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α-Glucopyranoimidazolines as intermediate analogue inhibitors of family 20 β-*N*-acetylglucosaminidases

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Abstract—The α-glucopyranoimidazolines, 2-methyl-(1,2-dideoxy-α-D-glucopyrano)[2,1-*d*]-1-imidazolines **1** and **2**, have been synthesized and evaluated as inhibitors of β-*N*-acetylglucosaminidases (NAGs). Compounds **1** and **2**, mimicking the oxazolinium ion intermediate in enzyme catalysis, served as potent and competitive inhibitors of family 20 NAGs with K_i as low as 0.1 μM, but showed no inhibitory activities toward family 3 NAGs. Due to structural and electrostatic resemblance to the oxazolinium ion intermediate, the α-glucopyranoimidazolines may lead to novel and selective inhibitors of mechanistically related glycosidases such as family 18 chitinases.

1. Introduction

β-*N*-Acetylglucosaminidases (NAGs) are glycosyl hydrolases that catalyze the hydrolysis of a β-1,4-linked *N*-acetylglucosamine residue from the nonreducing end of oligosaccharides and glycoconjugates.[1] The NAGs are involved in a number of physiologically important processes such as the catabolism of glycolipids, glycoproteins and glycosaminoglycans,[2] the binding of sperms to eggs,[3] and the complete degradation of chitin with a tandem action of chitinases.[4] Hence NAGs have attracted considerable research interest as therapeutic targets for some lysosomal storage diseases,[5] as antifungal agents,[6] and as catalysts for biomass degradation.[7] According to the classification of glycosyl hydrolases based on amino acid sequence similarity[8] (URL:http://afmb.cnrs-mrs.fr/CAZY/GH.html), most NAGs are classified into family 20. Family 20 NAGs catalyze the cleavage of a β-glycosidic bond of β-*N*-acetylhexosaminides with retention of configuration at the anomeric carbon, but are mechanistically distinct from conventional retaining β-glycosidases in that family 20 NAGs catalyze the reaction via the anchimeric assistance by the 2-acetamido group of the substrates (Fig. 1a).[9] Thus, a catalytic carboxy residue of family 20

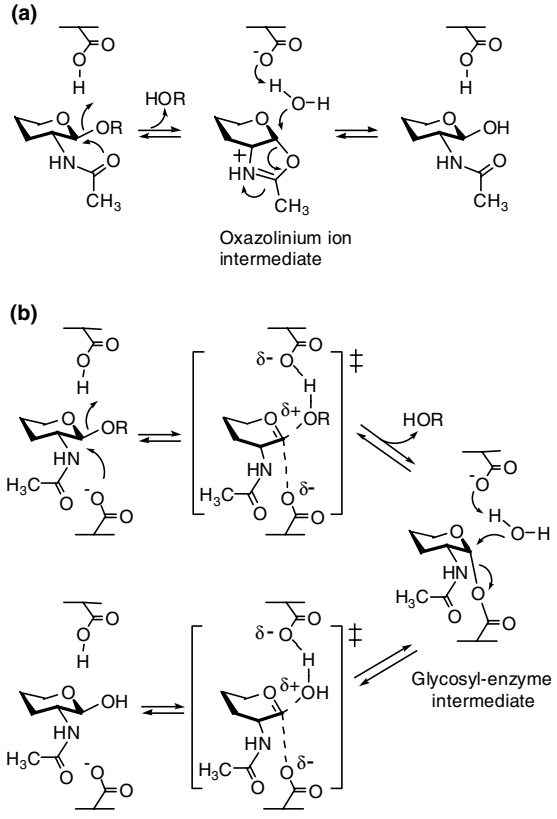

Figure 1. Proposed catalytic mechanisms for (a) family 20 β-*N*-acetylglucosaminidases and (b) family 3 β-*N*-acetylglucosaminidases.

Keywords: α-Glucopyranoimidazolines; Intermediate analogue inhibitors; Family 20 β-*N*-acetylglucosaminidases; Oxazolinium ion intermediate.
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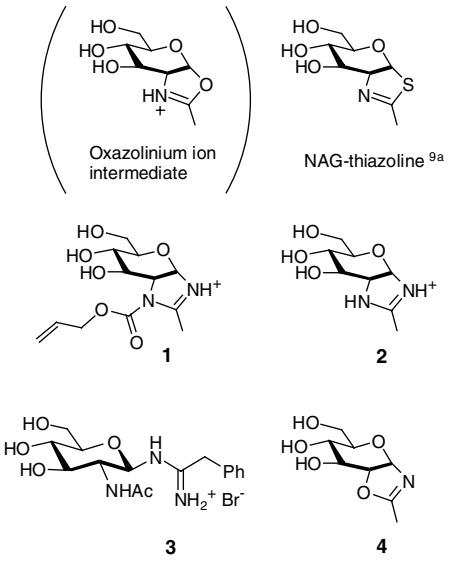
NAGs promotes the general-acid catalyzed cleavage of the glycosidic bond, while concomitant intramolecular nucleophilic attack by the substrate 2-acetamido oxygen results in the formation of an oxazolinium ion intermediate. The intermediate then undergoes base-catalyzed hydrolysis to yield β-*N*-acetylglucosamine with net retention of the configuration at the anomeric center.

