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Glycosyl azide—a novel substrate for enzymatic transglycosylations

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Abstract—Glycosyl azides are new efficient donors for glycosidases. Their high water solubility facilitates transglycosylations with comparable or better yields than common *O*-glycosides. The azido group totally changes the β-GalNAc-ase/β-GlcNAc-ase ratio in β-*N*-acetylhexosaminidases (from the usual 0.3–1.0 to <0.01), contrary to all known aglycons.

Enzymatic transglycosylation catalyzed by glycosidases is a respected method in carbohydrate synthesis.[1] The spectrum of acceptors is practically infinite, contrary to glycosyl donors, which are usually nitrophenyl glycosides.[2] Their major advantage is good reactivity, however, their low water solubility causes difficulties as high concentrations of substrates promote transglycosylation at the expense of donor hydrolysis. This is especially the case with modified glycosides.[3] Furthermore, commercial nitrophenyl glycosides are expensive and the yields of their syntheses may be unsatisfactory.[4] Therefore, alternative glycosyl donors are emerging, such as 3-nitro- and 5-nitro-2-pyridyl glycosides,[5] vinyl glycosides[6] and glycosyl fluorides.[7]

Glycosyl azides are easily prepared in high yield[8] and are highly water soluble (e.g., azide **1** is ca. 60 times better soluble than 4-nitrophenyl glycoside **3**). Moreover, the azide ion is easily removable, which strongly facilitates the purification of transglycosylation reaction mixtures. The only hint on cleavage of one of these substrates was published by Day and Withers (β-glucosidase)[9] and to the best of our knowledge, they have never been used as glycosyl donors in transglycosylation reactions.

Here, we tested 2-acetamido-2-deoxy-β-D-glucopyranosyl azide[8a] (**1**) and 2-acetamido-2-deoxy-β-D-galactopyranosyl azide[8b] (**2**) as glycosyl donors for a wide range of fungal β-*N*-acetylhexosaminidases from our enzymatic library.[10] The cleavage and transglycosylation potentials of these substrates were examined and the experimental data were compared with the results from molecular modelling. Three novel oligosaccharides were synthesized by transglycosylation with compound **1** as the glycosyl donor.

Substrates **1** and **2** were screened for cleavage by 20 β-*N*-acetylhexosaminidases selected on the basis of our previous chemoenzymatic studies[3b] with modified substrates. The results were compared to the standard substrates, 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**, Table 1, for its structure see Scheme 2: R = 4-nitrophenyl) and 4-nitrophenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**4**, for its structure see Scheme 2: R = 4-nitrophenyl). Due to the lack of a suitable chromophore in the glycosyl azides, the standard hydrolytic activity determination was no longer applicable[11] and, therefore, a new method of HPLC determination was developed (Polymer IEX H^+ column).

Substrate **1** (*gluco*-) was accepted by all the tested β-*N*-acetylhexosaminidases whereas substrate **2** (*galacto*-) was not hydrolyzed by any of the enzymes. It is a rather atypical phenomenon since the tolerance of β-*N*-acetylhexosaminidases towards the change of configuration at C-4 is one of their typical features.[12] This unexpected

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Table 1. Screening for the hydrolysis of substrate **1** by fungal β-*N*-acetylhexosaminidases

Source of enzyme	1[a]
Acremonium persicinum CCF 1850	+
Aspergillus awamori CCF 763	+
A. flavipes CCF 1895	+
A. flavus CCF 3056	+
A. oryzae CCF 1066	++
A. parasiticus CCF 1298	+
A. sojae CCF 3060	++
A. terreus CCF 2539	++
Fusarium oxysporum CCF 377	++
Hamigera avellanea CCF 2923	+
Penicillium oxalicum CCF 2315	++
P. chrysogenum CCF 1269	+++
P. funiculosum CCF 2985	+++
P. multicolor CCF 2244	++
P. brasilianum CCF 2155	++
P. pittii CCF 2277	++
P. spinulosum CCF 2159	++
Talaromyces flavus CCF 2686	+++
T. ohiensis CCF 2229	++
Trichoderma harzianum CCF 2687	++

[a] Enzymes were classified according to the ratio of specific activities (U mL^{-1}) towards substrate **1** and the standard substrate **3**: 16–11% (+++), 10–5% (++) or 4–1% (+); CCF: culture collection of fungi.

result could be explained by molecular modelling: substrates **1** and **2** were docked into the active site of the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 (a typical representative of eukaryotic β-*N*-acetylhexosaminidases) and the enzyme–substrate complex interaction energies were compared (Table 2).

