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Systematic synthesis and inhibitory activity of haloacetamidyl oligosaccharide derivatives toward cytoplasmic peptide:*N*-glycanase

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Abstract A series of glycosyl haloacetamides were synthesized as potential inhibitors of cytoplasmic peptide:*N*-glycanase (PNGase), an enzyme that removes *N*-glycans from misfolded glycoproteins. Chloro-, bromo-, and iodoacetamidyl chitobiose and chitotetraose derivatives exhibited a significant inhibitory activity. No inhibitory activity was observed with of fluoroacetamididyl derivatives. Moreover, *N*-acetylglucosamine derivatives, β-chloropropionamidyl chitobiose, and chloroacetamidyl cellooligosaccharide derivatives did not show any activity. These results underscore the importance of the *N*-acetyl groups of chitobiose for PNGase recognition. In addition, reactivity and position of the leaving group at the reducing end are also important factors.

Keywords Peptide:*N*-glycanase · Haloacetamidyl oligosaccharide · Inhibitor

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Introduction

Protein quality control is a ubiquitous process in eukaryotes, which is essential for the maintenance of cellular activity. It ensures that only correctly folded proteins are produced. Misfolded proteins are retrotranslocated into cytosol and eventually degraded by proteasomes, a pathway called the ER associated protein degradation (ERAD) [1]. In this pathway, cytoplasmic peptide:*N*-glycanase (PNGase) plays an important role, catalyzing the de-glycosylation of misfolded glycoproteins by cleaving the amide linkage between asparagine and *N*-linked oligosaccharide [2, 3]. Our recent research revealed that *N*-linked glycopeptides are degraded by 20S proteasome, but with drastically reduced rate compared to nonglycosylated peptides [4]. This result strongly suggests that PNGase activity facilitates the cytosolic degradation of glycoproteins. PNGase belongs to the transglutaminase superfamily with a conserved Cys, His, Asp, catalytic triad. Earlier studies revealed that PNGase binds to free oligosaccharides, derived from natural glycoprotein substrates [5, 6]. In order to investigate

the biological function of PNGase precisely, development of potent and specific inhibitors is desired. Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) is a known inhibitor of PNGase discovered by library screening [7]. The structure of PNGase-Rad23 complexes was solved previously [8] and the binding site for the peptide part of the substrate was identified using Z-VAD-fmk, its binds to the Cys residue of the catalytic triad. However, its activity and specificity is not optimal, due to its intrinsic property as a caspase inhibitor [9]. Recently, we found that iodoacetamide carrying high-mannose-type oligosaccharides ($Man_9GlcNAc_2$-IAc) and chitobiose ($GlcNAc_2$-IAc) covalently modify the catalytic Cys residue of PNGase (Fig. 1), strongly inhibiting its activity [10]. In addition, a hydrophobic fluorophore conjugated chitobiose derivative was efficiently introduced into cells [11]. Lately, Witte *et al*. reported an epoxysuccinate PNGase inhibitor [12]. Molecular modeling of the binding of chitobiose and PNGase indicated that *N*-acetyl groups in chitobiose interact with the binding site of PNGase [10]. In order to get insight into the information on recognition of the oligosaccharide structure by PNGase, we conducted a more systematic synthesis and comparison of the activities of a series of haloacetamidyl (F, Cl, Br, and I) chitooligosaccharides. Furthermore, cellooligosaccharide derivatives were synthesized to confirm the necessity of *N*-acetyl group.

Materials and methods

General methods

Starting materials and reagents were purchased from standard vendors and used without purification unless otherwise noted. Analytical thin layer chromatography (0.25 mm) was developed on silica gel 60 F plates (Merck, Darmstadt, Germany). ^{1}H NMR spectra were recorded at 400 MHz in D_2O with a JEOL AL-400 spectrometer. MALDI-TOF MS spectra were recorded in positive ion mode on an AXIMA-CFR Kompact MALDI (Shimazu/

