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NAG-thiazoline is a potent inhibitor of the *Vibrio campbellii* GH20 β -N-Acetylglucosaminidase

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Running Title

An inhibitor of GH-20 β -N-acetylglucosaminidase

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Abbreviations: GlcNAcase, β -N-acetylglucosaminidase; diNAG, di-N-acetylglucosamine, chitobiose; NGT, NAG-thiazoline; GlcNAc, N-acetylglucosamine; 4NP-GlcNAc, 4-nitrophenyl N-acetylglucosaminide.

Database: structural data are available in PDB database under the accession number 6K35.

ABSTRACT

Vibrio spp. play a vital role in the recycling of chitin in oceans, but several *Vibrio* strains are highly infectious to aquatic animals and humans. These bacteria require chitin for growth, thus potent inhibitors of chitin-degrading enzymes could serve as candidate drugs against *Vibrio* infections. This study examined NAG-thiazoline (NGT)-mediated inhibition of a recombinantly expressed GH20 β -N-acetylglucosaminidase, namely *Vh*GlcNAcase from *Vibrio campbellii* (formerly *V. harveyi*) ATCC BAA-1116. NGT strongly inhibited *Vh*GlcNAcase with an IC_{50} of $11.9 \pm 1.0 \mu\text{M}$ and K_i $62 \pm 3 \mu\text{M}$, respectively. NGT was also found to completely inhibit the growth of *V. campbellii* strain 650 with an MIC value of $0.5 \mu\text{M}$. ITC data analysis showed direct binding of NGT to *Vh*GlcNAcase with a K_d of $32 \pm 1.2 \mu\text{M}$. The observed $\Delta G^{\circ}_{\text{binding}}$ of $-7.56 \text{ kcal.mol}^{-1}$ is the result of a large negative enthalpy change and a small positive entropic compensation, suggesting that NGT binding is enthalpy-driven.

The structure complex shows that NGT fully occupies the substrate-binding pocket of *Vh*GlcNAcase and makes an exclusive hydrogen bond network, as well as hydrophobic interactions with the conserved residues around the -1 subsite. Our results strongly suggest that NGT could serve as an excellent scaffold for further development of anti-microbial agents against *Vibrio* infections.

INTRODUCTION

Vibrio spp. are Gram-negative bacteria that have a curved-rod shape and polar flagella with sheaths.^{1,2,3} Some of these bacteria, such as *Vibrio campbellii* (formerly *V. harveyi*)⁴ and *V. fischeri*⁵, are bioluminescent through a cellular mechanism that is regulated by quorum sensing.⁶ *Vibrio* spp. are facultative anaerobes⁷ that are widespread in coastal waters and ocean sediments. It is of practical interest that several *Vibrio* strains, such as *V. campbellii* and *V. salmonicida*, are highly pathogenic to fish and prawns, causing Vibriosis, which devastates seafood-based economies worldwide^{4,8} while *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus*^{9,10,11} cause severe illnesses in humans. Most

disease-causing strains are associated with gastroenteritis, but can also infect open wounds and cause septicemia⁹. *Vibrio* infections can be fatal if these conditions are left without prompt treatment and the risk of clinical disease and death increases dramatically in certain conditions, such as immunocompromised states, uncontrolled diabetes, liver cirrhosis and cancers^{12,13}. Cholera, another major life-threatening intestinal infection, is caused by *V. cholerae*, and affects an estimated 3-5 million people worldwide and causes 28,800-130,000 deaths a year^{14,15}. Countries with poor sanitation, including many in Africa and Southeast Asia, are at high risk of cholera infection as the disease is mostly spread by unclean water and undercooked seafood or by food contaminated with human feces containing the bacteria. Interestingly, *Vibrio spp.* mostly live in saltwater lacking glucose enrichment, yet these bacteria are able to grow because of their evolutionary adaptation to utilizing chitin, a sugar polymer that is highly abundant in oceans, as a nutrient. Since marine *Vibrio spp.* are dependent on the abundance of chitin in marine ecosystems, the activity of chitinolytic enzymes^{16,17,18}, chitin transporters and chitin-binding proteins in the chitin degradation pathway are crucial for the growth of these bacteria.^{17,19,20,21} Two major groups of glycoside hydrolases, endo-chitinases (EC 3.2.1.14) and *exo*- β -*N*-acetylglucosaminidases or GlcNAcases (EC 3.2.1.52), are required for the complete degradation of chitin by marine *Vibrio spp.* into products that enter metabolic pathways that enable the use of GlcNAc as their sole source of carbon, nitrogen and energy.^{16,17,21,22,23} Thus, both chitinases and GlcNAcases are excellent candidates for the development of anti-microbial agents directed against *Vibrio* infection, as suppression of the activity of these enzymes by inhibitors would reduce the growth of the bacteria.