A number of inhibitors have been prepared so far for the inhibition of NAGs.[6] The inhibitors of NAGs are potential antitumor[10] and antifungal agents[6] for use in pharmaceutical and agricultural applications, and also serve as biological tools to study the physiological function[11] and the mechanistic details of this class of enzymes.[9a,b,12] Despite the unique mechanistic features of family 20 NAGs, most of the NAG inhibitors reported so far are a GlcNAc version of the classical glycosidase inhibitors designed to mimic the charge and/or the shape of the oxocarbenium ion like transition states for conventional glycosidases (Fig. 1b). Thus, *N*-heterocycles such as nojirimycins,[13] isofagomins,[14] glyconoimidazoles,[15] triazoles,[16] glyconolactams,[17] glyconolactones,[18] and their oxime derivatives[19] are the most popular NAG inhibitors. However, a cyclic compound, NAG-thiazoline, is unique in that it mimics the oxazolinium ion intermediate and serves as a potent intermediate analogue inhibitor of jack bean β-*N*-acetylglucosaminidase (K_i = 0.28 μM).[9a] Due to its mechanistic relevance, this compound has been used successfully for the structural elucidation of the substrate-assisted catalysis by family 20 NAGs.[9b] We reasoned therefore that the mimicry of the oxazolinium ion intermediate is a key to generate selective inhibitors of family 20 NAGs and mechanistically related enzymes such as family 18 chitinases.[20] We previously reported that β-glycosylamidines served as highly potent and selective substrate analogue inhibitors of β-glycosidases, where the positively charged amidino group was essential for high inhibitory activities.[21,22] Here we report the synthesis, characterization, and evaluation of cyclic glucosylamidines, the α-glucopyranoimidazolines **1** and **2**, as intermediate analogue inhibitors of family 20 NAGs. The cyclic and positively charged amidino group (imidazoline) well mimicked the oxazolinium ion intermediate and showed potent and selective inhibitory activities toward family 20 NAGs, but did not inhibit family 3 NAGs, the enzymes that do not employ the substrate-assisted catalysis, but adopt a conventional double displacement mechanism via a glycosyl-enzyme intermediate (Fig. 1b). For comparison, a GlcNAc derivative of glycosylamidine **3** (an acyclic analogue) and a isoxazoline **4** (a cyclic, but neutral analogue) were also prepared for evaluating their inhibitory activities. The physicochemical properties of the imidazolines **1** and **2** are discussed in connection with base-catalyzed isomerization to imidazolosugars.

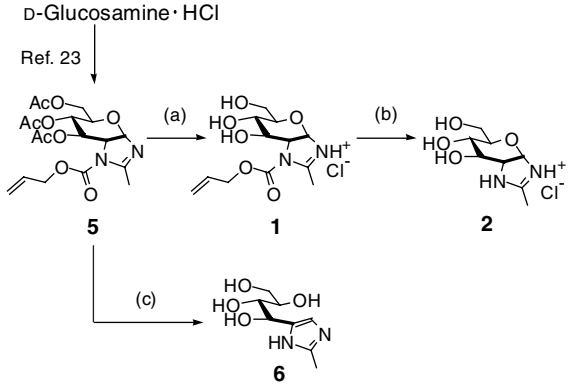


2. Results

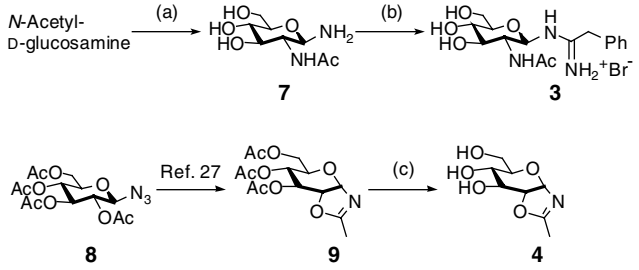
2.1. Synthesis

The α-glucopyranoimidazolines **1** and **2** were synthesized from compound **5** by deacetylation and subsequent Pd(0)-catalyzed removal of the allyloxycarbonyl (aloc) group, respectively (Scheme 1). Compound **5** was synthesized in five steps from D-glucosamine hydrochloride according to the literatures.[23] Our initial attempts to remove the acetyl groups of **5** under conventional basic conditions such as methanolic sodium methoxide, cat. NEt_3/MeOH and cat. Cs_2CO_3/MeOH were not successful and led to the formation of the imidazolosugar **6** as a major product (see Sec. 2.3 Chemical property). We therefore employed an acidic condition for deacetylation by treating **5** with HCl in MeOH to obtain the desired product **1** as colorless and hygroscopic crystals in 90% yield. The aloc group of **1** was then removed reductively by using $Pd(PPh_3)_4$ and formate to give crude **2** that contained ca. 9 mol% of **6** (^{1}H NMR). As described later, the unprotected imidazoline **2** was relatively unstable in aqueous media and was isomerized to the imidazolosugar **6** during purification. Hence crude **2** was used for the inhibition studies without further purification.

As an acyclic analogue, the β-glycosylamidine **3** was prepared from *N*-acetylglucosamine (Scheme 2). The syn-



Scheme 1. Reagents and conditions: (a) HCl, MeOH, rt, 90%; (b) $HCOO^-HNEt_3^+$, cat. $Pd(PPh_3)_4$, PPh_3, CH_3CN, rt, 85%; (c) cat. MeO^-Na^+, MeOH, rt, 33%.



Scheme 2. Reagents and conditions: (a) $NH_4^+HCO_3^-$ (1 equiv), sat. NH_3 in MeOH, 40 °C, 78%; (b) *S*-Methyl phenylthioacetimidate hydrobromide, pyridine, 0 °C, 45%; (c) cat. NEt_3, MeOH, rt, 63%.

thesis of a precursor β-glycosylamine **7** by the reported procedure[24] using a saturated aqueous ammonium bicarbonate solution followed by lyophilization was rather laborious and gave **7** in poor yield. We therefore modified our previous method[22] by using methanolic ammonia and 1 equiv of ammonium bicarbonate[25] to obtain a mixture of **7** and its carbamate salt quantitatively. The latter was decomposed smoothly by repeated evaporation of the mixture from MeOH to give pure **7** in 78% yield. The β-glycosylamine **7** was allowed to react with *S*-methyl phenylthioacetimidate hydrobromide[21,22] to give the β-glycosylamidine **3**.

The isoxazoline **4** is similar in shape to the putative oxazolinium ion intermediate, but has no positive charge. Hence, the isoxazoline **4** was expected to serve as a cyclic, but neutral analogue of **2** and was worth evaluating its inhibitory activity and physicochemical property. 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl azide (**8**)[26] was converted to the protected isoxazoline **9**,[27] followed by deacetylation with NEt_3/MeOH to afford the isoxazoline **4** (Scheme 2). In contrast to the imidazoline **2**, the isoxazoline **4** was extremely labile to acids, but was relatively stable under neutral to basic conditions and was purified by ODS reversed phase column chromatography. As expected from its structure, the isoxazoline **4** adopted almost the same conformation (distorted skew boat $^{O}S_2$) with that of **2** as determined by ^{1}H NMR.