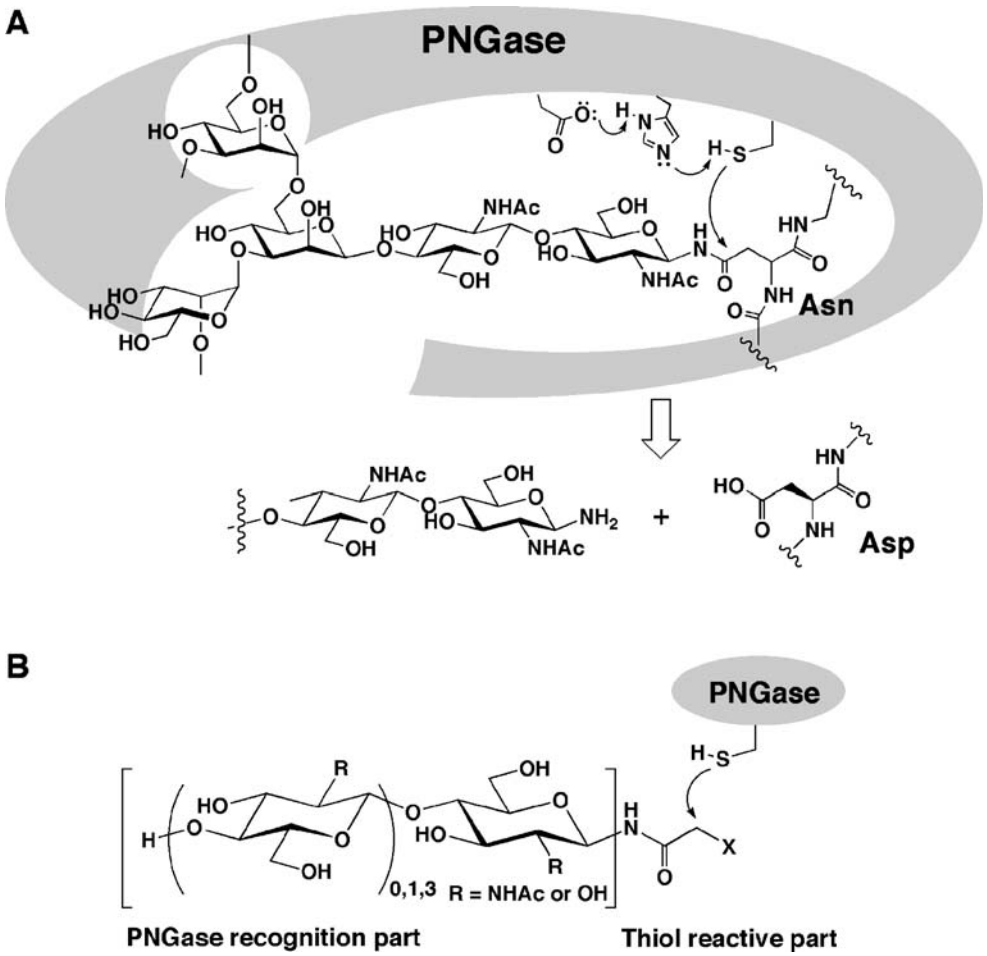


Fig. 1 Proposed mechanism of deglycosylating enzyme PNGase and design of its inhibitor. **a** Deglycosylation mechanism of Png1. **b** Haloacetamidyl sugar covalently modifies the catalytic PNGase

KRATOS). HPLC was performed by a Waters 2690 (separation module), Waters 996 (photodiode array detector).

Synthesis of haloacetamidyl oligosaccharides

*Iodoacetamidyl N-acetylglucosamine (**1**)*

N-Acetylglucosamine (52 mg, 0.24 mmol) was dissolved in saturated aqueous NH_4HCO_3 (5 mL) and stirred at 40°C for 48 h. Subsequently, the mixture was concentrated and co-evaporated with H_2O. The residue was dissolved in dioxane/H_2O (1:1) (2 mL) and treated with $NaHCO_3$ (126 mg, 1.50 mmol) and iodoacetic anhydrate (57 mg, 0.16 mmol) at 0°C. The mixture was stirred at 0°C, then iodoacetic anhydrate (415 mg, 1.17 mmol) was added. Consequently, the mixture was concentrated in vacuo. The residue was purified by reverse phase chromatography (Waters Sep-Pak C18, washed with water, then eluted with H_2O/MeOH (40/1) followed by gel filtration (Amersham Bioscience: Superdex 30 (20 mm × 600 mm), H_2O) to give compound **1** (34 mg, 38%, in 2 steps); ^{1}H NMR (400 MHz, D_2O): δ 4.9(d, 1H, *J*=9.76 Hz), 3.75–3.32 (m, 9H), 1.90 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.18, 173.60, 79.33, 78.50, 74.86, 70.16, 61.21, 55.15, 23.28; MALDI-TOF MS: calcd for $C_{10}H_{17}IN_2O_6$ $[M+Na]^+$ 411.00, found 410.95.

*Iodoacetamidyl di-N-acetylchitobiose (**2**)*

Glycosylamine derived from 50 mg (0.12 mmol) of chitobiose was treated with iodoacetic anhydrate (72 mg, 0.21 mmol) and $NaHCO_3$ (59 mg, 0.70 mmol) as described for **1** to give 42 mg of **2** (58%).