We previously identified a *nag* gene encoding a GH20 GlcNAcase, namely *VhGlcNAcase*, from the genome of the pathogen *V. campbelli* ATCC BAA-1116. The gene was cloned into a pET23a(+) expression vector and successfully expressed at high levels.¹⁷ *VhGlcNAcase* is an *exo*-acting enzyme that degrades chitooligosaccharides from the non-reducing end in a sequential manner, liberating GlcNAc units.^{17,22,24} In an effort to gain insight into the biochemistry and biological function of GlcNAc-processing enzymes, small-molecule inhibitors have been developed and a number of inhibitors of both bacterial and human GH20 GlcNAcases have been reported. For example, PUGNAc (*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate) has been characterized as a potent inhibitor of GlcNAcases from not only family GH20^{25,26} but also GH3²⁷ and

GH84.²⁸ NAG-thiazoline (1,2-dideoxy-2'-methyl- α -D-glucopyranoso-[2,1-d]- Δ 2'-thiazoline, NGT)²⁹ has been shown to be a potent inhibitor of both GH20³⁰ and GH84 GlcNAcases²⁶ and iminosugars, such as 2-acetamino-1,2-dideoxynojirimycin (NHAcDNJ) and 6-acetamido-6-deoxy-castanospermine (NHAcCAS) have also been found to be the potent inhibitors of GlcNAcases from GH3^{31,32}, GH20^{31,33,34,35,36} and GH84^{28,31,36}. Since NGT and its derivatives have received attention as possible therapeutics^{35,37} this report focused on this molecule as an inhibitor of *Vh*GlcNAcase, to gain insight into its mode of inhibition and potential anti-bacterial activity against *V. campbellii*. Structural studies and binding thermodynamics have provided insight into the details of the enzyme-inhibitor interactions and explain the high potency of NGT against the *Vibrio* enzyme.

RESULTS

Inhibitory effect of NGT on *Vh*GlcNAcase using a chromogenic substrate

NGT is an analog of the reaction-intermediate for bacterial and human GlcNAcases^{24,26} found in family GH20. Its chemical structure closely resembles that of the oxazoline reaction intermediate and NGT thus potentially acts as a strong inhibitor of *Vh*GlcNAcase (**Fig. 1A**). Using the chromogenic substrate 4NP-GlcNAc, we initially demonstrated that NGT reduced the specific activity of *Vh*GlcNAcase in a concentration-dependent manner (**Fig. 1B**). At a concentration of 100 μ M NGT, only a residual activity of 15% was retained. Dose-response experiments were then carried out to determine the half-maximal inhibitory concentration (IC_{50}) from a plot of the fractional (v_0/v_i) activity vs. the logarithm of the inhibitor concentration. From the data obtained the IC_{50} value for NGT was found to be 11.9 ± 1.0 μ M (**Fig. 1C**).

Inhibitory effect of NGT on *Vh*GlcNAcase in the presence of a natural chitooligosaccharide

To further confirm the inhibitory effects of NGT on the activity of *Vh*GlcNAcase, we conducted an assay utilizing chitotetraose [(GlcNAc)₄] as a small natural substrate in the presence or absence of NGT and used TLC to analyze the hydrolytic products formed. Enzymatic degradation of (GlcNAc)₄ by *Vh*GlcNAcase at different time points of 0, 5, 10, 30, and 60 min (Lane 1, 2, 3, 4, and 5, respectively) was easily observed (**Fig. 2**). In the reaction mixtures containing NGT (**Fig. 2A**),

starting material was still present after 10 minutes of incubation and after 60 minutes (GlcNAc)₂ was still present. In the reactions without NGT (**Fig. 2B**), *VhGlcNAcase* degraded the substrate much more efficiently, complete degradation of (GlcNAc)₄ being already observed after 10 minutes of reaction, while after 60 minutes, only the final degradation product GlcNAc was present. These results demonstrated that NGT was able to inhibit the activity of the enzyme in the presence of a small natural substrate.

Determination of binding affinity of NGT for *VhGlcNAcase* using ITC

ITC was performed to determine the equilibrium dissociation binding constant (K_d), stoichiometry (n), enthalpy change (ΔH°), entropy change (ΔS°) and change of Gibbs free energy of binding ($\Delta G^\circ_{\text{binding}}$) for the interaction of NGT with *VhGlcNAcase*. Heat generated after titrating NGT with *VhGlcNAcase* at 37 °C resulted in a typical thermogram (**Fig. 3A, lower thermogram**), with only a small heat of dilution observed when NGT was injected into the buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) without protein (**Fig. 3A, upper thermogram**). The binding reaction was observed to be exothermic, with overall negative heat integration (**Fig. 3B**). Curve fitting using a single-site binding model yielded the dissociation binding constant (K_d) of $32 \pm 1.2 \mu\text{M}$ and a stoichiometry (n) of 0.9 ± 0.1 (**Table 1**), agreeing with the molar ratio of 1:1 between NGT and *VhGlcNAcase*. Analysis of thermodynamic parameters indicated that the binding was exergonic ($-\Delta G^\circ_{\text{binding}}$) and driven by enthalpy. The negative $\Delta G^\circ_{\text{binding}}$ value ($-6.4 \text{ kcal}\cdot\text{mol}^{-1}$) is the net outcome of a large negative ΔH° value ($-18.4 \pm 3.5 \text{ kcal}\cdot\text{mol}^{-1}$) and a small positive ($-T\Delta S^\circ$) value ($+11.9 \pm 3.5 \text{ kcal}\cdot\text{mol}^{-1}$) (**Fig. 3C**).

