CH_2Cl_2) to give the ammonium compound **9**, followed by hydrogenolysis over $Pd(OH)_2$/C catalyst under acidic conditions to furnish the desired TMG-α-GlcNAc MP (*p*-methoxyphenyl) glycoside **10** (84% for two steps). Attempts to remove the anomeric MP group with CAN under various conditions were found to be futile. However, it was readily removed by the mild oxidizing agent $Ag(DPAH)_2$[22] to convert the MP glycoside **10** into the TMG-α-GlcNAc derivative **11**, in a satisfactory yield of 63%.

BF_3·Et_2O-promoted glycosylation of the glucosamine-4-OH derivative **13** (Scheme 2) with the 2-azido-glucopyranosyl-α-imidate **12** afforded the β-(1→4)-disaccharide **14** in 72% yield.[17,23] A procedure similar to that used for the previous conversion of disaccharide **5**→**8** was then employed to convert **14** into the disaccharide ammonium compound **16** (77%



Scheme 2. Synthesis of the TMG-β-GlcNAc derivative **17**. a) BF_3OEt_2, 72%; b) $H_2NCH_2CH_2NH_2$, EtOH, reflux; c) Ac_2O, DMAP, pyridine, RT, 88% for 2 steps; d) propane-1,3-dithiol, Et_3N, pyridine, H_2O, RT; e) MeI, *i*Pr_2NEt, THF, RT, 87% for 2 steps; f) H_2, $Pd(OH)_2$/C, MeOH, CH_2Cl_2, aq. HCl, RT, 81%.

for four steps). Hydrogenolysis of **16** over $Pd(OH)_2$/C under acidic conditions provided the desired TMG-β-GlcNAc MP glycoside **17** in 81% yield.

The TMG-β-chitobiose derivatives **22** and **23** were synthesized as shown in Scheme 3. Coupling of the disaccharide *ortho*-hexynylbenzoate **18**[17] with the glucosamine-4-OH derivative **4** in the presence of PPh_3AuOTf in CH_2Cl_2 at −30 °C afforded the β-linked trisaccharide **19** in an excellent 91% yield. Steps similar to those used for **5**→**10** were then employed for the conversion of **19** into the TMG-β-chitobiose MP glycoside **22** (six steps, 37%). Final removal of the anomeric MP group with $Ag(DPAH)_2$ gave the TMG-β-chitobiose **23** in 70% yield.

The TMG-tetraose derivatives **30** and **31** were assembled by use of a convergent 3+2 glycosidic coupling as a key step (Scheme 4). Treatment of the MP glycoside **19** with CAN, followed by condensation with *ortho*-hexynylbenzoic acid,[24] led to the trisaccharide *ortho*-hexynylbenzoate **24** (69% yield for two steps). Coupling of **24** with the disaccharide acceptor **25**[17] with PPh_3AuOTf catalysis in CH_2Cl_2 provided the β-linked pentasaccharide **27** in a modest yield of 51%, with the trisaccharide glycal **26** obtained as the major by-product (32%).[24] Steps similar to those used for **5**→**10** were then employed for the conversion of **27** into the TMG-β-tetraose MP glycoside **30** (six steps, 49%). Final removal of the anomeric MP group with $Ag(DPAH)_2$ furnished the TMG-β-tetraose **31** in 71% yield. The solubilities of the pentasaccharides **30** and **31** in methanol were poor, so MeOH/H_2O (1:1) was used as eluent for column chromatography (Sephadex LH-20).

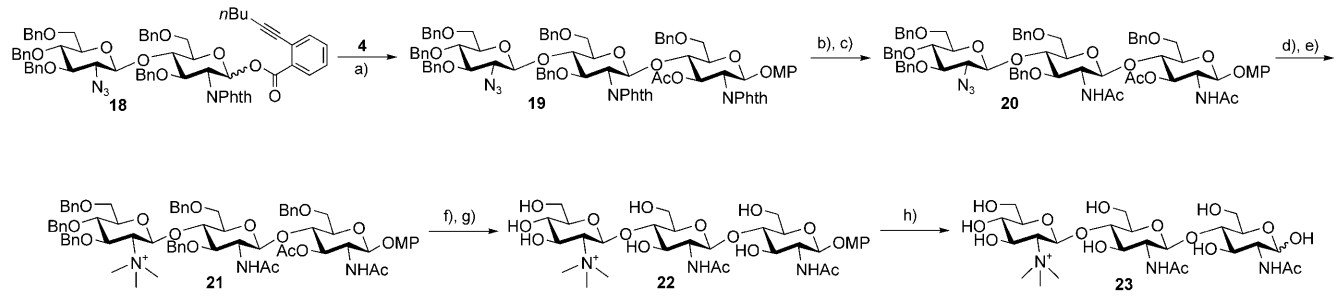
It should be noted that the chemical shifts of the anomeric protons of the β-TMG residues in these final oligosaccharide compounds (**1**,[17] **17**, **22**, **23**, **30**, **31**, and **33**[17]) appeared at $\delta = 5.23 \sim 5.42$ ppm with small $J_{1,2}$ values of 0~4.5 Hz, implying that a twist-boat conformation was adopted by the TMG residue rather than the normal chair conformation assumed by the glucosamine residue.[17]

Activity evaluation of TMG

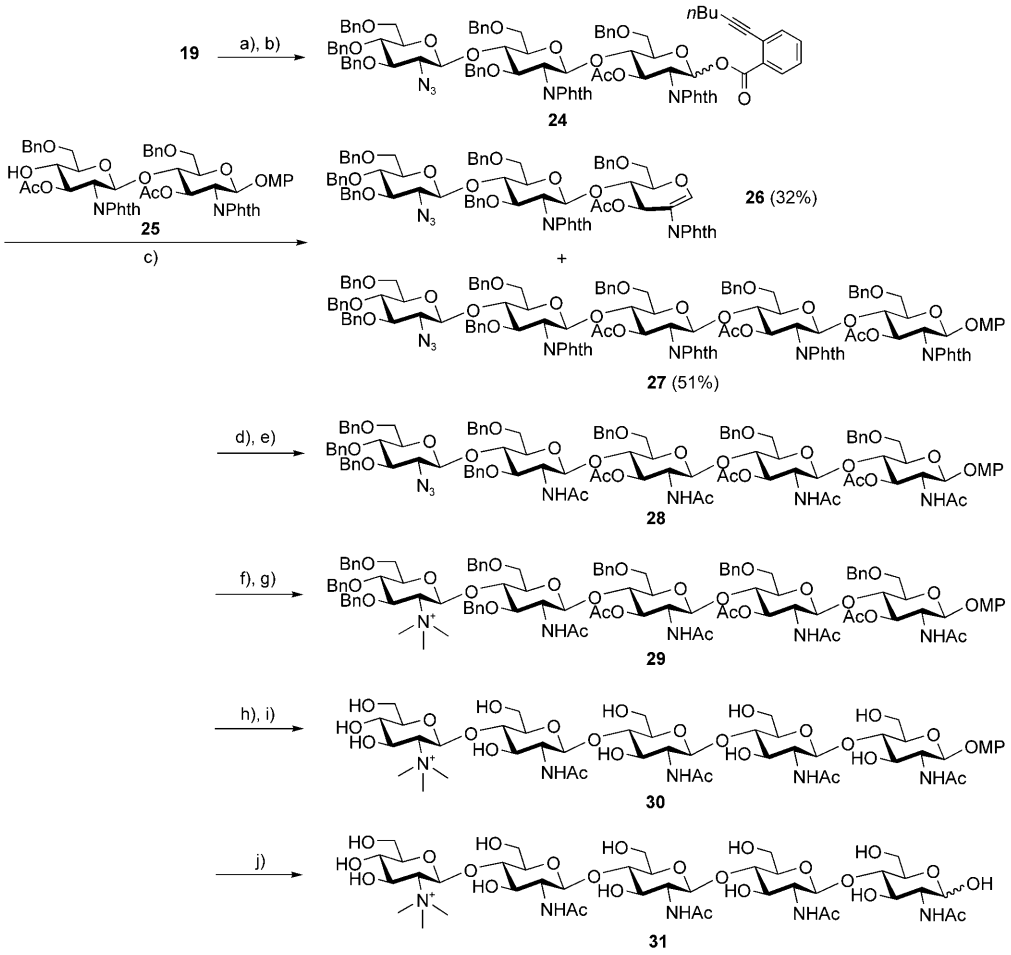
TMG was found to be inactive against β-*N*-acetyl-D-hexosaminidase from the insect *Spodoptera litura*,[16] but whether TMG is inactive against β-*N*-acetyl-D-hexosaminidases from other organisms was unclear. We evaluated its inhibitory activity against β-*N*-acetyl-D-hexosaminidases from bacteria (SpHex), insect (OfHex1), plant (CeHex), and mammal (BtHex). TMG showed no inhibition against any of the tested enzymes (Table 1). This result confirmed that it was the sugar component of TMG-chitotriomycin that was essential for its inhibitory activity against β-*N*-acetyl-D-hexosaminidases.