2.2. Inhibition study

Compounds **1**, **2**, **3**, and **4** were assayed for their ability to inhibit NAGs. We employed two different types of NAGs: family 20 NAGs from jack bean (NAG Jack bean) and from *Streptomyces thermoviolaceus* (NAG C),[28] and family 3 NAGs from *S. thermoviolaceus* (NAG A)[29] and from *Clostridium paraputrificum* (NAG 3A).[30] For comparison, β-glucosidases from *Aspergillus niger* and almond were also used for the inhibition studies. The results are summarized in Table 1. The α-glucopyranoimidazoline **1** strongly inhibited family 20 NAGs (K_i = 0.58 and 1.6 μM), but showed no inhibitory activities toward family 3 NAGs. The inhibition was competitive with respect to the substrate (*p*NP-β-GlcNAc) and was simply reversible without showing any slow-binding kinetics in its inhibition.[31] The inhibitory activities of **1** further increased by removal of the *N*-aloc group; the free imidazoline **2** inhibited family 20 NAGs with a K_i of less than 0.1 μM, but still did not inhibit family 3 NAGs. The observed inhibitory activities of **2** were probably underestimated, because the free imidazoline **2** was relatively unstable in aqueous solution and contained at least ca. 9 mol% of the imidazolosugar **6**. No inhibitory activities of **6** were observed with family 20 NAGs (data not shown). Neither of the imidazolines **1** and **2** was inhibitory toward β-glucosidases that do not employ the catalysis involving the cyclic oxazolinium ion intermediate.

The acyclic β-glycosylamidine **3** served as a very weak inhibitor of family 20 NAGs as compared to the imidazolines **1** and **2**. Contrary to our expectation, the β-glycosylamidine **3** did not inhibit family 3 NAGs significantly, although the amidine **3** has a β-*N*-acetylglucosamine moiety and was thought to be a substrate mimic. The inhibitory activity of the isoxazoline **4** was measured at pH 7 due to its unstable nature under

Table 1. Inhibitory activities of compounds **1**, **2**, **3**, and **4** against β-*N*-acetylglucosaminidases (NAGs) and β-glucosidases

Enzyme		K_i (μM)			
		1	**2**	**3**	**4**
Family 20	NAG Jack bean[a]	0.58 ± 0.06	0.087 ± 0.016	490 ± 10[b]	78 ± 5[b,c]
	NAG C[d]	1.6 ± 0.3	0.17 ± 0.03	25 ± 1[b]	—[e]
Family 3	NAG A[f]	N.I.[g]	N.I.	N.I.	—
	NAG 3A[h]	N.I.	N.I.	65 ± 1[b]	—
Aspergillus niger β-glucosidase		N.I.	N.I.	—	—
Almond β-glucosidase		N.I.	N.I.	—	—

[a] Family 20 NAG from jack bean (*Canavalia ensiformis*), K_m = 620 μM (*p*NP-β-GlcNAc).
[b] IC_{50} (μM).
[c] Measured at pH 7.
[d] Family 20 NAG from *S. thermoviolaceus*,[28] K_m = 400 μM (*p*NP-β-GlcNAc).
[e] Not determined.
[f] Family 3 NAG from *S. thermoviolaceus*,[29] K_m = 40 μM (*p*NP-β-GlcNAc).
[g] N.I: less than 50% inhibition at 1000 μM inhibitors.
[h] Family 3 NAG from *C. paraputrificum*,[30] K_m = 20 μM (*p*NP-β-GlcNAc).

acidic conditions. Despite the similarity in shape, the isoxazoline **4** was much less inhibitory against NAG Jack bean (IC_{50} = 78 μM) than the imidazolines **1** and **2**.

2.3. Chemical property

As described in the synthesis, the stability of the imidazolines **1** and **2** was highly dependent on pH: compounds **1** and **2** were stable to acid, but were labile under basic conditions and decomposed slowly to an imidazole derivative even in a neutral aqueous solution. To confirm the structure of the decomposed product, the imidazolosugar **6** was synthesized separately from D-glucosamine according to the reported procedure[32] with a slight modification. Thus, D-glucosamine hydrochloride was treated with ethyl acetimidate hydrochloride in 1 M carbonate buffer (pH 10) to afford the imidazolosugar **6** in 63% yield. The comparison of the spectroscopic data unambiguously identified the decomposition product from **1**, **2**, and **5** to be the imidazolosugar **6** (see Experimental).

Knowing that the imidazolosugar **6** was the sole product from **1** and **2**, the long-term stability of **1** and **2** was examined by incubating the compounds in an aqueous solution of varying pH to monitor the reaction with ^{1}H NMR (Fig. 2). The *N*-aloc imidazoline **1** was highly stable under acidic to neutral conditions; 90% of **1** remained after 30 days. Under basic condition (pH 11), however, compound **1** was converted to the imidazole **6** with a half-life ($t_{1/2}$) of 2 days. The reaction was clean and gave **6** and allyl alcohol as the sole products. On the other hand, the free imidazoline **2** was much less stable than **1** and was isomerized slowly to **6** even under acidic conditions ($t_{1/2}$ = 34 and 20 days at pH 1 and pH 7, respectively). Under a basic condition (pH 11), compound **2** was isomerized instantaneously to **6**.

The synthesis of the α-glucopyranoimidazoline **2** was reported for the first time by Fischer and Lewis in 1967.[33] To the best of our knowledge, this is the only literature to date about this compound. According to this literature, compound **2** was synthesized by the reaction of D-glucosamine and ethyl acetimidate hydrochloride in DMF, but, interestingly, this method is almost identical to what we used for the synthesis of the imidazolosugar **6** as described above. Hence we reinvestigated the synthesis of **2** according to their procedure. Despite several attempts including different solvent systems such as pyridine, MeOH, and water, the only product we obtained by the reaction of D-glucosamine and ethyl acetimidate was the imidazolosugar **6**, rather than the imidazoline **2**. After a careful comparison of the spectroscopic data written in the literature to those for compound **6** (see Experimental), we concluded that the compound they had obtained was not the imidazoline **2**, but instead the imidazolosugar **6**. Since the imidazole **6** is a structural isomer of the imidazoline **2**, they seemed to have identified the product mistakenly as the imidazoline **2**. Therefore the present study is the first report about the synthesis and characterization of the α-glucopyranoimidazoline **2**.

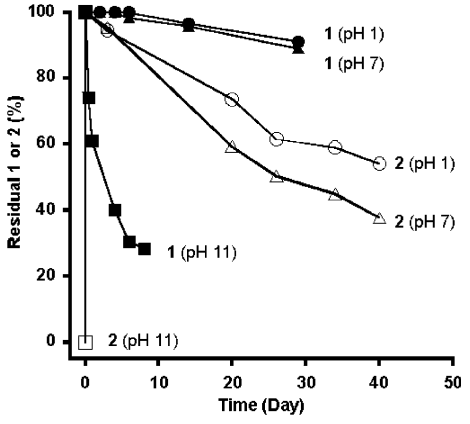


Figure 2. Stability of α-glucopyranoimidazolines **1** and **2** in aqueous solution (D_2O) of varying pH at 25 °C. ●, **1** in 0.35 M DCl; ▲, **1** in D_2O; ■, **1** in 84 mM Na_2CO_3; ○, **2** in 0.32 M DCl; △, **2** in D_2O; □, **2** in 40 mM Na_2CO_3.