Determination of the inhibitory constant (K_i) for NGT against *VhGlcNAcase*

The determination of the K_i of NGT against *VhGlcNAcase* was carried out in the presence of varied concentrations of the inhibitor using four concentrations of substrate (4NP-GlcNAc: 250, 500, 1000 and 2000 μM). From a plot of v_o vs $[S]_o$ at different concentrations of NGT, fitting of the Michaelis-Menten equation demonstrated that NGT inhibits *VhGlcNAcase* with a K_i value of $62 \pm 3 \mu\text{M}$ (**Fig. 4A**). A Lineweaver-Burk (LB) plot of the data (**Fig. 4B**) confirmed that NGT inhibits *VhGlcNAcase*

in a competitive fashion, in agreement with previously reported results of NGT inhibition of other GH20 enzymes.^{29,35}

Determination of the X-ray crystal structure of *VhGlcNAcase* co-crystallized with NGT

VhGlcNAcase was successfully co-crystallized with NGT in sitting-drop vapor diffusion wells containing 0.2 M sodium fluoride, 0.1 M Bis-Tris propane pH 6.5, 20% PEG3350. Single crystals of the complex diffracted with the synchrotron light source to the highest resolution of 2.36 Å (details of data collection and refinement statistics are in **Table 2**) with the crystal belonging to the space group $P2_1$. The *VhGlcNAcase* structure in complex with NGT was determined by the Molecular Replacement method as described (see **Supporting Information**) and refined to $R_{\text{work}}/R_{\text{free}}$ of 0.19/0.26. The root-mean-square deviations (RMSDs) of bond lengths and angles of the crystal structure were refined to 0.013 Å and 1.60°, respectively. The average B -factor was refined to 40.80 Å² and its coordinates and structure factors were deposited in the Protein Data Bank with PDB id **6K35**.

Since the crystal structure of *VhGlcNAcase* revealed two protein molecules per asymmetric unit, we carried out PISA analysis of molecule A vs. molecule B. The achieved Complex Formation Significance Score (CSS) of 0.00 suggests that the A-B interface does not play any role in complex formation and seems to be a result of crystal packing only.³⁸ A very small dimer interface is found between the C -terminus of molecule A and residues 245-248 of molecule B. Molecule A contains the carbohydrate-binding (CBD) domain (residues 1-114, purple) and is connected to the α + β domain (residues 148-264, blue) through a linker (residues 115-147, grey) (**Fig. 5A**). The catalytic (Cat) domain (residues 265-642, green) is composed of a typical $(\alpha/\beta)_8$ TIM-barrel fold, similar to the Cat domain of the previously-reported chitobiase (*SmChb*) from *Serratia marcescens*.³⁹ The catalytic pocket of *VhGlcNAcase* is observed on the surface of the Cat domain. Amino acid residues R274, D303, H373, D437, E438 D532, W546 and E584 are prominently located on the wall of the sugar

binding pocket (residues in sticks), with the conserved acidic pair D37/E438 being predicted to be crucial for catalysis.

The space-filling model shows that the active site of *VhGlcNAcase* is located on the surface of the Cat domain and has a small, open pocket (**Fig. 5B**), suitable for accommodating small chitooligosaccharides (2-4 sugar moieties). To identify the position of the bound NGT in the catalytic pocket of *VhGlcNAcase*, the structure of *VhGlcNAcase* complexed with NGT was superimposed on the structure of *SmChb* complexed with diNAG [(GlcNAc)₂], PDB id 1QBB). The structural alignment of the two enzymes yielded a root mean square deviation (RMSD) of 1.495 Å for C_α of 418 atoms, indicating a good fit. The diNAG molecule occupies two subsites; the inner site (-1 subsite) and the outer (+1 subsite), in the catalytic pocket of *SmChb* (**Fig. 5B, yellow**). When diNAG was modelled in the active site of *VhGlcNAcase*, the NGT molecule (**Fig. 5C, bright green**) was found to occupy the -1 subsite, where the inner ring of diNAG is located.

Structural analysis of *VhGlcNAcase*-inhibitor interactions

Specific interactions between the inhibitor and the enzyme were analyzed in detail. NGT occupies the -1 subsite (**Fig. 6, green**), as compared to the position of diNAG (**Fig. 6, yellow**) in the active site of *SmChb*.³⁹ There were no obvious steric clashes between NGT and the nearby residues on the surface of the catalytic pocket. As mentioned earlier, NGT is surrounded by R274, D303, H373, Q398, D437, E438, Y530, D532, E584 and W546, located on the surface of the GlcNAc-binding pocket, and the side chains of these residues are within 3.5 Å of NGT, indicating their capability to form strong H-bonds with the inhibitor (**Fig. 6A, broken lines**). The nitrogen and sulfur atoms at position C-2 of NGT were found to make contact through two H-bonds, one with D437 and the other with Y530, respectively. The thiazoline ring of NGT, which resembles the oxazolinium ring of the reaction intermediate of GH20 enzymes, is positioned close to the catalytic pair D437-E438 and makes an H-bond with the imidazole ring of H373, located between the two acidic groups. The 3-OH group of NGT forms two H-bonds; one directly with R274, while the other, with D303, is mediated by a nearby water molecule. The 4-OH group forms an H-bond with Glu584, while the 6-OH group of NGT is stabilized by two H-bonds with D532 and W546. The five aromatic residues, W487, W505, Y530, W546 and W582 form a hydrophobic wall surrounding the molecule (**Fig. 6B**) and