Inhibitory activity comparison of α-linked TMG-chitotriomycin analogues

All of the synthesized α-linked and β-linked TMG-chitotriomycin analogues with one to four GlcNAc components were tested for inhibition against representatives of bacterial, insect, plant, and mammal GH20 β-*N*-acetyl-D-hexosaminidases (Table 1). The α-linked TMG-chitotriomycin analogues all showed no inhibition against all the tested enzymes. On the other hand, all of the β-linked TMG-chitotriomycin analogues inhibited the bacterial and insect enzymes in a competitive mode with K_i values in the micro- to submicromolar ranges, respectively, whereas no inhibition of the plant and mammal



Scheme 3. Synthesis of the TMG-β-GlcNAc-GlcNAc derivatives **22** and **23**. a) PPh_3AuOTf (0.2 equiv), 4 Å MS, CH_2Cl_2, −30 °C–RT, 91%; b) $H_2NCH_2CH_2NH_2$, EtOH, reflux; c) Ac_2O, DMAP, pyridine, RT, 72% for 2 steps; d) propane-1,3-dithiol, Et_3N, pyridine, H_2O, RT; e) MeI, *i*Pr_2NEt, THF, RT, 84% for 2 steps; f) K_2CO_3, MeOH, CH_2Cl_2, RT; g) H_2, $Pd(OH)_2$/C, MeOH, CH_2Cl_2, aq. HCl, RT, 62% for 2 steps; h) $Ag(DPAH)_2$, CH_3CN, H_2O, RT, 70%.



Scheme 4. Synthesis of the TMG-β-$(GlcNAc)_3$-GlcNAc derivatives **30** and **31**. a) CAN, toluene, CH_3CN, H_2O, RT, 77%; b) *o*-hexnylbenzoic acid, DCC, DMAP, CH_2Cl_2, RT, 90%; c) PPh_3AuOTf (0.2 equiv), 4 Å MS, CH_2Cl_2, −30 °C–RT; d) $H_2NCH_2CH_2NH_2$, EtOH, reflux; e) Ac_2O, DMAP, pyridine, RT, 75% for 2 steps; f) propane-1,3-dithiol, Et_3N, pyridine, H_2O, RT; g) MeI, iPr_2NEt, THF, RT, 89% for 2 steps; h) K_2CO_3, MeOH, CH_2Cl_2, RT; i) H_2, $Pd(OH)_2/C$, MeOH, CH_2Cl_2, aq. HCl, RT, 74% for 2 steps; j) $Ag(DPAH)_2$, CH_3CN, H_2O, RT, 71%.

Table 1. Inhibitory activities of β-linked TMG-chitooligosaccharides against β-*N*-acetyl-D-hexosaminidases from different organisms.

TMG-chito-oligosaccharides	Origins of β-*N*-acetyl-D-hexosaminidases			
	Chitin-processing organism		Non-chitin-containing organism	
	bacterium[a] K_i [μM]	insect[b] K_i [μM]	plant[c] K_i [μM]	mammal[d] K_i [μM]/IC_{50}
TMG (**32**)[25]	n.d.[e]	n.d.	n.d.	n.d.
TMG-β-$(GlcNAc)_2$ (**23**)	1.1	0.077	n.d.	n.d.
TMG-β-$(GlcNAc)_3$ (**1**)	1.0	0.065	n.d.	n.d.
TMG-β-$(GlcNAc)_4$ (**31**)	1.1	0.058	n.d.	n.d.

[a] *Streptomyces plicatus*. [b] *Ostrinia furnacalis*. [c] *Canavalia ensiformis*. [d] *Bos taurus*. [e] n.d. = inhibition not detected at 0.1 mM.

enzymes was observed, even at 0.1 mM concentration. The results therefore demonstrated that only the β-linked TMG-chitotriomycin analogues could act as efficient inhibitors against GH20 β-*N*-acetyl-D-hexosaminidases.

Inhibitory activity of β-linked TMG-chitotriomycin analogues

To evaluate how the GlcNAc components affected the selective inhibition potency of TMG-chitotriomycin, we synthesized TMG-β-$(GlcNAc)_2$ (**23**), TMG-β-$(GlcNAc)_3$ (**1**), and TMG-β-$(GlcNAc)_4$ (**31**) and evaluated them against β-*N*-acetyl-D-hexosaminidases from bacterium (SpHex), insect (OfHex1), and plant (CeHex), as well as mammal (BtHex) (Table 1). All of these analogues selectively inhibited SpHex and OfHex1 but not CeHex and BtHex, meaning that the number of GlcNAc units had no effect on the selective potency. However, TMG-β-$(GlcNAc)_3$ (**1**) and TMG-β-$(GlcNAc)_4$ (**31**) inhibited SpHex and OfHex1 with the same magnitude of K_i as TMG-β-$(GlcNAc)_2$ (**23**), thus implying that a minimum of two GlcNAc units were needed for full activity, but that additional GlcNAc units (up to

two) can also be accommodated with no effect on the activities of these inhibitors (Table 1). Furthermore, the K_i values varied over the different organisms, suggesting the existence of selective inhibition of β-*N*-acetyl-D-hexosaminidases by TMG-chitotriomycin and its analogues. TMG-β-$(GlcNAc)_3$ (**1**) inhibited OfHex1 with a K_i value of 0.065 μM, which was more than ten times lower than the K_i values with β-*N*-acetyl-D-hexosaminidases from bacterium (*Streptomyces plicatus*) and fungus (*Aspergillus oryzae*).[16]

Table 2. Inhibitory activity of *p*-methoxyphenyl-derivatized β-linked TMG-chitooligosaccharides against β-*N*-acetyl-D-hexosaminidases from different organisms.

TMG-chito-oligosaccharides	Origins of β-*N*-acetyl-D-hexosaminidases			
	Chitin-processing organism		Non-chitin-containing organism	
	bacterium[a] K_i [μM]	insect[b] K_i [μM]	plant[c] K_i [μM]	mammal[d] K_i [μM]
TMG (**32**)[25]	n.d.[e]	n.d.	n.d.	n.d.
TMG-β-GlcNAc-OMP (**17**)	5.7	0.86	n.d.	n.d.
TMG-β-GlcNAc-GlcNAc-OMP (**22**)	1.3	0.073	n.d.	n.d.
TMG-β-$(GlcNAc)_2$-GlcNAc-OMP (**33**)	1.2	0.07	n.d.	n.d.
TMG-β-$(GlcNAc)_3$-GlcNAc-OMP (**30**)	1.1	0.066	n.d.	n.d.

[a] *Streptomyces plicatus*. [b] *Ostrinia furnacalis*. [c] *Canavalia ensiformis*. [d] *Bos taurus*. [e] n.d. = inhibition not detected at 0.1 mM.

TMG-chitiotriomycin analogues with *p*-methoxyphenyl substituents

We also investigated the effects of the sugar component at the reducing end of TMG-chitiotriomycin. *p*-Methoxyphenyl-substituted (MP-substituted) analogues, the intermediates in the synthesis of TMG-chitiotriomycin, were chosen. TMG-β-GlcNAc-OMP (**17**) inhibited SpHex and OfHex1 with K_i values five and 11 times higher, respectively, than those achieved by TMG-β-$(GlcNAc)_2$ (**23**; Tables 1 and 2), demonstrating that the MP group could not serve as the second GlcNAc. On the other hand, TMG-β-GlcNAc-GlcNAc-OMP (**22**) and TMG-β-$(GlcNAc)_2$-GlcNAc-OMP (**33**) inhibited SpHex and OfHex1 with the same magnitude as TMG-β-$(GlcNAc)_3$ (**1**) and TMG-β-$(GlcNAc)_4$ (**31**), as revealed by the range of K_i values (Tables 1 and 2), suggesting that the third and the fourth GlcNAc residues can be replaced by other substituents with little effect on the inhibitory activities of these compounds.

Binding modes of TMG-chitotriomycin in the SpHex and HsHexB by molecular docking

The modes by which TMG-chitotriomycin binds to SpHex and to human HsHexB were compared by molecular docking (Figure 1). The best docking results were obtained by setting the side chains of several amino acid residues of the enzymes