Both resonance structures of the azido group (–N=N^+=N^- and –N^-–N^+≡N) were considered in the calculations. The real structure is a hybrid of the two. In the case of the *gluco*-substrate **1**, the docking

Table 2. Docking of 4-nitrophenyl- and azido-hexosaminides into the active site of β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066

Substrate[a]	Steric contribution (kJ mol^{-1})	Electrostatic contribution (kJ mol^{-1})	Total interaction energy[b] (kJ mol^{-1})
1-A	−29	−237	−266
1-B	−57	−200	−257
2-A	−72	−95	−167
2-B	−62	−102	−164
3	−84	−216	−300
4	−66	−155	−221

[a] A: Azide calculated as –N=N^+=N^-; B: azide calculated as –N^-–N^+≡N.

[b] The drop in the enzyme–substrate complex interaction energy reflects improved binding of the substrate into the enzymatic active site.

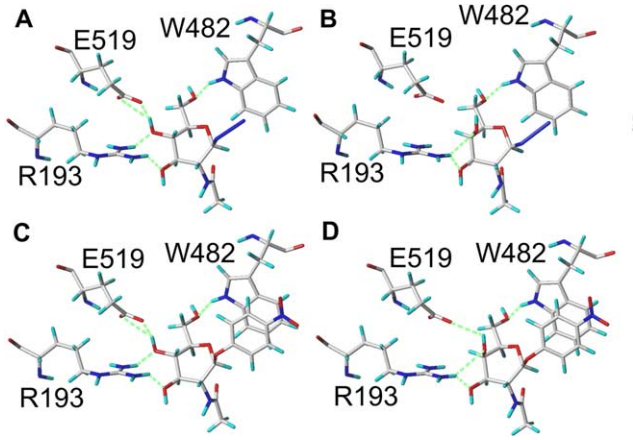


Figure 1. Substrates **1** (A), **2** (B), **3** (C) and **4** (D) docked into the active site of the β-*N*-acetylhexosaminidase from *A. oryzae* CCF 1066. Hydrogen bonding (in green) to glutamic acid 519, arginine 193 and tryptophan 482 fixes the glycosides. The azido group (substrates **1** and **2**) is depicted as a blue stick.

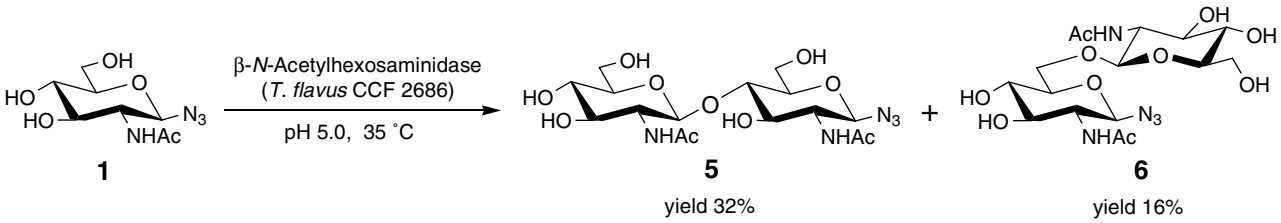
(Fig. 1) shows rather similar total interaction energies for both resonance structures (−266 and −257 kJ mol^{-1}), which are comparable to the standard substrate **3** (−300 kJ mol^{-1}). The minor difference is mainly caused by the steric contribution. On the contrary, docking of both resonance structures of the *galacto*-substrate **2** exhibited a more significant drop in total interaction energies (−167 and −164 kJ mol^{-1} compared to −221 kJ mol^{-1} for the standard substrate **4**), which is essentially caused by the difference in electrostatic energy contributions. A closer look at the binding pocket arrangement shows that the hydrogen bonding with the carboxyl group of glutamic acid 519 with the standard substrate **4** cannot occur with substrate **2** due to the distance between the respective moieties of the latter and the protein amino acids being too large (Fig. 1). These results strongly suggest that substrate **2** is not bound into the enzymatic active site due to the lack of hydrogen bonding, as reflected by the electrostatic energy contribution (in this case the dominating interaction). This is in accordance with experimental data (i.e., no cleavage).

The hydrolysis of substrate **1** by β-*N*-acetylhexosaminidases from *Aspergillus oryzae* CCF 1066 (cleavage rate 7% related to **3**) and from *Talaromyces flavus* CCF 2686 (the best one from the screening, cleavage rate 16% related to **3**) was characterized by basic kinetic constants (K_m, V_{max}; Table 3).