importantly, W582 stacks directly against the plane of its pyranose ring. The C_α traces of the TIM-barrel catalytic domains of three bacterial GlcNAcases complexed with NGT, including that of *VhGlcNAcase*, were superimposed (**Fig. 7A**). Structural alignment demonstrated that the fold of *VhGlcNAcase* is most similar to that of the β -hexosaminidase from *Paenibacillus* sp. TS12 (β -HEX1) (RMSD of 0.72 Å over 240 residues, PDB ID 3SUR)³⁴, followed by the β -N-acetylhexosaminidase from *Streptomyces plicatus* (*SpHex*) (RMSD 0.98 Å over 251 residues, PDB ID 1HP5)³⁰, respectively. The unbiased electron density maps of NGT and the surrounding binding residues of *VhGlcNAcase* are displayed as a close-up view inside a black circle. The NGT molecule was modelled in the electron density map with full occupancy and the pyranose ring of NGT in the -1 subsite clearly adopts the energetically favored 4C_1 conformation. The side-chains of residues R274, D303, H373, D437, E438, D532, W505, W546 and E584 of *VhGlcNAcase* (equivalent to that of R170, D199, H258, D321, E322, D397, W370, W410 and E443 in *Paenibacillus* sp. TS12 β -HEX1 and R162, D199, H250, D313, E314, D395, W361, W408 and E444 in *SpHex*) are also completely conserved among the three bacterial GlcNAcases studied (**Fig. 7B**).

Anti-bacterial activity of NGT against *Vibrio campbellii* (formerly *V. harveyi*) strain 650

We used disk diffusion assays to further test the bactericidal activity of NGT against *V. campbellii* strain 650 grown on agar plates containing Vibrio Complex Medium (VCM), supplemented with 1% (w/v) NaCl. After incubation the development of a clear zone around the disks soaked with different concentrations of NGT was observed, with the diameter of the zone increasing in a dose-dependent manner (**Fig. 8A**). On the other hand, NGT (at the same concentrations) had no visible effect on the growth of *E. coli* DH5 α , which lacks this specific GlcNAcase activity (**Fig. 8B**). Broth microdilution was carried out to determine the minimal inhibitory concentration (MIC) of NGT against the bacterium. The results show that the growth of *V. campbellii* strain 650 was inhibited by NGT with an MIC value of 0.5 μ M (0.11 μ g mL⁻¹) (**Fig. 8C**) while no inhibition of growth was seen when *E. coli* DH5 α cells were treated with the same concentration range of NGT (**Fig. 8D**). These results demonstrate the key role of *VhGlcNAcase* in the growth of *V. campbellii* strain 650.

DISCUSSION

Vibrio spp. are known to play a major role in the efficient utilization of chitin.⁴⁰ *Vibrio*-chitin interactions have also been proposed to be critical for the pathogenicity of the bacteria during infection of human hosts.⁴¹ *Vibrio* infections in farmed fish and prawn have caused vast economic losses for seafood industries worldwide, and outbreaks of *V. cholerae* have become a crucial global public health problem, especially in poorer countries.

VhGlcNAcase is a chitin recycling enzyme that helps to complete chitin degradation.¹⁷ The catalytic mechanism of *VhGlcNAcase* involves substrate-assisted catalysis⁴² and several compounds such as sodium azide⁴³ and the putative transition-state analogues PUGNAc, NHAcCAS, and NHAcDNJ have been previously shown to inhibit the activity of *VhGlcNAcase*²⁵, as well as that of other GH20 and GH84 GlcNAcases.^{28,33} NGT bound to *VhGlcNAcase* with a K_d of $32 \pm 1.2 \mu\text{M}$, as determined by ITC. This value is similar to the K_i of $62 \pm 3 \mu\text{M}$, determined by steady-state kinetics under the same buffer conditions. NGT also inhibited *SpHex* from *Streptomyces plicatus*³⁴ and β -HEX1 from *Paenibacillus* sp. TS12⁴⁴ at micromolar concentrations, suggesting that NGT may be a general inhibitor of GH-20 GlcNAcases.

The crystal structure of *VhGlcNAcase* shows the active site, with an open, narrow pocket that can accommodate a linear chitooligosaccharide chain containing 2-4 GlcNAc units.¹⁷ NGT was found to fully occupy the -1 subsite, which is located in the inner part of the catalytic pocket. Bound NGT was found to make strong H-bonds with several charged and polar residues, and also to form hydrophobic interactions with five aromatic residues (W487, W505, Y530, W546, W582), all of which are conserved among GH20 enzymes. The pyranose ring of NGT was observed in a full 4C_1 conformation, similar to the conformation found in the active site of other GH20 GlcNAcases.^{30,34}

In our activity assays, NGT was found to suppress the hydrolytic activity of *VhGlcNAcase* against both a synthetic substrate (4NP-GlcNAc) and a natural substrate (chitotetraose), consistent with this compound being an inhibitor of these enzymes. In growth assays against *V. campbellii*, dose-dependent inhibition of bacterial growth was observed, consistent with the bacterium requiring the enzyme for growth. Importantly we did not see any effect of NGT on the viability of *E. coli* DH5 α , with a similar result reported previously.⁴⁵ Our results suggest that NGT targets *VhGlcNAcase* in the

chitin degradation pathway of *Vibrio*, and thus serves as an effective potential antimicrobial agent against *Vibrio* infections.