^{1}H NMR (400 MHz, D_2O): δ 4.90 (d, 1H, *J*=9.76 Hz), 4.44 (d, 1H, *J*=8.29 Hz), 3.79–3.25 (m, 15H), 1.94 (s, 3H), 1.86 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.21, 175.13, 173.63, 102.00, 79.36, 79.15, 77.00, 76.56, 74.08, 73.39, 70.35, 61.20, 60.53, 56.25, 54.45, 23.11, 22.83; MALDI-TOF MS: calcd for $C_{18}H_{30}IN_3O_{11}$ $[M+Na]^+$ 614.08, found 614.18.

*Iodoacetamidyl tetra-N-acetylchitotetraose (**3**)*

Glycosylamine derived from 50 mg (0.06 mmol) of chitotetraose was treated with iodoacetic anhydrate (99 mg, 0.28 mmol) and $NaHCO_3$ (59 mg, 0.70 mmol) as described for **1** to give 36 mg of **3** (67%); ^{1}H NMR (400 MHz, D_2O): δ 4.89 (d, 1H, *J*=9.28 Hz), 4.43–4.41 (m, 3H), 3.77–3.28 (m, 26H), 1.9–1.88 (m, 12H); ^{13}C NMR (100 MHz, D_2O): δ 175.15, 175.07, 173.62, 102.12, 101.91, 101.82, 79.82, 79.61, 79.19, 77.12, 76.62, 75.23, 74.16, 73.41, 72.78, 70.42, 61.28, 60.71, 56.35, 55.79, 54.58, 23.25, 22.97; MALDI-TOF MS: calcd for $C_{34}H_{56}IN_5O_{21}$ $[M+Na]^+$ 1020.24, found 1020.49.

*Bromoacetamidyl N-acetylglucosamine (**4**)*

Glycosylamine derived from 51 mg (0.23 mmol) of *N*-acetylglucosamine was treated with bromoacetic anhydrate (265 mg, 1.02 mmol) and $NaHCO_3$ (136 mg, 1.62 mmol) as described for **1** to give 50 mg of **4** (65%); ^{1}H NMR (400 MHz, D_2O): δ 4.95 (d, 1H, *J*=9.76 Hz), 3.81–3.70 (m, 4H), 3.63 (dd, 1H, *J*=12.44, 4.64 Hz), 3.50 (t, 1H, *J*=8.54Hz), 1.89 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.24, 171.23, 79.49, 78.49, 74.72, 70.17, 61.20, 55.10, 28.26, 22.93; MALDI-TOF MS: calcd for $C_{10}H_{17}BrN_2O_6$ $[M+Na]^+$ 363.02, found 362.93.

*Bromoacetamidyl di-N-acetylchitobiose (**5**)*

Glycosylamine derived from 50 mg (0.12 mmol) of chitobiose was treated with bromoacetic anhydrate (140 mg, 0.54 mmol) and $NaHCO_3$ (66 mg, 0.79 mmol) as described for **1** to give 51 mg of **5** (75%); ^{1}H NMR (400 MHz, D_2O): δ 4.93 (d, 1H, *J*=9.76 Hz), 4.46 (d, 1H, *J*=8.54 Hz), 3.79–3.33 (m, 15H), 1.92 (s, 3H); 1.86 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.73, 175.55, 171.70, 102.45, 79.90, 79.78, 77.46, 77.00, 74.53, 73.72, 70.81, 61.66, 61.00, 56.71, 54.88, 28.62, 23.30, 23.25; MALDI-TOF MS calcd for $C_{18}H_{30}BrN_3O_{11}$ $[M+Na]^+$ 566.10, found 566.19.

*Bromoacetamidyl tetra-N-acetylchitotetraose (**6**)*

Glycosylamine derived from 50 mg (0.06 mmol) of chitotetraose was treated with bromoacetic anhydrate (66 mg, 0.25 mmol) and $NaHCO_3$ (34 mg, 0.40 mmol) as described for **1** to give 35 mg of **6** (67%); ^{1}H NMR (400 MHz, D_2O): δ 4.95 (d, 1H, *J*=9.76 Hz), 4.48–4.44 (m, 3H), 3.83–3.32 (m, 30H) 1.94 (d, 10H), 1.88 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 174.79, 174.63, 170.82, 101.48, 101.27, 101.19, 100.00, 79.11, 78.91, 78.70, 78.52, 76.43, 75.92, 74.52, 73.44, 72.58, 72.12, 72.06, 69.69, 60.53, 59.94, 59.85, 55.58, 55.02, 53.81, 27.40, 22.11, 22.07; MALDI-TOF MS calcd for $C_{34}H_{56}BrN_5O_{21}$ $[M+Na]^+$ 972.25, found 972.12.