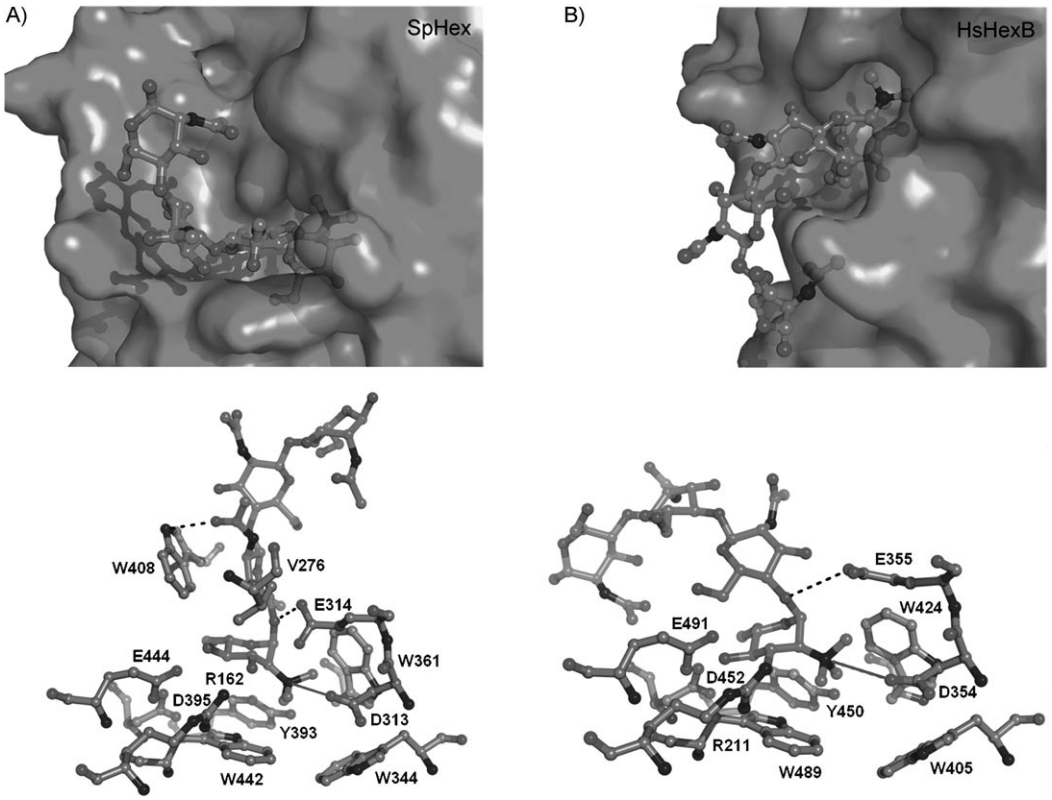


Figure 1. Binding modes of TMG-chitotriomycin in A) SpHex, and B) HsHexB. Upper figures show the active pocket surface and the bottom figures show the residues in the active pocket. Hydrogen bonds and salt bridges are shown as dashed and solid lines, respectively.

(including Glu314, Trp361, and Trp408 of SpHex, and Glu355 and Trp424 of HsHexB) as flexible. From the free energies of binding, the binding between TMG-chitotriomycin and SpHex was more favorable ($\Delta G = -7.48$ kcal mol^{-1}) than that between TMG-chitotriomycin and HsHexB ($\Delta G = -0.13$ kcal mol^{-1}).

In both SpHex and HsHexB, the TMG component of TMG-chitotriomycin is bound to the -1 subsite, which is enclosed by three conserved Trp residues (Trp344, Trp361, and Trp442 in SpHex, and Trp405, Trp424, and Trp489 in HsHexB) in a skewed chair conformation, positioning TMG in direct interaction with the conserved residues (Figure 1). TMG-chitotriomycin seemed to mimic the substrate because the acid/base catalyst (Glu314 in SpHex, Glu355 in HsHexB) is close to the oxygen atom forming the glycosidic bond between TMG and the first GlcNAc (Figure 1). It is noteworthy that the positively charged nitrogen atom on the $N(CH_3)_3$ group is close to Asp313 in the case of SpHex and to Asp354 in the case of HsHexB (Figure 1), suggesting that a salt bridge might form when this catalytic Asp is deprotonated during catalysis. The main difference in the binding between TMG-chitotriomycin and SpHex-37 versus TMG-chitotriomycin and HsHexB-2 at the -1 subsite is that the stacking of the pyranose ring (in TMG) against Trp442 of SpHex was tighter than that against Trp489 in HsHexB (Figure 1).

In the $+1$ subsite, which is present in SpHex but absent in HsHexB, the GlcNAc adjacent to TMG is stacked against Trp408 and the oxygen atom on the 2-acetamido group is hydrogen-bonded to the nitrogen atom of the indolyl group of Trp408 (Figure 1 A). The binding of this GlcNAc seemed to push the TMG component into the -1 subsite so as to form tighter stacking against Trp442. This is consistent with our observation that a conserved Trp408 at the $+1$ subsite of a TMG-chitotriomycin-sensitive enzyme is essential for binding with TMG-chitotriomycin.

As for SpHex-37 and HsHexB-2, no intermolecular interaction was found between the second or third GlcNAc units of TMG-chitotriomycin and the enzymes (Figure 1). This is in good agreement with our experimental data. It is interesting to note that in the case of SpHex, the K_i value for TMG-β-$(GlcNAc)_2$ (**23**) was five times lower than that for TMG-β-GlcNAc-OMP (**17**). The reason is not known. We deduced that there might be a $+2$ subsite in SpHex for binding the second GlcNAc of TMG-β-$(GlcNAc)_2$ through hydrogen bonding. Because the MP group does not contain hydroxy substituents, MP was not able to bind and be stabilized by the $+2$ subsite. However, the precise mechanism requires further work.

Discussion

GH20 β-*N*-acetyl-D-hexosaminidases are enzymes with different physiological roles. To achieve selective inhibition for each of these enzymes would provide insight into the specialized functions of these enzymes in vivo. Selective inhibition would also be an advantage for disease control and plant protection. Little progress has been made as far as selective inhibitors are concerned.

By comparing the structures of two bacterial chitinolytic β-*N*-acetyl-D-hexosaminidases—SmCHB[6] and SpHex[7]—with that of lysosomal HsHexB,[8,9] we found that the bacterial chitinolytic enzymes each have an extra $+1$ subsite that contains two highly conserved amino acid residues in the active site. We speculated that this structural discrepancy between the bacterial and human enzymes could be a determining factor for selective inhibition.

TMG-chitotriomycin has recently been found to be an inhibitor that shows selective inhibition toward β-*N*-acetyl-D-hexosaminidases from chitin-containing organisms.[16] It is surprising that this naturally occurring compound contains four sugar units, because the extra two sugar units at the reducing end were not essential for selective inhibition. Although the structure of TMG-chitotriomycin has been established, its inhibition mechanism remains to be clarified. In addition, the complex structure of TMG-chitotriomycin makes it difficult to synthesize on large scales.

In this work, two series of α- and β-(1→4)-linked TMG-$(GlcNAc)_{1-4}$ derivatives were synthesized and their inhibitory potencies against several GH20 β-*N*-acetyl-D-hexosaminidases from different organisms were evaluated. The enzymatic assay showed that only β-(1→4)-linked TMG-$(GlcNAc)_{1-4}$ derivatives were effective. Glycosidases can be classified in term of their preference either for α- or for β-glycoside bonds in the substrates, so it is easy to understand why β-(1→4)- rather than α-(1→4)-linked TMG-chitotriomycin has been selected in nature as an inhibitor for the GH20 β-*N*-acetyl-D-hexosaminidase β-glycosidases. In addition to the previously reported NMR data,[17] here we have confirmed the structure of this inhibitor through an enzymatic approach.

The activity assay of the β-(1→4)-linked TMG-$(GlcNAc)_{1-4}$ derivatives showed that either one or two GlcNAc units are required for inhibition. Crystal structure comparison of bacterial (SmCHB and SpHex)[6,7] and human (HsHexB)[8,9] β-*N*-acetyl-D-hexosaminidases indicated that the bacterial enzymes have larger and longer active pockets than the human enzyme. The exceptional $+1$ subsite with conserved residues (Val493 and Trp685 of SmCHB and Val276 and Trp408 of SpHex) in the bacterial enzyme is ideal for binding long and linear substrates. It is interesting to note that the reducing end GlcNAc (NAGB) of chitobiose is tightly bound in the $+1$ subsite through a stacking interaction with the indolyl group of Trp685 in the SmCHB-chitobiose complex.[6] NAGB is twisted around 90° relative to the nonreducing end GlcNAc (NAGA). In SpHex, a glycerol is located at the $+1$ subsite and superimposed onto half of the NAGB in the SpHec-NAG-thiazoline complex,[7] so the binding of $+1$ GlcNAc might be required for stabilizing the binding conformation of the -1 sugar. We thus presume that the GlcNAc component next to TMG binds at the $+1$ subsite and stabilizes the binding conformation of TMG in TMG-chitotrimycin-sensitive enzymes. Furthermore, the docking of TMG-chitotriomycin to SpHex suggested that TMG-chitotriomycin could be tightly bound through interaction with the amino acid residues in both the -1 and the $+1$ subsites of SpHex. Because HsHexB does not contain a $+1$ subsite, docking of TMG-chitotriomycin at the -1 subsite in HsHexB probably occurred

through weak interaction. The higher ΔG value obtained for the binding of TMG-chitotriomycin to HsHexB confirmed that this binding was unstable. To this end, we speculate that the GlcNAc component of TMG is positioned at the +1 subsite through stacking against the conserved Trp residues and is hydrogen-bonded to the conserved Val residues of TMG-chitotrimycin-sensitive enzymes. In this way, TMG is stabilized and can interact with the catalytic residues at the −1 subsite.

TMG-chitotriomycin is a stronger inhibitor against insect β-*N*-acetyl-D-hexosaminidases (OfHex1 and β-*N*-acetyl-D-hexosaminidase from *Spodoptera litura*[16]) than against bacterial β-*N*-acetyl-D-hexosaminidase (SpHex) and fungal β-*N*-acetyl-D-hexosaminidase (β-*N*-acetyl-D-hexosaminidase from *A. oryzae*[16]). We deduced that the insect enzyme might contain a narrower substrate binding cleft than the bacterial and fungal enzymes.