3. Discussion

3.1. Inhibitory activity

We have designed and synthesized the α-glucopyranoimidazolines **1** and **2** as intermediate analogue inhibitors of family 20 NAGs. Family 20 NAGs are mechanistically unique in that they catalyze the cleavage of a β-glycosidic bond of *N*-acetylglucosamines and *N*-acetylgalactosamines through the anchimeric assistance by the 2-acetamido group of the substrates to form an oxazolinium ion intermediate (Fig. 1a). A positively charged imidazoline ring in **1** and **2** was expected to mimic the shape and the charge of the oxazolinium ion intermediate to serve as a key structure for selective inhibition of family 20 NAGs. As shown in Table 1, the α-glucopyranoimidazolines **1** and **2** strongly inhibited two family 20 NAGs with K_i of as low as 0.1 μM, but did not inhibit family 3 NAGs at all. The affinity with family 20 NAGs was up to 7000 times higher than that of the substrate. The observed selectivity is consistent with the differences in the catalytic mechanisms of family 20 and family 3 NAGs (Fig. 1a and b, respectively), where the oxazolinium ion intermediate is involved only in the catalysis by family 20 NAGs. We also synthesized the β-glycosylamidine **3** and the isoxazoline **4** as an acyclic and a neutral analogue, respectively, for evaluating their inhibitory activities. The acyclic analogue **3** was much less inhibitory than the imidazolines **1** and **2** toward family 20 NAGs, and the isoxazoline **4** was a rather weak inhibitor of NAG Jack bean (IC_{50} = 78 μM). These results cogently suggested that the imidazolines **1** and **2** well mimicked the shape and the positive charge of the putative oxazolinium ion intermediate, thereby exerting strong and highly selective inhibitory activities toward family 20 NAGs. It should be noted, however, that the *N*-acylated imidazoline **1** served as a fairly good inhibitor of family 20 NAGs (K_i = 0.58 and 1.6 μM), although the free imidazoline **2** was several times as strong as compound **1**. We anticipated initially that a substituent on the imidazoline N-3 nitrogen would greatly diminish the inhibition potency, because this nitrogen is corre-

sponding to the positively charged nitrogen in the oxazolinium ion intermediate, and the electrostatic interaction of this nitrogen with a highly conserved aspartate residue (Asp313 for *Streptomyces plicatus* β-*N*-acetylhexosaminidase[9d] and Asp354 for human β-*N*-acetylhexosaminidase[12]) was shown to be a key in stabilizing the intermediate during the catalysis of family 20 NAGs.[9,14] An electron-withdrawing group such as the aloc group attached to this nitrogen is most likely to decrease the basicity of the imidazoline ring as well as to increase the steric hindrance around the nitrogen, thereby negatively affecting the interaction with the enzymes. In fact, this was true, but to a limited extent: the *N*-aloc imidazoline **1** was several times less active than the free imidazoline **2**, but was still as good an inhibitor as other intermediate analogues such as NAG-thiazoline (K_i = 0.28 μM for NAG Jack bean).[9a] Since the *N*-aloc imidazoline **1** and NAG-thiazoline are less basic than the free imidazoline **2** and the conformation of **1** and **2** was very similar to each other (^{1}H NMR, see Experimental), the overall shape or the cyclic structure is probably more important than the electrostatic interaction for the inhibitors to bind to the enzymes. As regards the shape, the isoxazoline **4** is a fairly good mimic of the cyclic intermediate, but was actually a moderate inhibitor of the NAG Jack bean (IC_{50} = 78 μM). This is probably because the isoxazoline **4** is not protonated under the assay conditions (cf. pK_a = 5.52 for 2-methyloxazolinium ion[34]) and lacks a nitrogen atom at the C-2 position of the glucopyranose ring. The presence of NH and a positive charge at the C-2 position is essential for the interaction of the oxazolinium ion intermediate with the enzymes.[9b,d,12] In this regard, the α-glucopyranoimidazolines **1** and **2** meet the criteria for tight binding: the cyclic structure, the positive charge, and the presence of NH at C-2, to inhibit the enzymes strongly.

We reported previously that the β-glycosylamidines served as substrate analogue inhibitors that inhibit the corresponding β-glycosidases according to the glycon- and α-, β-specificities of the enzymes.[21] However, compound **3**, a GlcNAc-type β-glycosylamidine, was almost inactive toward family 3 NAGs (Table 1). This could be attributed to the substrate specificities of these enzymes that act primarily on oligosaccharides: NAG A shows the highest activity toward chitopentaose,[29] while NAG 3A is most active on chitobiose.[30] Hence the introduction of a hydrophilic substituent or a GlcNAc oligosaccharide chain into the aglycon part (or 'reducing' end) of compound **3** might increase the inhibitory activity toward family 3 NAGs.

3.2. Chemical property

The α-glucopyranoimidazolines **1** and **2** were stable under acidic conditions, but were isomerized exclusively into the imidazolosugar **6** under alkaline conditions. The free imidazoline **2** was extremely labile to alkali, whereas the *N*-aloc imidazoline **1** was much more stable (Fig. 2). The proposed mechanism for their conversion is shown in Figure 3. The isomerization of **2** is probably initiated by the abstraction of a proton from the N-3

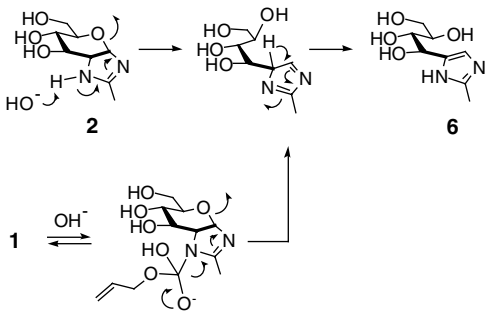


Figure 3. Putative mechanism for the decomposition of α-glucopyranoimidazolines **1** and **2**.

