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Inhibition of microbial β-*N*-acetylhexosaminidases by 4-deoxy- and *galacto*-analogues of NAG-thiazoline

Jana Krejzová [a,b], Lubica Kalachova [a], Petr Šimon [a,†], Helena Pelantová [a], Kristýna Slámová [a,*], Vladimír Křen [a]

[a] Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ 14220 Praha 4, Czech Republic
[b] Department of Biochemistry and Microbiology, Institute of Chemical Technology Prague, Technická 5, CZ 16628 Praha 6, Czech Republic

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ABSTRACT

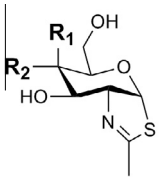
NAG-thiazoline is a well-established competitive inhibitor of two physiologically relevant glycosidase families—β-*N*-acetylhexosaminidases (GH20) and β-*N*-acetylglucosaminidases (GH84). Based on the different substrate flexibilities of these enzyme groups, we designed and synthesized the 4-deoxy derivative of NAG-thiazoline aiming at the selective inhibition of GH20 β-*N*-acetylhexosaminidases. One GH84 and two GH20 microbial glycosidases were employed as model enzymes for the inhibition assays. Surprisingly, the new compound 4-deoxy-thiazoline exhibited no activity inhibition with either of the enzyme families of interest. Unlike with the substrates, the 4-hydroxyl group of the inhibitor's sugar ring seems to be crucial for binding the inhibitor to the active sites of these enzymes.

Lysosomal β-*N*-acetylhexosaminidases (β-Hex; GH20) and cytoplasmatic β-*N*-acetylglucosaminidases (*O*-GlcNAcases; GH84) were identified as structurally and functionally related glycosidases with different abundances, substrate specificities and therefore biological functions.[1] In humans, a lack of these enzymes causes severe neurodegenerative disorders such as Tay-Sachs' and Sandhoff's lysosomal storage disorders (GH20) and Alzheimer's disease (GH84). For the study of the individual enzymes in vivo, inhibitors specific for just one of these enzyme families are required in order to avoid the generation of complex phenotypes. With respect to the substrate-assisted catalytic mechanism and active site architecture shared by both of these glycosidase families,[2] the design and synthesis of group-specific inhibitors has been developing rapidly in last few decades.

One of the most studied inhibitors of the GH20 and GH84 glycosidases is NAG-thiazoline (2′-methyl-2-acetamido-2-deoxy-α-D-glucopyranosyl-[2,1-*d*]-Δ2′-thiazoline, **1**),[3] which acts as a stable mimic of the reaction intermediate NAG-oxazoline. Since its first synthesis by Knapp and co-workers, it has been used as a lead structure for various derivatizations.[2,4–6] However, with the single exception of a highly selective derivative named thiamet G,[7] no other synthetic analogue of the inhibitor **1** reached the effectivity of the parent compound, moreover, the selectivity of the new derivatives was too low for their practical application.

In this work we focused on the inhibition of GH20 and GH84 glycosidases based on their fundamental difference in substrate specificity: while the GH84 *O*-GlcNAcases are strictly specific for the hydrolysis of GlcNAc units, the typical feature of GH20 β-Hex is that they also accept the respective *galacto*-epimer (GalNAc)[8] and, as a special case, the 4-deoxy-derivative phenyl 2-acetamido-2,4-dideoxy-β-D-*xylo*-hexopyranoside can be utilized by some fungal β-*N*-acetylhexosaminidases with no significant loss of activity.[9] Based on this finding, the *galacto*- (**2**) and 4-deoxy (**3**) derivatives of NAG-thiazoline (**1**) (Fig. 1) were synthesized and tested as inhibitors of the bacterial and fungal β-Hex and bacterial *O*-GlcNAcase employed as model enzymes.

Whereas the known NAG-thiazoline (**1**)[3] as well as Gal-thiazoline (**2**)[10] were prepared according to Knapp's procedure, the newly designed 4-deoxy-thiazoline (**3**) was prepared from 2-acetamido-2-deoxy-D-glucose (GlcNAc; **4**) by the multistep synthesis shown



	R_1	R_2
1: NAG-thiazoline	H	OH
2: Gal-thiazoline	OH	H
3: 4-deoxy-thiazoline	H	H

Figure 1. Structures of inhibitors used in this study.

* Corresponding author. Tel.: +420 296442766; fax: +420 296442509.
E-mail addresses: slamova@biomed.cas.cz, slamova.kristyna@gmail.com (K. Slámová).
† Present address: Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, CZ 16610 Praha 6, Czech Republic.

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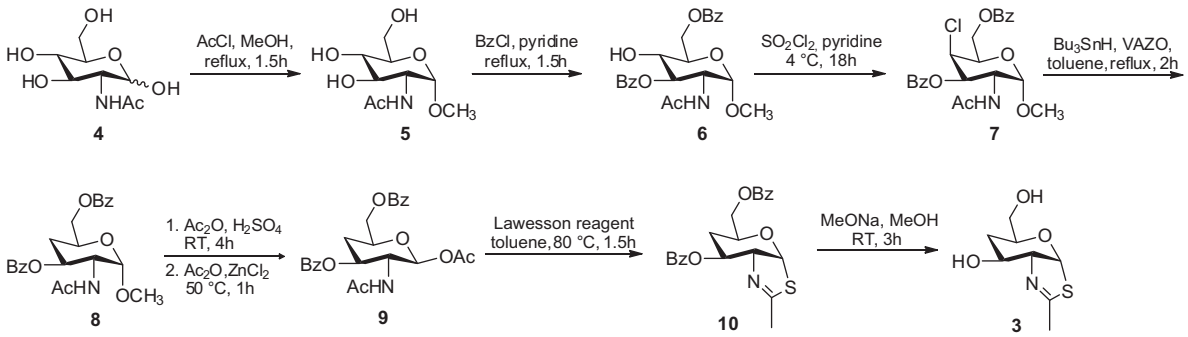

Scheme 1. Synthesis of 4-deoxy-thiazoline **3**.