In conclusion, we have demonstrated that NGT is a potent inhibitor of the GH20 β -N-acetylglucosaminidase, *VhGlcNAcase* from the *Vibrio* pathogen. NGT is an inhibitor that may possess suitable biocompatible properties allowing for its use in agricultural, as well as pharmaceutical, applications. Its high potency offers a considerable opportunity for it to be used as a scaffold for further development of highly effective anti-microbial agents against *Vibrio* infections of both aquatic animals and humans.

MATERIALS AND METHODS

Recombinant protein expression and purification of *VhGlcNAcase*

The *nag* gene encoding *VhGlcNAcase* (VIBHAR_01265 ORFs) was previously identified in the genome of the marine bacterium *V. campbellii* (formerly *V. harveyi*) ATCC BAA-1116.¹⁷ The gene was cloned into the pQE60 expression vector and expressed in *E. coli* M15 (pREP4) host cells with a C-terminal (His)₆-tag. The bacterial cells were cultured with shaking at 37 °C in Luria-Bertani (LB) broth containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin and 25 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin until the optical density at 600 nm reached 0.6, and recombinant protein was overexpressed overnight at 20°C by the addition of isopropyl thio- β -D-galactoside (IPTG) to a final concentration of 0.4 mM. Cells were harvested by centrifugation and the cell paste suspended in extraction buffer (20 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5% (v/v) glycerol, 1 $\text{mg}\cdot\text{mL}^{-1}$ lysozyme and 1 $\text{mg}\cdot\text{mL}^{-1}$ DNase I) at 25 °C for 30 min, after which the cells were lysed by ultrasonication. Cell debris was removed by centrifugation at 12000 x g at 4 °C for 1 h. The supernatant was immediately applied to a TALON[®] metal affinity resin (Clontech Laboratories, Inc. A Takara Bio Company, USA) run under gravity at 4 °C following the manufacturer's instructions (<http://www.clontech.com>). The column was washed with 20 column-volumes (CV) of 50 mM potassium phosphate buffer, pH 7.0, followed by 7 CV of 5 mM imidazole in potassium phosphate buffer, pH 7.0, then cobalt-bound proteins were eluted with 150 mM imidazole in potassium

phosphate buffer, pH 7.0. The purity of the protein was analyzed by 12% SDS-PAGE with Coomassie brilliant blue staining. Fractions of purified *Vh*GlcNAcase were then pooled and concentrated using Vivaspin-20 ultrafiltration membrane concentrators (M_r 10,000 cut-off, Vivascience AG, Hannover, Germany) and then further purified on a Sephadex 200 column (Amersham Bioscience, Piscataway, NJ, USA) connected to an ÄKTAprime Plus system with the applied flow rate 1.3 ml/min.

*Vh*GlcNAcase-containing fractions were pooled and concentrated in 50 mM potassium phosphate buffer, pH 7.0 using the same type of Vivaspin membrane concentrator. Protein concentration was determined by absorption at 280 nm using a molar extinction coefficient of $119470 \text{ M}^{-1}\text{cm}^{-1}$.⁴⁶ The freshly prepared protein was immediately frozen in liquid nitrogen and stored at -80°C until used.

Enzyme activity assay

The activity of GlcNAcase against 4NP-GlcNAc (Sigma-Aldrich (MO, USA) was determined with a colorimetric assay. Assays were carried out in a 96-well microtiter plate in triplicate, the reaction mixtures (200 μL) containing 0.1 μg of *Vh*GlcNAcase, 125 μM 4NP-GlcNAc and 0, 1, 10 and 100 μM of NGT, prepared in 100 mM phosphate buffer, pH 7.0. The plates were incubated at 37°C for 10 min with constant agitation in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany), and the enzymatic reaction was terminated by addition of 3 M Na_2CO_3 (100 μL). The amount of 4NP released was determined at 405 nm in a microtiter plate reader (Bio-Rad Laboratories Ltd., Hercules, CA, USA). The molar quantity of liberated 4NP was calculated from a calibration curve of 4NP standards varying from 0 to 20 nmol. The initial rate of the reaction was expressed as nmol of 4NP released per min per μg protein at 37°C . The activity of *Vh*GlcNAcase using the natural substrate $(\text{GlcNAc})_4$ was carried out in a 1.5 mL Eppendorf tube. The master mix (1.4 mL) contained 20 μg of *Vh*GlcNAcase, 2.5 mM chitotetraose and 250 μM of NGT, prepared in 100 mM Tris-HCl buffer, pH 7.5 containing 150 mM NaCl, and was incubated at 37°C with constant agitation in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany). 200 μL of the reaction mixture was then removed to new tubes after 5, 10, 30, and 60 min of incubation and flash frozen in liquid nitrogen. The degradation products released at each time point were analyzed by TLC following the method described by previously.^{47,48}

Determination of IC_{50} value

Dose-response experiments were carried out using 4NP-GlcNAc as substrate. The reaction mixture (200 μ L) contained 0.1 μ g *Vh*GlcNAcase, 125 μ M 4NP-GlcNAc and various concentrations of NGT in a ten-fold dilution series, to obtain a concentration range of 10^{-10} to 10^{-2} M in 100 mM phosphate buffer, pH 7.0. The reaction mixtures were treated as described previously. The IC_{50} value was obtained from a plot of the logarithm of NGT concentration vs. fractional activity of the enzyme using the three-parameter curve fit function in GraphPad Prism v. 5.01. (GraphPad Software, California, USA).