nitrogen atom of the imidazoline ring and is facilitated by aromatization to form a stable imidazole ring. The protection of this nitrogen atom with an aloc group prevents the initiation to decrease the isomerization rate, but the cleavage of the protective group follows facile isomerization to **6**. This mechanism is consistent with the observation that the incubation of **1** under acidic conditions gave the free imidazoline **2** and allyl alcohol along with the isomerized product **6**, and that the amount of allyl alcohol was equal to the total amount of **2** and **6** (^{1}H NMR, data not shown). Consequently, all our attempts to prepare **1** from **5** by deacetylation under basic conditions resulted in the formation of **6**. This type of isomerization from a glucoimidazoline to an imidazole derivative was reported by Yoshimura et al.[35]

In contrast to the stability of **1** and **2** under acidic conditions, the isoxazoline **4** was extremely labile to acid and was hydrolyzed instantaneously at pH 1. Furthermore, the isoxazoline **4** was hydrolyzed slowly even under neutral and basic conditions ($t_{1/2}$ = 24 and 195 h at pH 7 and 9, respectively). The fate of **4** was dependent on the pH of the reaction: the isoxazoline **4** was converted to β-glucosylamine **10** under an acidic condition probably via 2-*O*-acetyl-α-glucosylamine,[36] but gave the α-glucosylamide **11** under neutral to basic conditions (Fig. 4). The behavior of **4** is well understood by a general mechanism for imidates hydrolysis, where imidates yield amines and esters under acidic conditions, whereas amides and alcohols are formed under basic conditions via a tetrahedral intermediate.[37] Interestingly, the hydrolysis of the isoxazoline **4** did not accompany the opening of the pyranose ring that leads to the formation

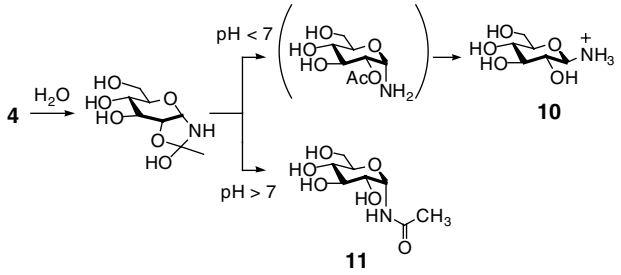


Figure 4. Hydrolysis of isoxazoline **4**.

of an oxazole derivative, as observed with the alkaline treatment of the imidazolines **1** and **2** to form the imidazole **6**.

In conclusion, the α-glucopyranoimidazolines **1** and **2** designed to mimic the oxazolinium ion intermediate served as highly potent and selective inhibitors of family 20 NAGs. Since the *N*-aloc imidazoline **1** is fairly stable as compared to the free imidazoline **2**, compound **1** is a promising lead for further structural elaboration for the inhibitors of other family 20 NAGs and mechanistically related enzymes such as family 18 chitinases.[20] This aspect of study is in progress.

4. Experimental

4.1. General

Flash column chromatography was performed on silica gel 60 (Merck, No. 9385, 40–63 μM). A reversed phase ODS column (ULTRA PACK ODS-S-50B and 50D, Yamazen Co., Osaka, Japan) was used for medium-pressure column chromatography. Analytical thin layer chromatography (TLC) was performed with Merck TLC plate silica gel 60 F_{254} (No. 5715). ^1H and ^{13}C NMR spectra were recorded on a JOEL JNM-AL 300 (300 MHz for ^1H) and JOEL JNM-AL 400 (400 MHz for ^1H) spectrometers with tetramethylsilane (for CD_3OD) and sodium 3-trimethylsilylpropanesulfonate (for D_2O) as an internal and an external standard, respectively. Infrared spectra (IR) were recorded on a Hitachi U-215 infrared spectrophotometer. UV spectra were recorded on a Shimadzu UV-3101PC UV–VIS–NIR scanning spectrophotometer. Mass spectra (MS) were obtained on a JEOL JMS 700 spectrometer. Elemental analyses were performed on a Yanaco MT-5. Melting points were recorded on a Mettler FP62 and were corrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter.

β-*N*-Acetylglucosaminidase from jack bean (*Canavalia ensiformis*) and β-glucosidases from almond and from *Aspergillus niger* were purchased from Sigma and were used without further purification. β-*N*-Acetylglucosaminidase from *S. thermoviolaceus* OPC-520[29] (Nag A, family 3) and a plasmid pNagC containing the gene of β-*N*-acetylglucosaminidase from *S. thermoviolaceus* OPC-520[28] (Nag C, family 20) were kind gifts from Prof. Hiroshi Tsujibo, Osaka University of Pharmaceutical Sciences. A plasmid pNag3A containing the gene of β-*N*-acetylglucosaminidase from *C. paraputrificum* M-21[30] (Nag 3A, family 3) was kindly provided by Prof. Kunio Ohmiya, Faculty of Bioresources, Mie University.

4.1.1. 2-Methyl-3-allyloxycarbonyl-(1,2-dideoxy-α-D-glucopyrano)[2,1-*d*]-1-imidazoline hydrochloride (1). To a solution of **5**[23] (6.44 g, 15.6 mmol) in $CHCl_3$ (30 mL) was added dropwise a solution of ca. 0.8 M HCl in MeOH (80 mL) (prepared by adding acetyl chloride (4.95 g, 63.0 mmol) to cold MeOH (80 mL)) at room temperature. The mixture was stirred for 23 h at room temperature. Another portion of 0.8 M HCl/MeOH (60 mL) was added, and the mixture was stirred for further 3 h to complete the reaction (TLC, *n*-BuOH/AcOH/H_2O 3:1:2). The reaction mixture was concentrated to dryness under vacuum to afford **1** as colorless and hygroscopic crystals (4.58 g, 90%). The analytical sample was purified by reversed phase medium-pressure ODS column chromatography eluted with a linear gradient of MeOH (0 to 10%) in water. The elution was monitored by absorbance at 240 nm. The fractions containing **1** (eluted at 1.2–3.3% MeOH) were collected and lyophilized to afford **1** as colorless and hygroscopic crystals. mp 119.2–121.1 °C; $[\alpha]_D^{23}$ −94.1° (*c* 0.3, H_2O); UV (H_2O), λ_{max} 217 nm (ε 7230); IR (KBr) ν_{max} 3330 (br), 2950, 1770, 1610, 1390, 1330, 1260, 1180, 1030, 960, 890, 760, and 630 cm^{-1} ; ^1H NMR (400 MHz, D_2O) δ_H 6.27 (d, 1H, J = 7.2 Hz, H-1), 6.04 (ddt, 1H, J = 17.6, 10.4 and 6.0 Hz, CH_2=C*H*CH_2), 5.47 (dq, 1H, J = 17.6 and 1.2 Hz, CH*H*=C*H*CH_2), 5.39 (dq, 1H, J = 10.4 and 1.2 Hz, C*H*H=C*H*CH_2), 4.91 (d, 1H, J = 7.2 Hz, H-2), 4.89 (d, 2H, J = 6.0 Hz, CH_2=CHCH_2), 4.68 (d, 1H, J = 2.8 Hz, H-3), 3.99 (ddd, 1H, J = 8.4, 5.2 and 2.8 Hz, H-5), 3.84 (dd, 1H, J = 8.4 and 2.8 Hz, H-4), 3.80 (dd, 1H, J = 12.0 and 2.8 Hz, H-6a), 3.64 (dd, 1H, J = 12.0 and 5.2 Hz, H-6b), 2.70 (s, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ_C 175.3 (NCOO), 152.5 (C=N), 133.2 (CH_2=*C*HCH_2), 123.2 (*C*H_2=CHCH_2), 91.72 (C-1), 82.30 (C-4), 76.01 (C-3), 73.02 (C-2), 72.67 (CH_2=CH*C*H_2), 70.82 (C-5), 66.02 (C-6), 18.26 (CH_3); Anal. Calcd for $C_{12}H_{19}ClN_2O_6{\cdot}0.3H_2O$: C, 43.92; H, 6.02; N, 8.54. Found: C, 43.91; H, 6.09; N, 8.67; FABHRMS Calcd for $C_{12}H_{19}N_2O_6$ (MH^+) 287.1244, found 287.1252.