This study has shed more light on the mechanisms of the selectivity of TMG-chitotriomycin with respect to the inhibition it exerts on GH20 β-*N*-acetyl-D-hexosaminidases. Furthermore, the inhibition of these enzymes by TMG-β-$(GlcNAc)_2$, which has one GlcNAc unit fewer than TMG-chitotriomycin, also validated our hypothesis that selective inhibitors can be designed according to the structural difference at the active pockets of GH20 β-*N*-acetyl-D-hexosaminidases.

Experimental Section

Synthesis

***p*-Methoxyphenyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-acetamino-2-deoxy-β-D-glucopyranoside (6):** Compound **5** (1.00 g, 1.16 mmol) was dissolved in EtOH (13 mL) at room temperature, followed by addition of ethylenediamine (4 mL). After having been heated at reflux at 100 °C for 14 h, the mixture was concentrated in vacuo to give a residue, which was used in the next step without further purification.

Ac_2O (8 mL) was added to a solution of the residue and DMAP (80 mg, 0.66 mmol) in pyridine (14 mL). After having been stirred at room temperature overnight, the solution was diluted with CH_2Cl_2 and washed with aqueous HCl (1 N), saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (petroleum ether/EtOAc 1:1→1:1.5) to give **6** (841 mg, 93% for two steps) as a white solid. $[\alpha]_D^{27} = +54.7$ ($c = 0.50$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.32$ (m, 5H), 6.94 (d, $J = 9.0$ Hz, 2H), 6.78 (d, $J = 8.7$ Hz, 2H), 5.93 (d, $J = 9.3$ Hz, 1H), 5.38 (t, $J = 10.2$ Hz, 1H), 5.30 (d, $J = 3.9$ Hz, 1H), 5.17 (t, $J = 9.0$ Hz, 1H), 5.01 (t, $J = 9.9$ Hz, 1H), 4.92 (d, $J = 7.5$ Hz, 1H), 4.56 (dd, $J = 12.0$, 14.7 Hz, 2H), 4.33 (dd, $J = 9.6$, 16.8 Hz, 1H), 4.18 (dd, $J = 3.6$, 12.6 Hz, 1H), 4.05 (m, 1H), 3.97 (m, 1H), 3.78 (m, 7H), 3.42 (dd, $J = 3.9$, 10.8 Hz, 1H), 2.08 (s, 6H), 2.03 (s, 3H), 2.02 (s, 3H), 1.97 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.8$, 170.4, 170.1, 170.0, 169.5, 155.4, 151.1, 137.7, 128.4, 127.8, 127.6, 118.3, 114.5, 100.1, 98.0, 74.8, 73.7, 73.6, 70.3, 69.0, 68.5, 68.1, 61.3, 61.0, 55.6, 53.2, 23.2, 20.8, 20.6, 20.5 ppm; HRMS (MALDI): *m/z*: calcd for $C_{36}H_{44}N_4O_{15}Na$: 795.2706 $[M+Na]^+$; found: 795.2695.

***p*-Methoxyphenyl 3,4,6-tri-*O*-acetyl-2-trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-acetamino-2-deoxy-β-D-glucopyranoside (8)**: Et_3N (2 mL) and propane-1,3-dithiol (4 mL) were added to a solution of **6** (600 mg, 0.78 mmol) in pyridine/H_2O (40 mL/10 mL). The solution was stirred at room temperature for 1 h and then concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N 80:1:1) to furnish **7** (532 mg, 92%) as a white solid.^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.31$ (m, 5H), 6.97 (d, $J = 9.3$ Hz, 2H), 6.78 (d, $J = 9.0$ Hz, 2H), 5.94 (d, $J = 9.3$ Hz, 1H), 5.20 (d, $J = 3.3$ Hz, 1H), 5.15 (t, $J = 8.7$ Hz, 1H), 4.93 (m, 3H), 4.58 (dd, $J = 11.7$, 19.8 Hz, 2H), 4.25 (m, 1H), 4.20 (m, 1H), 4.11 (m, 2H), 3.83 (m, 2H), 3.74 (m, 5H), 2.89 (dd, $J = 3.3$, 9.9 Hz, 1H), 2.08 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.96 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 171.6$, 170.8, 170.5, 170.2, 169.6, 155.4, 151.2, 137.8, 128.4, 127.7, 127.5, 118.4, 114.5, 100.5, 100.3, 75.0, 74.3, 73.5, 69.1, 68.7, 68.4, 61.8, 55.6, 54.3, 54.0, 23.2, 21.4, 20.8, 20.7, 20.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{36}H_{46}N_2O_{15}Na$: 769.2798 $[M+Na]^+$; found: 769.2790.

$(iPr)_2Net$ (0.4 mL) and CH_3I (2 mL) were added to a solution of the amine **7** (103 mg, 0.14 mmol) in dry THF (3 mL). After having been stirred at room temperature for 40 h, the mixture was filtered, concentrated in vacuo, and passed through a chromatography column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) to afford **8** (87 mg, 80%) as a slightly yellow solid. $[\alpha]_D^{27} = +40.4$ ($c = 0.30$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.29$ (m, 5H), 7.01 (d, $J = 8.7$ Hz, 2H), 6.76 (d, $J = 9.3$ Hz, 2H), 5.96 (t, $J = 8.4$ Hz, 1H), 5.69 (m, 2H), 5.58 (d, $J = 7.5$ Hz, 1H), 4.99 (t, $J = 9.0$ Hz, 1H), 4.67 (d-like, $J = 11.7$ Hz, 1H), 4.53 (d-like, $J = 11.4$ Hz, 1H), 4.40 (m, 2H), 4.24 (m, 2H), 4.10 (m, 1H), 3.88 (m, 2H), 3.80 (m, 2H), 3.72 (s, 3H), 3.53 (s, 9H), 2.16 (s, 3H), 2.12 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.90 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 171.9$, 170.6, 170.4, 169.3, 169.2, 155.4, 150.9, 137.6, 128.4, 127.9, 127.4, 118.9, 114.5, 99.4, 92.3, 75.7, 73.7, 73.1, 70.5, 69.9, 69.1, 68.9, 68.3, 68.0, 60.6, 55.6, 55.3, 54.8, 23.2, 21.9, 21.2, 20.7, 20.4 ppm; HRMS (MALDI): *m/z*: calcd for $C_{39}H_{53}N_2O_{15}$: 789.3452 $[M]^+$; found: 789.3441.

***p*-Methoxyphenyl 2-trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranoside (10)**: K_2CO_3 (23 mg, 0.17 mmol) was added to a solution of **8** (102 mg, 0.13 mmol) in MeOH/CH_2Cl_2 (4 mL/0.8 mL). The mixture was stirred at room temperature for 4 h and then neutralized with Dowex-50 (H^+). Filtration, concentration in vacuo, and elution through a chromatography column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) provided **9** (75 mg, 94%) as a white solid. $[\alpha]_D^{25} = +30.1$ ($c = 1.00$, MeOH); ^{1}H NMR (300 MHz, CD_3OD): $\delta = 7.32$ (m, 5H), 6.99 (d, $J = 8.7$ Hz, 2H), 6.79 (d, $J = 9.3$ Hz, 2H), 6.34 (d, $J = 2.1$ Hz, 1H), 5.07 (d, $J = 7.5$ Hz, 1H), 4.56 (s, 2H), 4.11 (m, 2H), 4.03 (m, 2H), 3.84 (m, 2H), 3.73 (s, 3H), 3.63 (m, 5H), 3.40 (s, 9H), 2.04 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 172.8$, 155.5, 151.4, 138.1, 127.9, 127.5, 117.3, 118.1, 114.1, 100.0, 94.3, 75.3, 74.0, 73.0, 72.9, 72.5, 71.4, 70.5, 68.9, 68.4, 60.2, 56.7, 54.7, 53.6, 21.7 ppm; HRMS (MALDI): *m/z*: calcd for $C_{31}H_{45}N_2O_{11}$: 621.3019 $[M]^+$; found: 621.3018.

Compound **9** (45 mg, 0.072 mmol) was dissolved in MeOH (5 mL) at room temperature, followed by addition of aqueous HCl (37%, two drops) and $Pd(OH)_2$/C (200 mg, 20%). After having been hydrogenated at room temperature for 22 h, the suspension was filtered and neutralized with Dowex-1X8 (OH^-). Filtration, concentration in vacuo, and elution through a chromatography column (Sephadex LH-20, MeOH) gave **10** (34 mg, 89%) as a colorless solid. $[\alpha]_D^{27} = +61.8$ ($c = 1.00$, MeOH); ^{1}H NMR (300 MHz, CD_3OD): $\delta = 6.97$ (d, $J = 9.0$ Hz, 2H), 6.82 (d, $J = 9.0$ Hz, 2H), 6.30 (d, $J = 2.1$ Hz, 1H), 5.03 (d, $J = 7.5$ Hz, 1H), 4.11 (m, 2H), 3.92 (m, 5H), 3.73 (m, 5H), 3.62 (m, 2H), 3.47 (m, 1H), 3.41 (s, 9H), 2.02 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 172.8$, 155.4, 151.6, 117.8, 114.1, 100.0, 94.5, 75.2, 74.8, 73.0, 72.6, 71.1, 70.9, 68.9, 60.6, 60.4, 56.7,

54.7, 53.6, 21.7 ppm; HRMS (MALDI): *m/z*: calcd for $C_{24}H_{39}N_2O_{11}$: 531.2552 $[M]^+$; found: 531.2548.