Table 1
Inhibition of microbial glycosidases by NAG-thiazoline-based inhibitors (substrate *p*NP-GlcNAc, pH 7, 25 °C)

Enzyme		K_I (μM)	K_I (μM)	K_I (μM)
		NAG-thiazoline (**1**)[6]	Gal-thiazoline (**2**)	4-Deoxy-thiazoline (**3**)
β-Hex	*T. flavus*	42.7 ± 1.9	393 ± 33	1888 ± 474
	S. plicatus	24.0 ± 5.0	90.8 ± 6.3	9900 ± 7000
O-GlcNAcase	*B. thetaiotaomicron*	0.029 ± 0.003	n.d.	1831 ± 97

n. d. = not determined.

in Scheme 1. The synthesis of 4-deoxy-thiazoline (**3**) is based on two key steps: 4-deoxygenation and thiazoline ring formation, both requiring the synthesis of suitable protected precursors, formed in a series of protection and deprotection reactions.

In the first step, the anomeric hydroxyl group of *N*-acetylglucosamine (**4**) was selectively methylated with methanol under catalysis with acetyl chloride to yield **5**,[11] which was then selectively benzoylated using benzoyl chloride in pyridine under reflux to afford the 3,6-di-*O*-benzoyl derivative **6**.[12] Deoxygenation of the hydroxyl group at C-4 in compound **6** was accomplished in two steps employing reductive dechlorination.[13] Compound **6** was treated with sulfuryl chloride in pyridine at 0 °C to yield the 4-chloro derivative (**7**, 62%) in the *galacto*-configuration. Compound **7** was then dechlorinated with tri-*n*-butyltin hydride in the presence of a catalytic amount of 1,1′-azobis(cyclohexanecarbonitrile) in anhydrous toluene, yielding the key intermediate methyl 2-acetamido-3,6-di-*O*-benzoyl-2,4-dideoxy-α-D-glucopyranoside (**8**, 93%). Two-step conversion of compound **8** into acetate followed by anomerization resulted into the corresponding β-acetate (**9**, 64%) required for the thiazoline ring formation.[3] Compound **9** was then converted with Lawesson's reagent in anhydrous toluene[3] at 80 °C into the protected 4-deoxy-thiazoline (**10**, 82%). In the final step, Zemplén deacylation of the benzoyl groups gave the desired product 4-deoxy-thiazoline (**3**, 76%). To avoid any inconsistent results of the enzymatic assays, we performed the ^{1}H NMR stability experiment in D_2O at ambient temperature confirming that compound **3** is stable for at least 24 h, which is sufficient for the kinetic experiments. Experimental details and spectral data of the new compounds can be found in the Supplementary material.

Three microbial model enzymes of the studied glycosidase families were used to test the inhibition potency of Gal-thiazoline (**2**) and 4-deoxy-thiazoline (**3**): β-*N*-acetylhexosaminidase (GH20) from the filamentous fungus *Talaromyces flavus* and the one from the bacterium *Streptomyces plicatus*; and the β-*N*-acetylglucosaminidase (GH84) from *Bacteroides thetaiotaomicron*. The enzymes were expressed and purified as described in our previous work.[6] NAG-thiazoline (**1**) was used as the benchmark inhibitor here, the inhibition constants for the studied enzymes with NAG-thiazoline were reported recently.[6] The results of the inhibition assays performed at pH 7, 25 °C using *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucosaminide (*p*NP-GlcNAc) as a chromogenic substrate are summarized in Table 1, the Lineweaver–Burk plots of the experiments with inhibitors **2** and **3** are shown in the Supplementary material (Figs. S1–S5).

In this study, we aimed at designing an inhibitor based on the key difference in substrate specificities between the two otherwise closely related glycosidase families 20 and 84. We employed the fact that only the GH20 β-*N*-acetylhexosaminidases utilize the *galacto*-configured substrates and some of these enzymes, mainly those of fungal origin, were shown to also readily accept the 4-deoxy substrate.[9] As it has already been shown previously,[14] Gal-thiazoline (**2**) is a specific inhibitor of GH20 glycosidases, even though its inhibition potency is somehow lower than that of NAG-thiazoline, which we also observed in our experiments. What we found interesting was the lack of inhibition of both tested enzyme groups by the newly designed compound 4-deoxy-thiazoline (**3**). With respect to our previous results with the 4-deoxy substrates, we expected that the GH20 β-*N*-acetylhexosaminidases would be inhibited by this modified thiazoline, however, it seems that the 4-hydroxyl of the glycosyl moiety is instrumental for the proper binding of the inhibitor in the active sites of the respective enzymes.

In summary, we have designed and synthesized the 4-deoxy derivative of NAG-thiazoline, a generally recognized strong competitive inhibitor of GH20 β-*N*-acetylhexosaminidases and GH84 β-*N*-acetylglucosaminidases, aiming at the selective inhibition of GH20 enzymes. Unlike the known 4-deoxy substrates, the analogous transition state mimicking thiazoline **3** was found to be a poor inhibitor of all model enzymes from both glycosidase families employing the substrate-assisted catalytic mechanism. We suppose that the 4-hydroxyl moiety in the equatorial configuration of the inhibitor molecule is crucial for its strong binding to the active sites of these glycosidases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.09.066.

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