4.1.2. 2-Methyl-(1,2-dideoxy-α-D-glucopyrano)[2,1-*d*]-1-imidazoline hydrochloride (2). To a solution of **1** (122 mg, 0.38 mmol) in CH_3CN (10 mL) purged with argon were added formic acid (52 mg, 1.13 mmol), triethylamine (42 mg, 0.42 mmol), triphenylphosphine (22 mg, 0.084 mmol) and tetrakis(triphenylphosphine)palladium(0) (23 mg, 0.020 mmol) at room temperature. The mixture was stirred for 10 h at room temperature under an argon atmosphere with protection from light. Another portion of tetrakis(triphenylphosphine)palladium(0) (46 mg, 0.040 mmol) was added, and the mixture was stirred for further 16 h to drive the reaction to completion (TLC, *n*-BuOH/AcOH/H_2O 3:1:2 and 2-PrOH/AcOH/MeOH/H_2O/$CHCl_3$ 4:1:3:3:4). The reaction mixture was concentrated to dryness, and the residual gummy syrup was washed successively with CH_3CN and $CHCl_3$. The supernatant was removed by decantation, and the residue was evaporated under a high vacuum to afford **2** as colorless and hygroscopic foam (80 mg, 85%). The product was used for enzyme assay without further purification. ^1H NMR (400 MHz, D_2O) δ_H 6.16 (d, 1H, J = 7.2 Hz, H-1), 4.62 (d, 1H, J = 7.2 Hz, H-2), 4.43 (d, 1H, J = 2.4 Hz, H-3), 3.96 (ddd, 1H, J = 8.8, 5.6, and 2.8 Hz, H-5), 3.79 (dd, 1H, J = 8.8 and 2.4 Hz, H-4), 3.80 (dd, 1H, J = 12.0 and 2.8 Hz, H-6a), 3.64 (dd, 1H, J = 12.0 and 5.6 Hz, H-6b), 2.30 (s, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ_C 172.6 (C=N), 92.96 (C-1), 82.01 (C-4), 76.04 (C-3), 70.92 (C-2), 70.87 (C-5),

66.12 (C-6), 14.82 (CH_3); FABHRMS Calcd for $C_8H_{15}N_2O_4$ (MH^+) 203.1033, found 203.1023.

4.1.3. N^1-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-phenylacetamidine hydrobromide (3). 2-Acetamido-2-deoxy-β-D-glucopyranosylamine (**7**) was prepared from *N*-acetyl-D-glucosamine according to a modified procedure of our previous method.[22] *N*-Acetyl-D-glucosamine (22.1 g, 0.1 mol) and ammonium bicarbonate (7.91 g, 0.1 mol) were added to MeOH (100 mL) saturated with ammonia gas at 0 °C in a pressure bottle. The bottle was tightly closed, and the mixture was stirred at 40 °C for 3 days. The suspension became a solution in 6 h, and colorless precipitate was then formed in 12 h. Ethanol (150 mL) was added to the reaction mixture, and the mixture was kept at 4 °C to precipitate out the product. The crystalline precipitate was collected by filtration and washed with EtOH and ether, successively, to give a mixture of **7** and its carbamate (1:0.8, ^{1}H NMR in D_2O) (20.6 g). The crude product was dissolved in MeOH (500 mL) and was evaporated to dryness to decompose the carbamate. Evaporation was repeated for three times to give pure glycosylamine **7** as colorless glassy hygroscopic foam (17.2 g, 78%). ^{1}H NMR (300 MHz, D_2O) δ_H 4.14 (d, 1H, J = 9.0 Hz, H-1), 3.88 (dd, 1H, J = 12.2 and 2.0 Hz, H-6a), 3.70 (dd, 1H, J = 12.3 and 5.6 Hz, H-6b), 3.61 (dd, 1H, J = 9.8 and 9.2 Hz, H-4), 3.51 (m, 1H, H-2), 3.4 (m, 2H, H-3 and H-5), 2.04 (s, 3H, CH_3CO). The glycosylamine **7** (560 mg, 2.54 mmol) suspended in dry pyridine (10 mL) was cooled to 0 °C. *S*-Methyl phenylthioacetimidate hydrobromide (prepared from phenylthioacetamide and bromomethane in acetone, 100%) (630 mg, 2.56 mmol) was added to the suspension. The mixture was stirred at 0 °C for 5 h under an argon atmosphere, during which the glycosylamine **7** was dissolved to give a clean solution. The reaction mixture was evaporated, and the crude product dissolved in water was applied to a medium-pressure ODS column. The column was eluted with a linear gradient of MeOH (0 to 50%) in water and the fractions absorbing at 254 nm were collected and freeze-dried to afford **3** as colorless and hygroscopic powder (473 mg, 45%). $[\alpha]_D^{25}$ −10.8° (*c* 1.0, H_2O); ^{1}H NMR (300 MHz, D_2O) δ_H 7.46–7.30 (m, 5H, aromatic), 4.93 (d, 1H, J = 9.3 Hz, H-1), 3.95–3.72 (m, 5H), 3.63–3.45 (m, 3H), 1.78 (s, 3H, CH_3); ^{13}C NMR (75 MHz, D_2O) δ_C 177.6 (C=O), 171.5 (C=N), 134.7, 132.2, 131.7, 131.2 (aromatic), 83.30 (C-1), 80.45 (C-5), 76.10 (C-3), 71.89 (C-4), 63.03 (C-6) 56.59 (C-2), 41.48 (CH_2Ph), 24.61 (CH_3CO); Anal. Calcd for $C_{16}H_{24}BrN_3O_5 \cdot 1.4H_2O$: C, 43.33; H, 6.09; N, 9.47. Found: C, 43.33; H, 5.89; N, 9.56; FABHRMS Calcd for $C_{16}H_{24}N_3O_5$ (MH^+) 338.1717, found 338.1711.