2-Trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-D-glucopyranose (11): $Ag(DPAH)_2$ (30 mg, 0.065 mmol) was added to a solution of **10** (16 mg, 0.03 mmol) in CH_3CN/H_2O (0.7 mL/0.7 mL). After having been stirred at room temperature for 4 min, the solution was filtered and neutralized with Dowex-1X8 (OH^-). The mixture was filtered and concentrated in vacuo. The residue was eluted through a chromatography column (Sephadex LH-20, MeOH) and then a Dowex-1X8 (Cl^-) column (H_2O) to afford **11** (8 mg, 63%) as a white solid. $[\alpha]_D^{26} = +79.4$ (c = 0.30, MeOH); ^{1}H NMR (300 MHz, CD_3OD): δ = 6.28 (brs, 1H), 5.08 (d, J = 3.3 Hz, 0.5H), 4.68 (d, J = 8.1 Hz, 0.4H), 4.15 (m, 1H), 3.96 (m, 3H), 3.83 (m, 3H), 3.70 (m, 2H), 3.54 (m, 1H), 3.40 (s, 9H), 2.00 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.5, 94.6, 90.9, 72.9, 72.6, 72.5, 71.7, 70.9, 69.8, 68.9, 60.6, 60.5, 54.8, 54.4, 53.4, 21.2 ppm; HRMS (MALDI): *m/z*: calcd for $C_{17}H_{33}N_2O_{10}$: 425.2139 $[M]^+$; found: 425.2130.

***p*-Methoxyphenyl 3,4,6-tri-*O*-benzyl-2-trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-acetamino-2-deoxy-β-D-glucopyranoside (16)**: Et_3N (0.2 mL) and propane-1,3-dithiol (0.4 mL) were added to a solution of **15**[17] (97 mg, 0.10 mmol) in pyridine/H_2O (4 mL/1 mL). The solution was stirred at room temperature for 1 h and was then concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (petroleum ether/EtOAc/Et_3N 1:2:0.03) to give a white solid (88 mg, 94%). (iPr)$_2$Net (0.3 mL) and CH_3I (2 mL) was added to a solution of the white solid (85 mg, 0.09 mmol) in dry THF (3 mL). After having been stirred at room temperature for 40 h, the mixture was filtered, concentrated in vacuo, and eluted through a chromatography column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) to provide **16** (83 mg, 93%) as a white solid. $[\alpha]_D^{25} = -20.3$ (c 0.70, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.29 (m, 21H), 7.14 (m, 4H), 6.97 (d, J = 8.7 Hz, 2H), 6.77 (d, J = 8.7 Hz, 2H), 5.33 (m, 2H), 4.76 (s, 2H), 4.64 (m, 2H), 4.53 (m, 3H), 4.45 (m, 5H), 4.07 (m, 5H), 3.74 (m, 6H), 3.59 (m, 1H), 3.49 (m, 1H), 3.33 (m, 1H), 2.95 (s, 9H), 1.99 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.8, 155.3, 151.3, 138.4, 137.8, 137.7, 136.7, 136.3, 128.7, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 127.7, 118.8, 114.5, 99.8, 93.5, 79.5, 78.3, 76.5, 74.9, 74.0, 73.6, 73.4, 73.3, 72.9, 72.6, 72.3, 69.8, 69.6, 55.7, 53.8, 23.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{59}H_{69}N_2O_{11}$: 981.4906 $[M]^+$; found: 987.4896.

***p*-Methoxyphenyl 2-trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranoside (17)**: Compound **16** (68 mg, 0.07 mmol) was dissolved in MeOH (5 mL) at room temperature, followed by addition of aqueous HCl (37%, two drops) and $Pd(OH)_2$/C (200 mg, 20%). After having been hydrogenated at room temperature for 4 h, the suspension was filtered and neutralized with Dowex-1X8 (OH^-). Filtration, concentration in vacuo, and elution through a chromatography column (Sephadex LH-20, MeOH) gave **17** (30 mg, 81%) as a colorless solid. $[\alpha]_D^{27} = -9.6$ (c = 0.90, MeOH); ^{1}H NMR (300 MHz, CD_3OD): δ = 6.94 (d, J = 9.0 Hz, 2H), 6.82 (d, J = 9.0 Hz, 2H), 5.39 (d, J = 4.5 Hz, 1H), 4.94 (d, J = 7.5 Hz, 1H), 3.93 (m, 5H), 3.83 (m, 2H), 3.73 (m, 6H), 3.58 (m, 2H), 3.34 (s, 9H), 2.01 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 172.5, 155.4, 151.6, 117.8, 114.1, 100.4, 95.7, 78.6, 77.6, 77.2, 75.0, 71.0, 69.9, 69.5„ 61.5, 60.6, 56.5, 54.7, 53.4, 21.7 ppm; HRMS (MALDI): *m/z*: calcd for $C_{24}H_{39}N_2O_{11}$: 531.2561 $[M]^+$; found: 531.2548.

***p*-Methoxyphenyl 3,4,6-tri-*O*-benzyl-2-azido-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranoside (19)**: Newly prepared PPh_3AuOTf in CH_2Cl_2 (0.05 M, 2.4 mL) was added dropwise under argon at −30 °C to a stirred mixture of the donor **18**[17] (775 mg, 0.68 mmol), the acceptor **4** (312 mg, 0.57 mmol), and freshly activated MS (4 Å, 1 g) in dry CH_2Cl_2 (20 mL). After 0.5 h, the mixture was allowed to warm up naturally to room temperature and the stirring was continued overnight. The mixture was then filtered and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether/EtOAc 3:1→2:1) to afford **19** (762 mg, 91%) as a white solid: $[\alpha]_D^{25} = +7.3$ (c = 0.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.71 (m, 8H), 7.27 (m, 25H), 6.92 (m, 2H), 6.75 (m, 5H), 6.63 (d, J = 9.0 Hz, 2H), 5.73 (t, J = 9.9 Hz, 1H), 5.71 (d, J = 9.0 Hz, 1H), 5.25 (d, J = 8.7 Hz, 1H), 4.80 (m, 4H), 4.62 (d, J = 11.4 Hz, 1H), 4.53 (d, J = 10.8 Hz, 1H), 4.39 (m, 8H), 4.19 (m, 2H), 4.08 (m, 2H), 3.91 (dd, J = 2.1, 10.5 Hz, 1H), 3.67 (m, 6H), 3.56 (m, 2H), 3.45 (m, 2H), 3.36 (m, 2H), 3.23 (t, J = 9.3 Hz, 1H), 3.12 (d-like, J = 9.6 Hz, 1H), 1.87 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.2, 155.5, 150.7, 138.7, 138.2, 138.1, 138.0, 137.9, 137.8, 134.2, 133.6, 131.6, 128.5, 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 126.9, 123.5, 123.2, 118.9, 114.3, 100.9, 97.5, 97.4, 83.2, 77.7, 77.5, 77.3, 75.4, 74.8, 74.7, 74.6, 74.5, 74.2, 73.3, 73.1, 72.6, 71.4, 68.3, 67.8, 67.6, 66.9, 56.1, 55.5, 55.0, 20.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{85}H_{81}N_5O_{19}Na$: 1498.5435 $[M+Na]^+$; found: 1498.5418.

***p*-Methoxyphenyl 2-trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranoside (22)**: Compound **19** (200 mg, 0.14 mmol) was dissolved in EtOH (6 mL) at room temperature, followed by addition of ethylenediamine (1.2 mL). After having been heated at reflux at 100 °C for 14 h, the mixture was concentrated in vacuo to give a residue, which was used in the next step without further purification.

Ac_2O (2 mL) was added to a solution of the residue and DMAP (36 mg, 0.30 mmol) in pyridine (6 mL). After having been stirred at room temperature overnight, the solution was diluted with CH_2Cl_2 and washed with aqueous HCl (1 N), saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (CH_2Cl_2/MeOH 80:1→60:1) to give **20** (126 mg, 72% for two steps) as a slightly yellow solid: $[\alpha]_D^{26} = -39.4$ (c = 0.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.25 (m, 30H), 6.92 (d, J = 8.8 Hz, 2H), 6.77 (d, J = 9.2 Hz, 2H), 6.09 (d-like, J = 9.2 Hz, 1H), 5.07 (t, J = 8.0 Hz, 1H), 4.88 (m, 2H), 4.79 (m, 3H), 4.56 (m, 5H), 4.45 (m, 2H), 4.35 (m, 4H), 4.07 (t, J = 8.8 Hz, 1H), 3.96 (t, J = 7.2 Hz, 1H), 3.89 (dd, J = 2.8, 10.8 Hz, 1H), 3.76 (m, 4H), 3.62 (m, 7H), 3.49 (m, 1H), 3.38 (m, 2H), 3.24 (t, J = 9.2 Hz, 1H), 3.14 (dd, J = 1.2, 9.6 Hz, 1H), 1.97 (s, 3H), 1.96 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.2, 170.4, 170.3, 155.2, 151.3, 139.1, 138.0, 137.9, 137.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 118.2, 114.5, 100.9, 100.2, 100.0, 83.0, 79.1, 77.6, 77.3, 76.5, 75.4, 74.9, 74.8, 74.7, 74.4, 73.8, 73.7, 73.3, 73.2, 73.1, 72.5, 68.4, 68.2, 66.9, 55.9, 55.6, 52.9, 23.3, 23.2, 20.8 ppm; HRMS (MALDI): *m/z*: calcd for $C_{73}H_{81}N_5O_{17}Na$: 1322.5537 $[M+Na]^+$; found: 1322.5520.

