NAG-thiazoline, An *N*-Acetyl-β-hexosaminidase Inhibitor That Implicates Acetamido Participation

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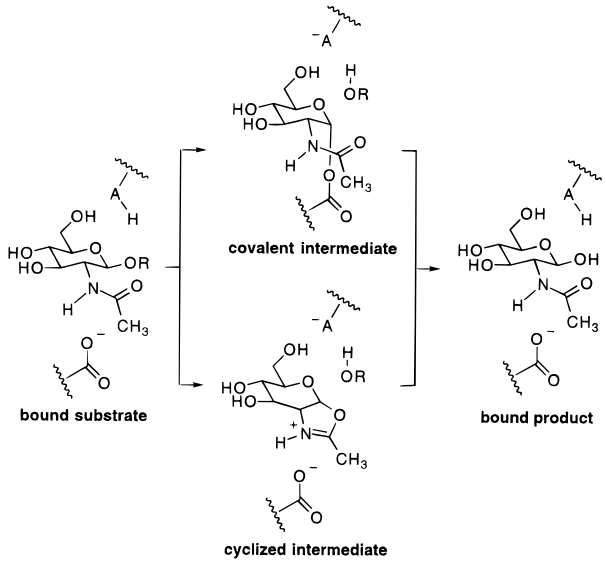
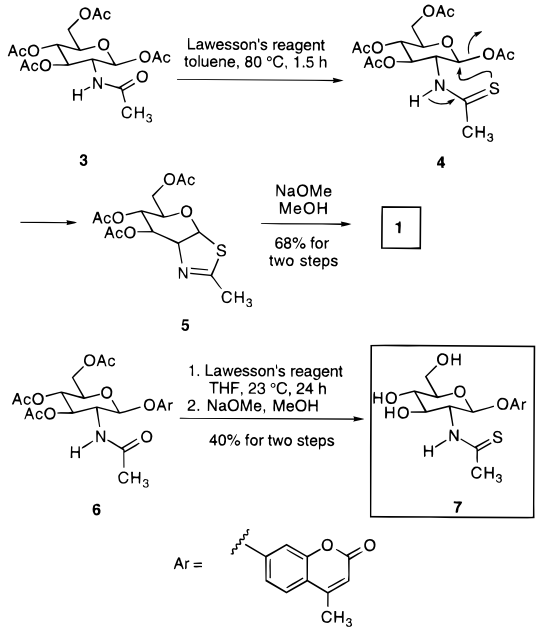
Received March 14, 1996

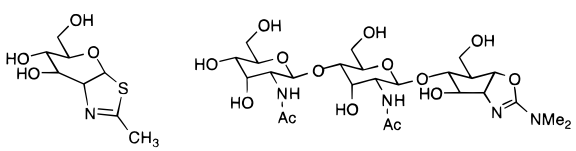
N-Acetylhexosaminidases (NAGases) are enzymes that promote the cleavage of *N*-acetylhexosaminides, as during glycoprotein processing and glycolipid catabolism.[1] They are highly specific inasmuch as *gluco*- and *galacto*pyranosides lacking the 2-acetamido functionality are poor substrates.[2] A number of inhibitors of NAGases have been discovered; these typically mimic some aspect of an enzyme-protonated 2-acetamidopyranoside substrate or a derived transition state (flattened C-1 conformation, positive or partially positive charge), and they uniformly possess an acetamido group at the C-2 site.[3−6] By analogy to a widely cited mechanism for retaining β-glucosidases,[7] retaining *N*-acetyl-β-hexosaminidases may be said to operate by stabilizing a transition state leading to a covalent enzyme−substrate complex (Scheme 1, upper path).[8] Alternatively, the configuration-retaining aspect of certain NAGases may be attributed to participation of the neighboring C-2 acetamido group, leading initially to a cyclized oxazoline intermediate (Scheme 1, lower path).[9] None of the existing NAGase inhibitors points to a choice between these two mechanistic possibilities, as the latter both feature similar transition state characteristics in the vicinity of C-1.[6] However, allosamidin (**2**), an inhibitor of chitinase, incorporates a cyclic isourea functionality that, when protonated, resembles the cyclized oxazoline shown in Scheme 1. A recent crystallographic study of a chitinase/**2** complex shows a position of the inhibitor and a hydrogen-bonding pattern consistent with acetamido participation in the natural substrate.[10] Although the "NAG-oxazoline" itself is too hydrolytically unstable for use as an inhibitor of NAGases,[6] we have prepared a stable version, "NAG-thiazoline" **1**, and report its powerful inhibitory effect on jack bean *N*-acetyl-β-hexosaminidase and its enzyme-mediated formation from a precursor substrate.

Scheme 1. Proposed *N*-Acetyl-β-hexosaminidase Mechanisms



Scheme 2. Synthesis of NAG-Thiazoline and a Precursor Substrate





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S0002-7863(96)00826-8 CCC: $12.00

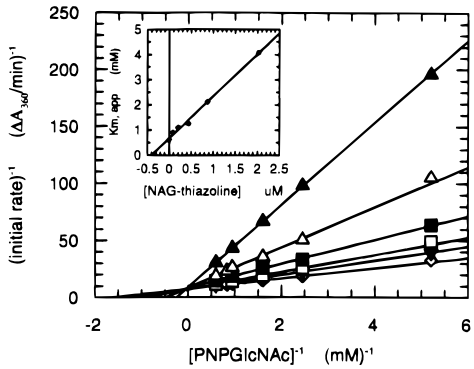

Figure 1. Inhibition of jack bean NAGase catalyzed hydrolysis of PNPGlcNAc by NAG-thiazoline **1**. The concentrations of **1** used were 0.000 (◇), 0.087 (▼), 0.203 (□), 0.434 (■), 0.868 (△), and 2.026 (▲) μM. Inset: graphical analysis of K_i by plotting K_m apparent against [NAG-thiazolin].