4.1.4. 2-Methyl-(1,2-dideoxy-α-D-glucopyrano)[2,1-*d*]-oxazoline (4). The peracetylated oxazoline **9**[27](1.02 g, 3.10 mmol) prepared from 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl azide (**8**) was dissolved in MeOH (10 mL) containing triethylamine (2 mL). The mixture was stirred for 15 h at room temperature and was concentrated in vacuo. The residue was dissolved in cold water (2 mL) and was passed through an ODS Sep-Pak® Cartridge (Waters). Lyophilization of the pass-through fractions gave the oxazoline **4** as colorless powder (494 mg, 63%). ^{1}H NMR (300 MHz, CD_3OD) δ_H 5.74 (d, 1H, J = 7.5 Hz, H-1), 4.37 (dd, 1H, J = 7.5 and 5.1 Hz, H-2), 3.76 (dd, 1H, J = 12.0 and 3.0 Hz, H-6a), 3.71 (dd, 1H, J = 6.6 and 5.1 Hz, H-3), 3.70 (dd, 1H, J = 12.0 and 5.1 Hz, H-6b), 3.48 (dd, 1H, J = 8.7 and 6.6 Hz, H-4), 3.29 (ddd, 1H, J = 8.7, 5.1 and 3.0 Hz, H-5), 2.05 (d, 3H, J = 0.9 Hz, CH_3); ^{13}C NMR (75 MHz, CD_3OD) δ_C 171.1 (C=N), 93.48 (C-1), 82.36 (C-4), 75.38 (C-3), 74.77 (C-2), 69.52 (C-5), 63.06 (C-6), 14.11 (CH_3); FABHRMS Calcd for $C_8H_{14}NO_5$ (MH^+) 204.0872, found 204.0874.

4.1.5. 2-Methyl-4-(1′,2′,3′,4′-tetrahydroxybutyl)imidazole (6) from compound 5. To a solution of **5** (1.95 g, 4.73 mmol) in dry MeOH (50 mL) was added 1 M sodium methoxide (0.24 mL, 0.24 mmol) at room temperature. The mixture was stirred at room temperature for 38 h. After the reaction was complete (TLC, *n*-BuOH/AcOH/H_2O 3:1:2, anisaldehyde), the reaction mixture was concentrated to dryness. The residue was crystallized from MeOH to afford the imidazolosugar **6** (free base) as colorless powder (315 mg, 33%). ^{1}H NMR (400 MHz, D_2O) δ_H 6.97 (s, 1H, H-5), 4.84 (d, 1H, J = 4.4 Hz, H-1′), 3.82 (dd, 1H, J = 6.8 and 4.4 Hz, H-2′), 3.76 (dd, 1H, J = 11.6 and 3.2 Hz, H-4′a), 3.68 (dt, 1H, J = 6.8 and 3.2 Hz, H-3′), 3.59 (dd, 1H, J = 11.6 and 6.8 Hz, H-4′b), 2.33 (s, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ_C 148.7 (C-2), 139.8 (C-4), 118.9 (C-5), 76.59 (C-2′), 74.10 (C-3′), 69.62 (C-1′), 65.28 (C-4′), 15.15 (CH_3). The imidazolosugar **6** (0.21 g, 1.04 mmol) was treated with 12 M HCl (0.095 mL, 1.14 mmol) and was lyophilized to afford the hydrochloride salt of **6** as colorless powder (0.25 g). mp 158.0–160.8 °C; $[\alpha]_D^{23}$ −14.0° (*c* 0.2, H_2O); UV (H_2O), λ_{max} 210 nm (ε 5100); IR (KBr) ν_{max} 3180 (br), 1640, 1560, 1340, 1300, 1270, 1210, 1120, 1090, 1050, 950, 890, 780, 750, 620 and 500 cm^{-1} ; ^{1}H NMR (400 MHz, D_2O) δ_H 7.23 (d, 1H, J = 0.8 Hz, H-5), 5.11 (dd, 1H, J = 2.4 and 0.8 Hz, H-1′), 3.85 (dd, 1H, J = 11.6 and 2.8 Hz, H-4′a), 3.79 (ddd, 1H, J = 8.4, 5.6 and 2.8 Hz, H-3′), 3.70 (dd, 1H, J = 8.4 and 2.4 Hz, H-2′), 3.67 (dd, 1H, J = 11.6 and 5.6 Hz, H-4′b), 2.59 (s, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ_C 147.3 (C-2), 135.7 (C-4), 117.8 (C-5), 75.55 (C-2′), 73.40 (C-3′), 66.96 (C-1′), 65.52 (C-4′), 13.30 (CH_3); Anal. Calcd for $C_8H_{15}ClN_2O_4$: C, 40.26; H, 6.33; N, 11.74. Found: C, 40.03; H, 6.36; N, 11.49; FABHRMS Calcd for $C_8H_{15}N_2O_4$ (MH^+) 203.1033, found 203.1027.