Et_3N (0.3 mL) and propane-1,3-dithiol (0.6 mL) were added to a solution of the trisaccharide azide **20** (120 mg, 0.09 mmol) in pyridine/H_2O (4 mL/1 mL). The solution was stirred at room temperature for 3 h and then concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N 100:2:1) to give the corresponding amine as a white solid. (iPr)$_2$Net (0.6 mL) and CH_3I (4 mL) was added to a solution of the above white solid in dry THF (6 mL). After having been stirred

at room temperature for 40 h, the mixture was filtered, concentrated in vacuo, and eluted through a column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) to provide **21** (102 mg, 84% for two steps) as a slightly yellow solid: $[\alpha]_D^{26} = -25.1$ ($c = 0.3$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.26$ (m, 30H), 6.95 (d, $J = 8.8$ Hz, 2H), 6.74 (d, $J = 8.8$ Hz, 2H), 6.54 (d-like, $J = 9.2$ Hz, 1H), 5.26 (d, $J = 5.2$ Hz, 1H), 5.20 (t, $J = 8.0$ Hz, 1H), 5.04 (d, $J = 7.6$ Hz, 1H), 4.78 (dd, $J = 4.8$, 13.2 Hz, 1H), 4.70 (br s, 2H), 4.59 (m, 4H), 4.45 (m, 6H), 4.27 (dd, $J = 8.8$, 16.8 Hz, 1H), 4.20 (t, $J = 8.4$ Hz, 1H), 4.09 (m, 1H), 4.01 (m, 4H), 3.82 (m, 3H), 3.70 (m, 6H), 3.52 (m, 2H), 3.43 (m, 1H), 3.33 (br s, 1H), 2.92 (s, 9H), 1.95 (s, 3H), 1.94 (s, 3H), 1.88 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 169.9$, 169.7, 169.6, 154.2, 150.2, 137.4, 136.8, 136.5, 135.7, 135.4, 127.6, 127.5, 127.4, 127.3, 127.0, 126.9, 126.8, 126.7, 126.6, 126.5, 126.4, 117.5, 113.5, 98.8, 92.4, 78.4, 78.1, 76.4, 75.4, 73.9, 73.6, 73.2, 72.6, 72.3, 72.2, 72.1, 71.8, 71.6, 71.3, 68.7, 68.0, 54.9, 54.6, 52.6, 52.2, 22.4, 22.2, 19.9 ppm; HRMS (MALDI): *m/z*: calcd for $C_{76}H_{90}N_3O_{17}$: 1316.6244 $[M]^+$; found: 1316.6265.

K_2CO_3 (50 mg, 0.36 mmol) was added to a solution of **21** (95 mg, 0.072 mmol) in MeOH/CH_2Cl_2 (5 mL/2.5 mL). The mixture was stirred at room temperature for 2 h and was then neutralized with Dowex-50 (H^+). The mixture was filtered and concentrated in vacuo to give a white solid for the next step, which was used without further purification.

The white solid was dissolved in MeOH/CH_2Cl_2 (3 mL/2 mL) at room temperature, followed by addition of aqueous HCl (37%, two drops) and $Pd(OH)_2$/C (300 mg, 20%). After having been hydrogenated at room temperature for 14 h, the suspension was filtered and neutralized with Dowex-1X8 (OH^-). Filtration, concentration in vacuo, and elution through a column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) provided **22** (32 mg, 62% for two steps) as a white solid: $[\alpha]_D^{24} = -13.3$ ($c = 0.25$, MeOH); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 6.85$ (d, $J = 9.2$ Hz, 2H), 6.72 (d, $J = 9.2$ Hz, 2H), 5.28 (d, $J = 4.4$ Hz, 1H), 4.80 (d, $J = 8.4$ Hz, 1H), 4.48 (d, $J = 8.0$ Hz, 1H), 3.85 (m, 2H), 3.76 (m, 6H), 3.63 (m, 7H), 3.55 (m, 3H), 3.46 (m, 2H), 3.37 (m, 1H), 3.24 (s, 9H), 1.95 (s, 3H), 1.90 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 172.5$, 172.3, 155.3, 151.6, 117.7, 114.1, 100.6, 100.5, 95.6, 79.6, 78.6, 77.6, 77.1, 75.1, 72.7, 70.9, 69.9, 69.3, 61.4, 60.5, 60.0, 56.3, 55.2, 54.7, 53.4, 21.7, 21.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{32}H_{52}N_3O_{16}$: 734.3365 $[M]^+$; found: 734.3342.

2-Trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-D-glucopyranose (23): $Ag(DPAH)_2$ (25 mg, 0.054 mmol) was added to a solution of the MP glycoside **22** (10 mg, 0.014 mmol) in CH_3CN/H_2O (0.5 mL/0.5 mL). After having been stirred at room temperature for 4 min, the solution was filtered and neutralized with Dowex-1X8 (OH^-). The mixture was filtered and concentrated in vacuo. The residue was eluted through columns [Sephadex LH-20, MeOH and then Dowex-1X8 (Cl^-), H_2O] to afford **23** (6 mg, 70%) as a white solid: $[\alpha]_D^{21} = -7.5$ ($c = 0.35$, MeOH); ^{1}H NMR (400 MHz, CD_3OD): $\delta = 5.36$ (d, $J = 4.0$ Hz, 1H), 5.08 (d, $J = 2.8$ Hz, 0.3H), 4.52 (d, $J = 7.6$ Hz, 1H), 4.49 (d, $J = 8.4$ Hz, 0.7H), 3.92 (m, 2H), 3.80 (m, 10H), 3.61 (m, 6H), 3.32 (s, 9H), 2.03 (s, 3H), 2.00 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): $\delta = 173.2$, 172.4, 101.6, 95.7, 78.6, 77.6, 77.2, 75.0, 71.0, 69.9, 69.5, 69.2, 61.4, 60.4, 60.3, 56.6, 56.4, 54.0, 53.4, 53.3, 21.6, 21.1 ppm; HRMS (ESI): *m/z*: calcd for $C_{25}H_{46}N_3O_{15}$: 628.2938 $[M]^+$; found: 628.2923.

3,4,6-Tri-*O*-benzyl-2-azido-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranosyl *ortho*-hexynylbenzoate (24): CAN (740 mg, 1.35 mmol) was added to a solution of the MP glycoside **19** (400 mg, 0.27 mmol) in toluene/CH_3CN/H_2O (2:3:2 mL). After having been stirred at room temperature for 30 min, the solution was poured into ice water and extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous $NaHCO_3$ and brine and dried over Na_2SO_4. Filtration, concentration in vacuo, and purification by silica gel column chromatography (petroleum ether/EtOAc 3:2) gave the corresponding lactol as a yellow solid (285 mg, 77%). *o*-Hexynylbenzoic acid (51 mg, 0.25 mmol), DCC (64 mg, 0.32 mmol), and DMAP (39 mg, 0.32 mmol) were added to a solution of the above yellow solid in dry CH_2Cl_2 (7 mL). The mixture was stirred at room temperature overnight and filtered. The filtrate was concentrated in vacuo and purified by silica gel column chromatography (petroleum ether/EtOAc 3:1) to give **24** (292 mg, 90%) as a white solid: $[\alpha]_D^{27} = +1.7$ ($c = 2.0$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.70$ (m, 8H), 7.30 (m, 29H), 6.92 (m, 2H), 6.75 (m, 3H), 6.56 (d, $J = 9.0$ Hz, 1H), 5.83 (dd, $J = 9.3$, 10.8 Hz, 1H), 5.26 (d, $J = 8.4$ Hz, 1H), 4.82 (m, 4H), 4.50 (m, 10H), 4.19 (m, 3H), 4.08 (m, 1H), 3.90 (m, 1H), 3.68 (m, 4H), 3.55 (m, 2H), 3.39 (m, 3H), 3.24 (t, $J = 9.6$ Hz, 1H), 3.12 (d-like, $J = 9.6$ Hz, 1H), 2.38 (t, $J = 7.5$ Hz, 2H), 1.85 (s, 3H), 1.54 (m, 2H), 1.39 (m, 2H), 0.89 ppm (t, $J = 7.2$ Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 170.1$, 167.5, 163.1, 138.7, 138.2, 138.1, 138.0, 137.9, 137.8, 134.5, 134.1, 133.6, 132.2, 131.7, 131.4, 130.7, 129.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 127.0, 126.9, 125.7, 125.5, 123.6, 100.9, 97.3, 97.1, 90.2, 83.2, 78.9, 77.7, 77.6, 77.3, 77.2, 75.4, 75.0, 74.8, 74.7, 74.5, 73.4, 73.1, 73.0, 72.7, 71.0, 68.3, 67.9, 67.5, 67.0, 56.1, 54.0, 30.6, 22.0, 20.5, 19.4, 13.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{91}H_{87}N_5O_{19}Na$: 1576.5887 $[M+Na]^+$; found: 1576.5888.