Determination of the binding constant of NGT using ITC

A MicroCal-PEAQ™ ITC system (Malvern Instruments Ltd.) was used for thermodynamic analysis of the interaction between NGT and WT *Vh*GlcNAcase. All samples were dissolved in buffer, passed through 0.22 μ m filters and degassed before the titrations. The binding of NGT to *Vh*GlcNAcase was carried out in triplicate at 37 °C with a stirring speed of 600 rpm. Aliquots of NGT solution (1.5 mM) were titrated into the 280- μ L sample cell containing 25 μ M purified *Vh*GlcNAcase in a buffer containing 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl. NGT injections (1.25 μ L) were repeated 29 times with 120 s intervals between additions. The control titration was of the corresponding ligand into the sample cell containing only the buffer. Measurement data were collected and analyzed by MicroCal-PEAQ ITC software, and the best fit generated according to a single-site binding model using a nonlinear least-squares method was then subtracted from the control profile. The binding stoichiometry (n), equilibrium binding association constant (K_a) and enthalpy change (ΔH°) were obtained from the fitted curve. The values of Gibbs free energy change (ΔG°) and the entropy change (ΔS°) were calculated from the equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = -RT \ln K$, where R is the gas constant (1.98 cal·K⁻¹·mol⁻¹) and T is the absolute temperature in Kelvin. Values presented are means $\pm$ S.D. obtained from experiments carried out in triplicate.

Determination of K_i value from steady-state kinetics

The reaction mixture (200 μ L) contained 1 μ g of freshly purified *Vh*GlcNAcase and various concentrations of 4NP -GlcNAc (0, 125, 250, 500, 1000, and 2000 μ M) in 100 mM Tris-HCl buffer pH 7.5 and 150 mM NaCl. Six different concentrations of NGT: 0, 2.5, 5, 10, 20, and 40 μ M, were tested at each concentration of substrate. The reaction was incubated at 37°C for 15 min in a 96-well plate with shaking at 300 rpm in a Thermomixer®C (Eppendorf (Thailand) Co. Ltd. Bangkok, Thailand). The reaction was terminated by adding 100 μ L of 1 M Na_2CO_3 to the reaction mixture. The amount of the 4NP released was determined as described above in Section 2. The reaction rate is expressed the product (4NP) formation per min per 1 μ g *Vh*GlcNAcase.

The mathematical expression for typical competitive inhibition is given by Eq. 1,⁴⁹

$$v_o = \frac{V_{max}}{K_m(1 + \frac{[I]}{K_i})} \cdot [S] \quad \text{Eq. 1}$$

where v_o is the initial rate, V_{max} is the maximum rate, K_m is the Michaelis constant, $[I]$ is NGT concentration, $[S]$ is initial substrate concentration (4NP-GlcNAc). Initial rates were fitted to this equation by non-linear regression, available in Graphpad Prism v. 5.0

Co-crystallization and structure determination

WT *Vh*GlcNAcase was freshly purified by size exclusion chromatography (SEC) as described above and was used for crystal screening. The enzyme (12 mg·mL⁻¹) in 10 mM HEPES buffer pH 7.5 containing 150 mM NaCl was co-crystallized with 2 mM NGT using the PACT *premier*TM-Screen from Molecular Dimensions at 20°C by sitting-drop vapor diffusion with a mosquito robot (TTP Labtech). Small plate-shaped crystals of *Vh*GlcNAcase were observed within 3 days under conditions

F1 [0.2 M sodium fluoride, 0.1 M Bis-Tris propane pH 6.5, 20% PEG3350] from PACT *premier*TM. Single crystals were harvested and directly flash-frozen in liquid nitrogen. A data set was collected at I24 at the Diamond Light Source (DLS), UK. Data were integrated with XDS⁵⁰ and scaled with Aimless⁵¹ in PHENIX.⁵² Initial phasing and modeling were performed using AUTOSOL within PHENIX. Model building was further performed using the program COOT.⁵³ Phases for *VhGlcNAcase*-NGT structure were obtained by molecular replacement (MR) with PHASER⁵⁴ using the crystal structure of the β -hexosaminidase from *Arthrobacter aureescens* (PDB id 3RCN; 35% identity to *VhGlcNAcase*; Midwest Center for Structural Genomics) as a search model. Five percent of the data were flagged for R_{free} and initial models were built using Auto Build⁵⁵ as part of PHENIX^{Error! Bookmark not defined.}, manually corrected with COOT⁵ and refined with phenix.refine in PHENIX⁵⁶. The final model was validated with MolProbity⁵⁷ and the graphical structures were generated and displayed by PyMOL (Schrödinger, LLC).