4.2. Compound 6 from D-glucosamine

D-Glucosamine hydrochloride (5.04 g, 23.4 mmol) was dissolved in 1 M carbonate buffer (pH 10) (140 mL). Ethyl acetimidate hydrochloride (4.33 g, 35.0 mmol) was added portionwise to the solution, and the mixture was stirred for 5 h at room temperature. Another portion of ethyl acetimidate hydrochloride (1.53 g, 12.4 mmol) was added. After 2 h, another portion of ethyl acetimidate hydrochloride (3.14 g, 25.4 mmol) was added, and the mixture was stirred for further 2 h

to drive the reaction to completion (TLC, *n*-BuOH/AcOH/H_2O 2:1:2, anisaldehyde). The reaction mixture was concentrated to dryness and was lyophilized to give a mixture of the product and the buffer salt as colorless powder. The product was extracted with MeOH (350 mL) and filtered. The filtrate was evaporated, and the residual solid was crystallized from EtOH to afford **6** (free base) as colorless powder (2.98 g, 63%). mp 174.9–175.7 °C; UV (H_2O), λ_{max} 208 nm (ε 7108); IR (KBr) ν_{max} 3000 (br), 2300, 1360, 1080, 1040, 950 and 880 cm^{-1} ; ^1H NMR (400 MHz, D_2O) δ_H 6.94 (s, 1H, H-5), 4.81 (d, 1H, J = 4.4 Hz, H-1′), 3.81 (dd, 1H, J = 6.8 and 4.4 Hz, H-2′), 3.73 (dd, 1H, J = 11.6 and 3.2 Hz, H-4′a), 3.67 (dt, 1H, J = 6.8 and 3.2 Hz, H-3′), 3.58 (dd, 1H, J = 11.6 and 6.8 Hz, H-4′b), 2.31 (s, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ_C 148.8 (C-2), 140.1 (C-4), 118.9 (C-5), 76.63 (C-2′), 74.12 (C-3′), 69.74 (C-1′), 65.25 (C-4′), 15.23 (CH_3); Anal. Calcd for $C_8H_{14}N_2O_4$: C, 47.52; H, 6.98; N, 13.85. Found: C, 47.33; H, 7.04; N, 13.75; FABHRMS Calcd for $C_8H_{15}N_2O_4$ (MH^+) 203.1033, found 203.1022. The imidazolosugar **6** (0.40 g, 1.98 mmol) was treated with 12 M HCl (0.18 mL, 2.18 mmol) and was lyophilized to afford the hydrochloride salt of **6** as colorless powder (0.47 g). mp 161.9–162.6 °C; $[\alpha]_D^{23}$ −15.6° (*c* 0.5, H_2O); UV (H_2O), λ_{max} 210 nm (ε 6830); IR (KBr) ν_{max} 3190 (br), 1640, 1560, 1340, 1300, 1270, 1210, 1120, 1090, 1050, 950, 890, 780, 750, 620 and 500 cm^{-1} ; ^1H NMR (400 MHz, D_2O) δ_H 7.23 (d, 1H, J = 0.8 Hz, H-5), 5.11 (dd, 1H, J = 2.4 and 0.8 Hz, H-1′), 3.83 (dd, 1H, J = 11.6 and 2.8 Hz, H-4′a), 3.78 (ddd, 1H, J = 8.4, 5.6 and 2.8 Hz, H-3′), 3.69 (dd, 1H, J = 8.4 and 2.4 Hz, H-2′), 3.66 (dd, 1H, J = 11.6 and 5.6 Hz, H-4′b), 2.59 (s, 3H, CH_3); ^{13}C NMR (100 MHz, D_2O) δ_C 147.3 (C-2), 135.7 (C-4), 117.9 (C-5), 75.59 (C-2′), 73.46 (C-3′), 67.01 (C-1′), 65.57 (C-4′), 13.37 (CH_3); Anal. Calcd for $C_8H_{15}ClN_2O_4{\cdot}0.2H_2O$: C, 39.66; H, 6.41; N, 11.56. Found: C, 39.75; H, 6.41; N, 11.57; FABHRMS Calcd for $C_8H_{15}N_2O_4$ (MH^+) 203.1033, found 203.1037.

4.3. Preparation of enzymes

A recombinant β-*N*-acetylglucosaminidase from *S. thermoviolaceus* OPC-520 (Nag C, family 20) was expressed in *E. coli* BL 21 by using the expression plasmid pNagC and was purified with a Ni-NTA column (Invitrogen).[28] β-*N*-Acetylglucosaminidase from *C. paraputrificum* M-21 (Nag 3A, family 3) was expressed as a recombinant in *E. coli* JM 109 by using the expression plasmid pNag3A and was purified according to the reported procedure.[30]

4.4. Enzyme assays

The following assay conditions were used for each enzyme (substrate, buffer, temperature, and K_m).

(1) β-*N*-Acetylglucosaminidase from jack bean [*p*NP-β-GlcNAc, 40 mM sodium citrate buffer (pH 5.0), 30 °C, K_m = 620 μM].
(2) Nag C from *S. thermoviolaceus* (family 20) [*p*NP-β-GlcNAc, 40 mM sodium phosphate buffer (pH 6.0), 30 °C, K_m = 400 μM].
(3) Nag A from *S. thermoviolaceus* (family 3) [*p*NP-β-GlcNAc, 40 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 40 μM].
(4) Nag 3A from *C. paraputrificum* (family 3) [*p*NP-β-GlcNAc, 40 mM sodium phosphate buffer (pH 7.0), 30 °C, K_m = 20 μM].
(5) β-Glucosidase from *A. niger* [*p*NP-β-Glc, 40 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 610 μM].
(6) β-Glucosidase from almond [*p*NP-β-Glc, 40 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 2.4 mM].

A typical assay method is as follows: An appropriate amount of β-*N*-acetylglucosaminidase from jack bean (36 μL) in 50 mM sodium citrate buffer (pH 5.0) containing 200 μg/mL bovine serum albumin (BSA) was added to a preincubated solution (564 μL, 30 °C) containing a varied volume of a stock solution of *p*NP-β-GlcNAc (10 mM in water) and 40 mM sodium citrate buffer (pH 5.0) (total volume of 600 μL, the final substrate concentration of 0.1–2.5 mM). The mixture was incubated for 6 min at 30 °C. The reaction was terminated by adding 1 M Na_2CO_3 (300 μL), and the absorbance at 405 nm was measured. The initial rate (v_0) was calculated using $\varepsilon(p\text{NP}) = 1.75 \times 10^4\ \text{L mol}^{-1}\text{cm}^{-1}$. K_m values were determined by v_0–[S] plot. Nonlinear regression analysis of kinetic data was performed directly by least-squares fit using KaleidaGraph program (Synergy Software). IC_{50} values were determined by varying the concentration of the inhibitor with the substrate concentration approximating to the K_m value. K_i values were determined by varying the concentration of the inhibitor with the substrate concentration approximating the K_m or $2K_m$ values.

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