***p*-Methoxyphenyl 3,4,6-tri-*O*-benzyl-2-azido-2-deoxy-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-*N*-phthalimido-2-deoxy-β-D-glucopyranoside (27)**: Newly prepared PPh_3AuOTf in CH_2Cl_2 (0.05 M, 0.17 mL) was added dropwise under argon at −30 °C to a stirred mixture of the donor **24** (130 mg, 0.084 mmol), the acceptor **25**[17] (81 mg, 0.084 mmol), and freshly activated MS (4 Å, 250 mg) in dry CH_2Cl_2 (5 mL). As the temperature was allowed to warm up naturally to −15 °C, further newly prepared PPh_3AuOTf in CH_2Cl_2 (0.05 M, 0.17 mL) was added dropwise to the mixture. The mixture was then allowed to warm up naturally to room temperature and the stirring was continued for 2 h. The mixture was then filtered and concentrated in vacuo. The residue was purified by silica gel column chromatography (toluene/EtOAc 6:1) to afford **27** (98 mg, 51%) and the glycal **26** (37 mg, 32%) as white solids.

Trisaccharide glycal 26: ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.81$ (m, 8H), 7.25 (m, 25H), 6.93 (m, 2H), 6.75 (m, 3H), 6.55 (s, 1H), 5.83 (d, $J = 5.2$ Hz, 1H), 5.36 (d, $J = 8.0$ Hz, 1H), 4.82 (m, 3H), 4.73 (m, 2H), 4.54 (d, $J = 10.8$ Hz, 1H), 4.41 (m, 5H), 4.30 (m, 1H), 4.16 (m, 6H), 3.93 (dd, $J = 2.4$, 11.2 Hz, 1H), 3.68 (m, 4H), 3.52 (m, 3H), 3.35 (dd, $J = 8.4$, 13.6 Hz, 1H), 3.25 (t, $J = 9.2$ Hz, 1H), 3.15 (m, 1H), 1.86 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 169.3$, 166.7, 147.9, 137.6, 137.1, 137.0, 136.9, 136.7, 133.1, 132.6, 130.7, 127.4, 127.3, 127.2, 126.9, 126.8, 126.7, 126.6, 126.5, 126.4, 125.9, 122.5, 122.1, 105.2, 99.9, 96.8, 82.1, 76.7, 76.4, 76.1, 76.0, 74.4, 74.0, 73.8, 73.7, 73.6, 72.4, 72.3, 71.9, 68.5, 67.2, 66.7, 66.0, 65.9, 54.8, 19.7 ppm; HRMS (ESI): *m/z*: calcd for $C_{78}H_{73}N_5O_{17}Na$: 1374.4958 $[M+Na]^+$; found: 1374.4894.

Pentasaccharide 27: $[\alpha]_D^{24} = -12.7$ ($c = 0.3$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.78$ (m, 16H), 7.24 (m, 29H), 7.05 (t, $J =$

7.6 Hz, 2H), 6.96 (t, J=7.6 Hz, 2H), 6.88 (m, 3H), 6.74 (m, 5H), 6.61 (m, 3H), 5.67 (d, J=8.4 Hz, 1H), 5.65 (dd, J=9.2, 10.8 Hz, 1H), 5.45 (dd, J=10.0, 19.6 Hz, 2H), 5.23 (d, J=8.0 Hz, 1H), 5.16 (d, J=8.4 Hz, 1H), 5.12 (d, J=8.8 Hz, 1H), 4.76 (m, 4H), 4.54 (dd, J=11.2, 15.6 Hz, 2H), 4.39 (m, 10H), 4.30 (m, 2H), 4.05 (m, 8H), 3.83 (m, 1H), 3.66 (m, 6H), 3.52 (m, 4H), 3.30 (m, 6H), 3.10 (t-like, J=8.4 Hz, 2H), 3.00 (d-like, J=10.0 Hz, 1H), 2.71 (d-like, J=9.6 Hz, 1H), 1.78 (s, 3H), 1.77 (s, 3H), 1.68 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=169.3, 169.2, 167.1, 166.9, 166.7, 166.3, 154.5, 149.7, 137.7, 137.2, 137.1, 137.0, 136.9, 136.8, 133.1, 132.7, 130.7, 130.5, 127.5, 127.4, 127.3, 127.2, 127.0, 126.9, 126.8, 126.7, 126.6, 126.5, 126.4, 126.3, 126.2, 126.1, 126.0, 125.8, 122.6, 122.5, 122.4, 117.9, 113.4, 99.8, 96.4, 95.8, 95.6, 95.0, 82.2, 76.7, 76.5, 76.2, 74.4, 73.8, 73.7, 73.5, 73.3, 73.0, 72.8, 72.5, 72.4, 72.0, 71.8, 71.6, 71.3, 71.2, 70.2, 69.9, 69.8, 67.4, 66.9, 66.7, 66.6, 66.5, 66.0, 55.1, 54.5, 54.4, 54.3, 53.9, 19.5, 19.4 ppm; HRMS (MALDI): *m/z*: calcd for $C_{131}H_{123}N_7O_{33}Na$: 2344.8098 $[M+Na]^+$; found: 2344.8054.

***p*-Methoxyphenyl 2-trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranoside (30)**: Compound **27** (150 mg, 0.065 mmol) was dissolved in EtOH (4.5 mL) at room temperature, followed by addition of ethylenediamine (0.9 mL). After having been heated at reflux at 100 °C for 14 h, the mixture was concentrated in vacuo to give a residue, which was used in the next step without further purification.

Ac_2O (1.5 mL) was added to a solution of the residue and DMAP (27 mg, 0.23 mmol) in pyridine (4.5 mL). After having been stirred at room temperature overnight, the solution was diluted with CH_2Cl_2 and washed with aqueous HCl (1 N), saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (CH_2Cl_2/MeOH 50:1→30:1) to give **28** (95 mg, 75% for two steps) as a white solid: $[\alpha]_D^{27}=-45.5$ (c=0.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ=7.30 (m, 40H), 6.92 (d, J=9.2 Hz, 2H), 6.78 (d, J=9.2 Hz, 2H), 6.24 (d, J=9.6 Hz, 1H), 5.44 (d, J=8.8 Hz, 1H), 5.06 (t, J=7.6 Hz, 1H), 4.94 (d, J=12.4 Hz, 1H), 4.88 (d, J=6.8 Hz, 1H), 4.79 (m, 6H), 4.68 (m, 2H), 4.52 (m, 5H), 4.36 (m, 8H), 4.23 (d, J=8.4 Hz, 1H), 4.17 (d, J=8.0 Hz, 1H), 4.02 (t, J=8.4 Hz, 1H), 3.89 (m, 5H), 3.76 (m, 4H), 3.60 (m, 12H), 3.33 (dd, J=8.8, 17.6 Hz, 2H), 3.21 (m, 3H), 3.10 (m, 1H), 2.05 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.90 (s, 3H), 1.75 (s, 3H), 1.67 (s, 3H), 1.66 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=170.3, 170.2, 169.9, 169.5, 169.3, 168.9, 168.8, 154.1, 150.3, 138.3, 137.0, 136.9, 136.8, 136.6, 136.3, 128.1, 128.0, 127.9, 127.8, 127.6, 127.5, 127.4, 127.3, 127.2, 127.0, 126.9, 126.8, 126.7, 126.6, 126.5, 126.3, 117.1, 113.5, 99.9, 99.8, 99.7, 99.2, 98.4, 82.0, 78.4, 76.6, 76.3, 75.4, 74.4, 73.9, 73.8, 73.6, 73.1, 72.9, 72.6, 72.5, 72.3, 72.2, 72.1, 72.0, 71.9, 71.8, 71.4, 67.5, 67.2, 66.5, 66.3, 65.9, 55.2, 54.6, 52.8, 51.5, 22.4, 22.2, 22.1, 22.0, 19.8, 19.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{107}H_{123}N_7O_{29}Na$: 1992.8244 $[M+Na]^+$; found: 1922.8258.