Agar disc diffusion assay

Agar disk diffusion assay was carried following the standard method recommended by Jorgensen and Turnidge.⁵⁸ *V. campbellii* strain 650 was grown using Vibrio Complex Medium (VCM) agar, containing 1%(w/v) NaCl with a chitin supplement. The overnight culture was diluted with 5 ml of 0.9% saline solution and incubated at 37°C at 180 rpm until reaching an OD_{600} of 0.5 (equivalent to cell density of $1.0 - 1.5 \times 10^8$ CFU·mL⁻¹ by MacFarland standard). A sterile cotton swab was dipped into the bacterial suspension and spread onto the VCM agar plate twice, and the plate was allowed to dry for a few minutes. Afterwards, sterile Whatman® Schleicher & Schuell® antibiotic-assay discs (Ø 6 mm, Merck Limited, Bangkok, Thailand) soaked with 5 µL of the NGT solution of a final concentration of 0.125, 0.25, 0.5 and 1 µM were placed on the agar plate (see Fig.8 on the main text). After the plate was incubated for 16h at 37 °C, the clear zone, indicating inhibition of bacterial growth, was measured. For *E. coli* DH5 α , the assay was carried out as described for *V. campbellii*, but Mueller-Hinton (MH) agar was used instead.

Anti-microbial susceptibility assay

Minimum inhibitory concentration (MIC) values were determined from 3 trials using the VCM medium for *V. campbellii* and MH medium for *E. coli* type strain DH5 α , supplemented with 1% (w/v) NaCl. Two-fold dilution experiments were set up, following the Clinical and Laboratory Standards Institute (CLSI) guidelines.⁵⁹ *E. coli* DH5 α or *V. campbellii* cells were spread onto an agar plate, and then incubated at 37°C for 16 h. Single colonies of the bacteria were grown in 10 mL of the specified medium and incubated at 37°C for an additional 16 h. To assess the effect of the inhibitor (NGT), 10 μ L aliquots of the cell culture (diluted to OD₆₀₀ = 0.1 or equivalent to 10⁴-10⁵ CFU mL⁻¹) were transferred into a 96-well plate, with each well containing 90 μ L of the MH medium, containing two-fold dilutions of NGT (0 - 1024 μ M). After incubation at 37°C for 24 h, MIC values were evaluated by measuring OD₆₀₀ of the bacterial culture with and without NGT.

TABLES

Table 1 Thermodynamic parameters obtained from ITC.

The ITC experiments were carried out in buffer containing 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl, at 37 °C. NGT (1.5 mM) was titrated into the freshly purified *Vh*GlcNAcase (25 μ M) as described in **Methods**. Values presented are mean $\pm$ S.D, averaged from three separate measurements.

Inhibitor (condition)	Isothermal microcalorimetric (ITC) parameters				
	K_d	$\Delta G^\circ_{\text{binding}}$	ΔH°	$-T\Delta S^\circ$	n
	(μ M)	(kcal.mol ⁻¹)	(kcal.mol ⁻¹)	(kcal.mol ⁻¹)	
NGT (37 °C)	32 $\pm$ 1.2	-6.4 $\pm$ 0.02	-18.4 $\pm$ 3.5	+11.9 $\pm$ 3.5	0.9 $\pm$ 0.1

Table 2. Data collection and refinement statistics.

WT-NGT (6K35)	
Data collection	
Wavelength (Å)	0.96862
Space group	$P2_1$
Unit-cell parameter	
a, b, c (Å)	62.3, 145.0, 88.7
α, β, γ (°)	90.0, 109.7, 90.0
Resolution range (Å)	29.35 - 2.36 (2.44 - 2.36)
R_{merge}	0.118 (0.695)
$I/\sigma(I)$	6.09 (1.27)
CC $\frac{1}{2}$	0.978 (0.500)
Completeness (%)	99.6 (99.9)
Redundancy	1.9 (1.9)
Refinement	
Resolution (Å)	2.36
No. of total reflections	117136 (11716)

No. of unique reflections	60693 (6032)
R_{work}	0.194 (0.287)
R_{free}	0.265 (0.379)
Number of atoms	
Protein	10292
NGT	28
Water	447
RMSD	
Bond lengths (Å)	0.013
Bond angles (°)	1.58
Average B -factors (Å ²)	
Protein	40.98
NGT	32.40
Water	37.21
Ramachandran plot	
Favoured regions (%)	96.00
Allowed Region (%)	3.61
Outlier regions (%)	0.39
Rotamer outliers (%)	0.00
Clash score	8.77

FIGURE LEGENDS

Figure 1. (A) Comparison of the chemical structures of the oxazolinium reaction intermediate and NGT. (B) Relative activity of *Vh*GlcNAcase using 4NP-GlcNAc as a substrate in the absence and presence of NGT (C) Dose-response analysis, showing the fractional activity of *Vh*GlcNAcase as a function of various concentrations of NGT. Values presented in (B) and (C) are means $\pm$ SD obtained from experiments carried out in triplicate.

Figure 2 Time-dependent effect of NGT on chitotetraose hydrolysis by *VhGlcNAcase*. Each reaction mix (200 μ L) included 20 μ g *VhGlcNAcase*, 2.5 mM chitotetraose with 250 μ M NGT (A) and without NGT (B) in 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl. Reactions were terminated by snap freezing in liquid nitrogen, and products analyzed by TLC. Lanes: Std, standard chitooligosaccharide mix; 1, reaction mix contained substrate without enzyme (set at 0 min); 2, 5 min; 3, 10 min; 4, 30 min; 5, 60 min of incubation. Figures (A) and (B) are representative TLC traces that were consistently obtained from the experiments carried out twice.