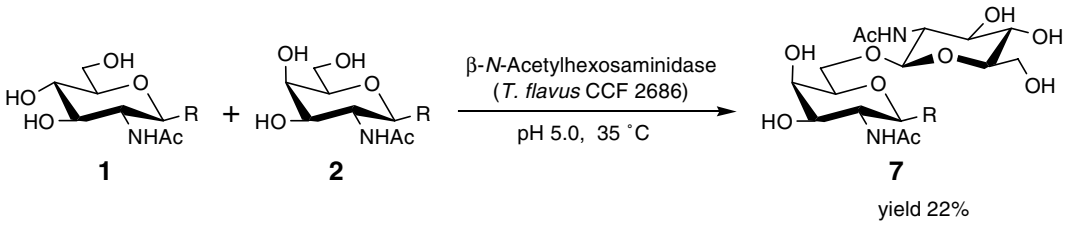
Results from the Michaelis–Menten plot that both tested enzymes were inhibited at higher concentrations of the standard substrate **3** were evident (K_i, Table 3),

Table 3. Hydrolysis of substrates **1** and **3** by selected enzymes

Enzyme source	Azido substrate **1**			4-Nitrophenyl substrate **3**		
	K_m (mM)	V_{max} (μM min^{-1})	K_i (mM)	K_m (mM)	V_{max} (μM min^{-1})	K_i (mM)
A. oryzae CCF 1066	3.1 ± 0.4	3.4 ± 0.2	0	0.75 ± 0.05	56 ± 2	7.0 ± 0.6
T. flavus CCF 2686	0.75 ± 0.09	4.4 ± 0.1	0	0.36 ± 0.04	67 ± 4	1.3 ± 0.1



Scheme 1. Enzymatic synthesis of compounds **5** and **6**.



Scheme 2. Enzymatic synthesis of compound **7**, R = N_3.

whereas no inhibition was observed with substrate **1**. This fact, together with the substantially improved water solubility (saturated water solution is 1.1 M for **1** and 0.02 M for **3**), enables very efficient transglycosylation reactions with a low enzyme consumption using substrate **1** as the glycosyl donor.

Glycosidases, which are typically retaining enzymes with *O*-glycosides,[1a] are inverting towards some unusual substrates like glycosyl fluorides (C–F bond).[7] Therefore, the hydrolysis of substrate **1** (C–N bond) was monitored by ^{1}H NMR to determine the anomeric configuration of the originating D-Glc*p*NAc. Since the signal of H-1β in D-Glc*p*NAc is hidden under the water resonance, D-Glc*p*NAc signals for H-1α ($\delta = 5.01$, d, $J = 3.5$ Hz) and H-2β ($\delta = 3.48$, dd, $J = 10.2$, 8.4 Hz) were compared. The concentration of β-D-Glc*p*NAc increases faster than that of its α-anomer, which is formed from the β-anomer in water due to mutarotation (Fig. 1 in the Supplementary data).[13] Substrate **1** remained stable under the conditions. As a result, the β-*N*-acetylhexosaminidase from *Aspergillus oryzae* CCF 1066 hydrolyzed substrate **1** as a retaining enzyme, analogously to *O*-glycosides.

The three best β-*N*-acetylhexosaminidases for hydrolysis (Table 1) were tested for their ability to transfer the 2-acetamido-2-deoxy-β-D-glucopyranosyl moiety from substrate **1** to different acceptors. The same transglycosylation products were detected (HPLC) in all three cases. The enzyme from *T. flavus* CCF 2686 exhibited the best yield of transglycosylation product and the highest specific activity. Therefore, it was used for semi-preparative transglycosylation reactions. Autocondensation reactions catalyzed by this enzyme yielded two isomeric disaccharides **5** and **6** (Scheme 1), which were separated by preparative HPLC. Compound **5** is a *p*-nitrophenyl β-chitobioside[14] analogue, which can be easily conjugated to complex biological structures, such as immunoactive glycoconjugates,[15] after reduction of the C-1 azido group to an amine.

Transfer of the β-D-Glc*p*NAc moiety to acceptor **2** (not hydrolyzed) catalyzed by β-*N*-acetylhexosaminidase from *T. flavus* CCF 2686 afforded disaccharide **7** in 22% yield (Scheme 2). For experimental data on all the compounds prepared, see the Supplementary data.

In summary, glycosyl azide **1** proved to be an efficient and easily preparable glycosyl donor for β-*N*-acetylhexosaminidase-catalyzed transglycosylation, as demonstrated by the preparation of three novel disaccharides **5**, **6** and **7**. Glycosyl azides can serve as interesting alternatives to traditional nitrophenyl glycosides.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.10.040.

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