Treatment of 2-acetamido-2-deoxy-1,3,4,6-tetra-*O*-acetyl-β-D-glucopyranose[11] (**3**, Scheme 2) with Lawesson's reagent[12] led to selective formation of the thioamide **4** (observable by TLC), which then cyclized by displacement of acetate to provide the thiazoline triacetate **5**. The structure of **5** was confirmed by the close match of its ^{1}H NMR spectrum with that of the corresponding NAG-derived *oxazoline* triacetate.[13] The α-anomer of **3** did not cyclize under these conditions, presumably because direct participation of the thiocarbonyl with inversion at C-1 would require a strained *trans*-fused transition state. Methanolysis of the acetyls and then column chromatography gave the NAG-thiazoline **1** as a hygroscopic off-white solid. Not only is **1** stable at basic pH, but it also survives protonation with trifluoromethanesulfonic acid in chloroform solution followed by neutralization.

NAG-thiazoline **1** was found to be a potent competitive inhibitor of jack bean *N*-acetylhexosaminidase as measured against *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (K_m = 0.62 mM).[14] Reciprocal replot of apparent K_m values vs inhibitor concentration, as shown in Figure 1, reveals a K_i for **1** of 280 nM. It therefore binds almost 20 000 times more tightly than the parent sugar *N*-acetyl-β,D-glucosamine (K_i = 5 mM) and may be compared to the best inhibitors of this enzyme, such as 2-acetamido-1,2-dideoxynojirimycin (K_i = 140−230 nM), and 2-acetamido-2-deoxynojirimycin (1.2 nM).[4a]

If the *N*-acetyl-β-hexosaminidase uses the *acetamido* group for anchimeric assistance in the cleavage of the β-glycosidic linkage, then a glycoside of 2-deoxy-2-*thioacetamido*-β-D-glucose might be converted by the enzyme to the stable, inhibitory, thiazoline **1**, resulting in a time-dependent loss of enzyme activity. The 4-methylumbelliferyl 2-thioacetamido glucoside **7** was prepared from 4-methylumbelliferyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-β-D-glucopyranoside[6,15] **6** by treatment with Lawesson's reagent and then sodium methoxide

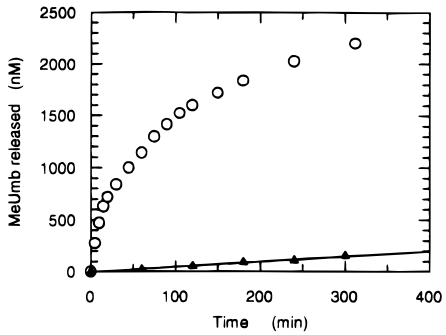

Figure 2. Time course of release of 4-methylumbelliferone from 0.65 mM MuTag (**7**) upon reaction with jack bean NAGase (○); Spontaneous decomposition of **7** (▲).

(Scheme 2). It was isolated after crystallization as a white solid, mp 144.5−146 °C, and then was further purified by HPLC to remove traces of thiazoline **1**. Incubation of jack bean *N*-acetylglucosaminidase with **7**, while monitoring the release of 4-methylumbelliferone fluorometrically,[16] resulted in slow, time-dependent loss of activity, as shown in Figure 2. Reaction over a 300 min period resulted in a 21-fold reduction in rate with release of 2.2 μmol of 4-methylumbelliferone, consistent with stoichiometric conversion of **7** to **1**. No loss of enzyme activity occurred in the absence of **7**, and the putative precursor **7** is itself a poor inhibitor of jack bean *N*-acetylglucosaminidase when measured over time periods too short for formation of significant quantities of **1**.

These results on jack bean NAGase provide strong evidence for a mechanism involving acetamido participation and an oxazoline intermediate (Scheme 1, lower path) and complement the chitinase crystallographic study.[9] Furthermore, 2-deoxy-2-thioacetamido glucosides such as **7** may prove valuable as enzyme-activated inhibitor precursors with adjustable properties according to their aglycon portions.

Acknowledgment. We are grateful to American Cyanamid, Berlex Corporation, Hoffmann-La Roche, the National Sciences and Engineering Council of Canada, and the Protein Engineering Network of Centres of Excellence for support of this research. We thank Rutgers undergraduates Peter Blomgren and Suzanne Moeller for assistance with the preparation of starting materials and American Cyanamid for a Faculty Award to S.K.

Supporting Information Available: Experimental procedures for the synthesis of **1** and **7** (2 pages). See any current masthead for ordering and Internet access instructions.

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(16) The time course of release of 4-methylumbelliferone from **7** by jack bean NAGase was followed by establishing a series of reaction mixtures, each containing NAGase (2.4 μg/mL) and **7** (0.65 mM) in 420 μL of 50 mM citrate buffer containing 100 mM NaCl and 0.1% BSA at pH 5.0. These mixtures were quenched at various incubation times by adding 1.26 mL of 0.2 M glycine buffer, pH 10.65. The fluorescence due to 4-methylumbelliferone was then measured at 450 nM. The resulting time-dependent decrease in activity was shown to be due to the buildup of a reversible inhibitor rather than covalent inactivation by repeating the experiment at a higher enzyme concentration and then diluting the sample prior to assay. Under these conditions essentially no time-dependent loss of enzyme activity was observed.