Figure 3. ITC analysis of NGT binding to *VhGlcNAcase* at 37°C. (A) Titration of NGT into *VhGlcNAcase* (lower thermogram) and titration of NGT into 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl (upper thermogram). (B) A representative integrated curve fit of the thermogram shown in panel A. (C) Thermodynamic parameters of NGT binding plotted as bar graphs with the error bars representing the standard deviations obtained from three independent experiments.

Figure 4. Determination of the K_i of NGT against *VhGlcNAcase* using 4NP-GlcNAc as substrate. The reaction mixture (200 μ L), contained 15 μ g *VhGlcNAcase* and 4NP-GlcNAc (0-2000 μ M) with increasing NGT concentrations in 100 mM Tris-HCl, pH 7.5 and 150 mM NaCl buffer. The release of 4NP was determined by measuring the absorption at 405 nm (A_{405}). (A) Michaelis plot of v_o vs. (S) at different concentrations of NGT. (B) Competitive inhibition of the enzyme by NGT was identified using a Lineweaver-Burk (LB) plot. Data presented in non-linear plots (A) and linear plots (B) are means $\pm$ SD that were obtained from experiments carried out in triplicate.

Figure 5. (A) Ribbon representation of three distinct domains of *VhGlcNAcase*, consisting of the CBD domain shown in purple, the linker in grey, the $\alpha+\beta$ domain in blue and the Cat domain in green. Amino acids represented by sticks are identified as the GlcNAc-binding residues (B) Surface representation of the substrate-binding pocket of *VhGlcNAcase* modelled with diNAG (space-filling, yellow, ligand id: CBS) from *Serratia marcescens* chitobiase (*SmChb*) (PDB id 1QBB), and (C) *VhGlcNAcase* in complex with NAG-thiazoline (space-filling, bright green) (PDB id: 6K35). The structures were edited by and displayed in PyMOL (Educational version).

Figure 6. Specific interactions between NGT and *VhGlcNAcase*. (A) The amino acid sidechains (green sticks) and a water molecule form the H-bond network with NGT (green ball-and-sticks) in the -1 subsite (colored by atom, blue for N and red for O). NGT in the *VhGlcNAcase* active site (cyan) is contoured at 1σ . A water molecule is shown as a red sphere and hydrogen bonds are shown as red broken lines. (B) Surface representations of the aromatic residues involved in hydrophobic interactions. The diNAG molecule (yellow) bound to *SmChb* was modelled into the active site of *VhGlcNAcase* to locate the position of the NGT molecule in the -1 subsite. The structures were edited by and displayed in PyMOL (Educational version).

Figure 7. Superimposition of bacterial GH20 GlcNAcases including *VhGlcNAcase* (SwissProt: D9ISE0), *Paenibacillus sp.* TS12 β -HEX1 (SwissProt: D2KW09) and *SpHex* (SwissProt: O85361). (A) Wire representation of the C_α trace of the TIM-barrel catalytic domains of *VhGlcNAcase* (green) complexed with NGT (green ball-and-stick) superimposed with *Paenibacillus sp.* TS12 β -HEX1 (pink) complexed with NGT (pink ball-and-stick) and *SpHex* (cyan) complexed with NGT (cyan ball-and-stick). In the circle, a composite simulated annealing $2F_o - F_c$ map contoured at 1.0 sigma (carve = 1.6) of the *VhGlcNAcase* active site after removal of the thiazoline inhibitor (green stick model with oxygens red, nitrogens blue and sulphur yellow). Selected residues interacting with the inhibitor are shown as stick models in green (oxygens red, nitrogens blue). For map generation, 5% of the structure was omitted at a time, and a simulated annealing temperature of 500 K was used. (B) A close-up from (A) of the active site residues. The active site residues of three bacterial GlcNAcases are completely conserved, of which R274, D303, H373, D437, E438, D532, W546 and E584 (in *VhGlcNAcase*) are the key residues with H-bond interaction. The structures were edited by and displayed in PyMOL (Educational version).

Figure 8. Agar disk diffusion and MIC assays. For disc assays (A) *V. campbellii* strain 650 and (B) *E. coli* DH5 α , standardized cultures were spread onto agar plates. Sterile disk papers ($\varnothing$, 6 mm) soaked with 0.125, 0.25, 0.5 and 1 μ M of NGT were placed on plates and incubated for 16 h at 37 °C and

zones of clearance indicating growth inhibition were assessed. Plot of bacterial growth (OD_{600}) of *V. campbellii* strain 650 (C) and *E. coli* DH5 α (D) vs. NGT at various concentrations. The MIC values are presented as mean values $\pm$ SD from two separate experiments, where each experiment the data were collected in triplicate.

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AUTHOR CONTRIBUTIONS

WS initiated the idea of the project and supervised functional characterization. PM performed functional characterization and took part in structural refinements. AA carried out the disk diffusion assays and microbial susceptibility tests and performed part of structural analysis. AK and AD performed *Vh*GlcNAcase expression, purification for kinetic experiments of NGT inhibition and carried out GlcNAcase activity assays with chitotetraose and the TLC analysis. KAS provided the inhibitor. WS expressed, purified, and co-crystallized *Vh*GlcNAcase with NGT. DMB helped with protein expression, purification, and crystallization. He also took part in model building and structural refinements. BVDB supervised protein crystallization and carried out X-ray data collection. WS, PM, KAS and BVDB co-wrote, and revised the manuscript.

CONFLICTS OF INTEREST

All authors declare no conflict of interest.

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