Et_3N (0.2 mL) and propane-1,3-dithiol (0.4 mL) were added to a solution of the pentasaccharide azide **28** (78 mg, 0.040 mmol) in pyridine/H_2O (3 mL/0.8 mL). The solution was stirred at room temperature for 3 h and then concentrated in vacuo to give a residue, which was purified by silica gel column chromatography (CH_2Cl_2/MeOH/Et_3N 90:3:1) to give the corresponding amine as a white solid. (iPr)$_2$Net (0.45 mL) and CH_3I (3 mL) were added to a solution of the above white solid in dry THF (4.5 mL). After having been stirred at room temperature for 40 h, the mixture was filtered, concentrated in vacuo, and eluted through a column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) to provide **29** (70 mg, 89%) as a white solid: $[\alpha]_D^{26}=-39.2$ (c=0.3, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1:1): δ=7.22 (m, 40H), 6.86 (d, J=9.2 Hz, 2H), 6.68 (d, J=8.8 Hz, 2H), 5.21 (d, J=4.8 Hz, 1H), 5.04 (t, J=9.6 Hz, 1H), 4.96 (m, 2H), 4.88 (d, J=8.4 Hz, 1H), 4.59 (m, 8H), 4.44 (m, 10H), 4.30 (dd, J=3.2, 12.0 Hz, 2H), 4.07 (m, 1H), 3.98 (m, 4H), 3.85 (m, 4H), 3.65 (m, 13H), 3.48 (m, 2H), 3.39 (m, 2H), 3.29 (m, 1H), 3.18 (m, 2H), 2.75 (s, 9H), 1.91 (s, 3H), 1.86 (s, 3H), 1.85 (s, 6H), 1.83 (s, 3H), 1.75 (s, 3H), 1.74 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD 1:1): δ=171.3, 171.1, 170.9, 170.8, 170.5, 170.4, 170.3, 154.7, 150.6, 137.7, 137.4, 137.3, 137.2, 136.9, 136.7, 136.0, 135.9, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 127.2, 127.1, 127.0, 126.9, 126.8, 126.7, 126.6, 126.5, 117.6, 113.7, 99.5, 99.4, 98.7, 98.4, 93.1, 78.8, 78.7, 77.1, 75.9, 74.5, 74.1, 73.9, 73.7, 73.5, 73.4, 73.3, 73.0, 72.6, 72.5, 72.4, 72.3, 72.2, 72.0, 71.9, 71.8, 69.2, 68.9, 67.5, 67.3, 67.2, 56.1, 54.6, 54.2, 53.9, 53.1, 52.3, 22.0, 21.7, 21.6, 21.5, 19.8, 19.7, 19.6 ppm; HRMS (MALDI): *m/z*: calcd for $C_{110}H_{132}N_5O_{29}$: 1986.9026 $[M]^+$; found: 1986.9003.

K_2CO_3 (60 mg, 0.43 mmol) was added to a solution of **29** (100 mg, 0.050 mmol) in MeOH/CH_2Cl_2 (6:3 mL). The mixture was stirred at room temperature for 6 h and was then neutralized with Dowex-50 (H^+). Filtration, concentration in vacuo, and elution through a column (Sephadex LH-20, CH_2Cl_2/MeOH 1:1) gave a white solid (83 mg, 88%) for the next step. The white solid (70 mg, 0.038 mmol) was dissolved in MeOH/CH_2Cl_2 (3:2 mL) at room temperature, followed by addition of aqueous HCl (two drops, 37%) and $Pd(OH)_2$/C (300 mg, 20%). After having been hydrogenated at room temperature for 14 h, the suspension was filtered and neutralized with Dowex-1X8 (OH^-). Filtration, concentration in vacuo, and elution through a column (Sephadex LH-20, MeOH/H_2O 1:1) provided **30** (36 mg, 84%) as a white solid: $[\alpha]_D^{22}=-27.6$ (c=0.1, MeOH/H_2O 2:1); ^{1}H NMR (400 MHz, CD_3OD/D_2O 1:1): δ=7.02 (d, J=7.6 Hz, 2H), 6.94 (d, J=8.0 Hz, 2H), 5.42 (br s, 1H), 5.00 (d, J=7.2 Hz, 1H), 4.61 (br s, 3H), 3.80 (m, 33H), 3.37 (s, 9H), 2.09 (s, 9H), 2.06 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD/D_2O 1:1): δ=173.9, 154.9, 151.3, 118.1, 114.7, 101.3, 100.4, 95.6, 79.2, 79.1, 78.3, 77.0, 76.6, 74.8, 74.6, 72.3, 72.2, 70.8, 69.6, 69.1, 61.3, 60.4, 59.9, 56.1, 55.5, 55.1, 53.8, 44.5, 22.0, 21.9 ppm; HRMS (ESI): *m/z*: calcd for $C_{48}H_{78}N_5O_{26}$: 1140.4944 $[M]^+$; found: 1140.4930.

2-Trimethylammonium-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamino-2-deoxy-D-glucopyranose (31): $Ag(DPAH)_2$ (25 mg, 0.054 mmol) was added to a solution of the MP glycoside **30** (11 mg, 0.0096 mmol) in CH_3CN/H_2O (0.7:0.7 mL). After having been stirred at room temperature for 3 min, the solution was filtered and neutralized with Dowex-1X8 (OH^-). The mixture was filtered and concentrated in vacuo. The residue was eluted through columns [Sephadex LH-20 MeOH/H_2O 1:1 and then Dowex-1X8 (Cl^-), H_2O] to afford **31** (7 mg, 71%) as a white solid: $[\alpha]_D^{23}=-12.4$ (c=0.2, MeOH/H_2O 1:1); ^{1}H NMR (400 MHz, CD_3OD/D_2O 1:1): δ=5.42 (d, J=4.0 Hz, 1H), 5.18 (d, J=1.6 Hz, 0.4H), 4.68 (d, J=8.0 Hz, 0.4H), 4.60 (m, 3H), 4.05 (t, J=8.0 Hz, 1H), 3.91 (m, 15H), 3.67 (m, 12H), 3.55 (m, 2H), 3.37 (s, 9H), 2.10 (s, 3H), 2.09 (s, 6H), 2.07 ppm (s, 3H); ^{13}C NMR (125 MHz, CD_3OD/D_2O 1:1): δ=175.3, 175.2, 102.7, 97.0, 80.6, 79.7, 78.4, 78.0, 76.0, 73.6, 72.2, 71.4, 70.9, 70.6, 70.4, 62.8, 61.7, 61.3, 57.5, 56.5, 55.1, 46.1, 23.4 ppm; HRMS (MALDI): *m/z*: calcd for $C_{41}H_{72}N_5O_{25}$: 1034.4547 $[M]^+$; found: 1034.4511.

Expression and purification of recombinant OfHex1: OfHex1 was overexpressed in *Pichia pastoris* strain GS115 as a C-terminal His_6-tagged fusion protein and purified by ammonium sulfate precipita-

tion, metal chelating chromatography, and anion exchange chromatography as described previously.[26]

Enzymatic assay: The activities of β-*N*-acetyl-D-hexosaminidases were measured with *p*-nitrophenyl *N*-acetyl-D-glucosaminide (*p*NP-β-GlcNAc, Sigma–Aldrich, USA) as substrate at 25 °C. β-*N*-Acetyl-D-hexosaminidase (BtHex) from *Bos taurus* and β-*N*-acetyl-D-hexosaminidase (CeHex) from *Canavalia ensiformis* were purchased from Sigma–Aldrich. β-*N*-Acetyl-D-hexosaminidase (SpHex) from *Streptomyces plicatus* was purchased from New England Biolabs (USA). BtHex, CeHex, and SpHex were assayed in sodium citrate buffer (40 mM) at their optimal pH values (BtHex 4.5; CeHex 5.0; SpHex 4.0). OfHex1 was assayed in sodium phosphate buffer (40 mM, pH 7.0).[26,27] The reactions were terminated by addition of equal amounts of Na_2CO_3 (0.5 M) and absorbance at 405 nm was monitored with a microplate reader (TECAN, Switzerland). For determinations of K_i values, different amount of inhibitors were preincubated with the enzymes for 10 min before the addition of substrate. The K_i values were calculated with Dixon plots. The values represent the means of three independent experiments.

Molecular docking: The crystal structures of SpHex from bacteria (PDB ID: 1HP5) and HsHexB from human (PDB ID: 1NOU) were used as 3D models for docking studies. The newest AutoDock 4.2 program was applied to dock the TMG-chitotriomycin into the binding sites of the two enzymes.[28] The Gasteiger charges were used for TMG-chitotriomycin and the active torsions were assigned with the aid of the newest ADT program. The residues at the entrance of each binding pocket were designated as flexible (SpHex: Glu314, Trp361, and Trp408, and HsHexB: Glu355 and Trp424) and allowed for conformational search in the docking process. The geometric centers of the binding sites were chosen as the grid center, the grid size was set to 80×80×80, and the used grid space was the default value of 0.375 Å. In the docking process, a conformational search was performed for the TMG-chitotriomycin by the Solis and Wets local search method, and the Lamarkian genetic algorithm (LGA) was applied for conformational searching of the binding complexes of TMG-chitotriomycin with SpHex and HsHexB. To achieve optimal docking results, the population size of the Lamarkian genetic algorithm was set as 150 and the maximum number of evaluations was set as 25 000 000. The interaction energies that resulted from probing the TMG-chitotriomycin with SpHex and HsHexB were assessed by the newest adopted AutoDock scoring function. Cluster analysis was performed on a set of 50 candidates for the docked complex structures, and the best one was selected according to the interaction energy and the complementarity in the binding pocket inspected visually.

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