*Chloroacetamidyl N-acetylglucosamine (**7**)*

Glycosylamine derived from 134 mg (0.61 mmol) of *N*-acetylglucosamine was treated with chloroacetyl chloride (242 μL, 3.04 mmol) and $NaHCO_3$ (303 mg, 3.61 mmol) as described for **1** to give 113 mg of **7** (62%); ^{1}H NMR (400 MHz, D_2O): δ 4.95 (d, 1H, *J*=9.77 Hz), 4.03–3.95 (m, 2H), 3.76–3.67 (m, 2H), 3.61 (dd, 1H, *J*=12.45, 4.88 Hz), 3.49 (t, 1H, *J*=8.06Hz), 3.42–3.31 (m, 2H), 1.86 (s, 1H); ^{13}C NMR (100 MHz, D_2O): δ 175.30, 170.88,

79.58, 78.44, 74.63, 70.20, 61.22, 55.01, 42.86, 22.80; MALDI-TOF MS: calcd for $C_{10}H_{17}ClN_2O_6$ $[M+Na]^+$ 319.07, found 319.41.

*Chloroacetamidyl di-N-acetylchitobiose (**8**)*

Glycosylamine derived from 50 mg (0.12 mmol) of chitobiose was treated with chloroacetyl chloride (94 μL, 1.23 mmol) and $NaHCO_3$ (112 mg, 1.33 mmol) as described for **1** to give 34 mg of **8** (58%); ^{1}H NMR (400 MHz, D_2O): δ 4.96 (d, 1H, *J*=9.76 Hz), 4.47 (d, 1H, *J*=8.54 Hz), 4.04–3.81 (m, 13H), 1.94 (s, 3H), 1.87 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.29, 175.02, 170.90, 102.04, 79.49, 77.03, 76.61, 74.14, 73.25, 70.41, 61.27, 60.62, 56.33, 54.44, 42.83, 22.97, 22.92, 22.88, 22.79, 22.75; MALDI-TOF MS: calcd for $C_{18}H_{30}ClN_3O_{11}$ $[M+Na]^+$ 522.15, found 522.22.

*Chloroacetamidyl tetra-N-acetylchitotetraose (**9**)*

Glycosylamine derived from 50 mg (0.06 mmol) of chitotetraose was treated with chloroacetyl chloride (20 μL, 0.25 mmol) and $NaHCO_3$ (33 mg, 0.39 mmol) as described for **1** to give 28 mg of **3** (52%); ^{1}H NMR (400 MHz, D_2O): δ 4.96 (d, 1H, *J*=9.76 Hz), 4.47–4.45 (m, 3H), 4.00 (d, 2H, *J*=2.9 Hz), 3.78–3.34 (m, 28H), 1.94 (d, 9H), 1.88 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 174.89, 174.63, 170.49, 101.48, 101.27, 101.20, 79.11, 78.90, 78.79, 78.56, 76.39, 75.91, 74.55, 74.52, 73.44, 72.50, 72.12, 72.06, 69.69, 60.52, 59.93, 59.85, 55.58, 55.02, 53.73, 42.04, 22.11, 21.93; MALDI-TOF MS: calcd for $C_{34}H_{56}ClN_5O_{21}$ $[M+Na]^+$ 928.31, found 928.64.

*Fluoroacetamidyl N-acetylglucosamine (**10**)*

Method A: Glycosylamine derived from 26 mg (0.12 mmol) of *N*-acetylglucosamine was treated with sodium fluoroacetate (44 mg, 0.44 mmol) and DMT-MM (101 mg, 0.36 mmol) in MeOH at r.t. for 21 h. Consequently, the mixture was concentrated in vacuo. The residue was purified by HPLC (Intakt Unison US-C18) eluted with 1% CH_3CN to give compound **10** (20 mg, 58%).

^{1}H NMR (400 MHz, D_2O): δ 5.0 (d, 1H, *J*=9.76H), 4.86–4.78 (m, 1H), 4.74–4.70 (m, 1H), 3.77–3.72 (m, 2H), 3.62 (dd, 1H, *J*=12.44, 4.88 Hz), 3.5 (t, 1H, *J*=8.59Hz), 3.43–3.33 (m, 2H), 1.86 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.54, 172.19, 79.42(d, *J*=180.52 Hz), 78.00, 77.53, 73.78, 69.31, 60.31, 54.07, 22.70; MALDI-TOF MS: calcd for $C_{10}H_{17}FN_2O_6$ $[M+Na]^+$ 303.10, found 303.38.

Method B: Iodoacetamidyl *N*-acetylglucosamine (**1**) (20 mg, 0.05 mmol) was treated with CsF (190 mg, 1.25 mmol) in dimethylacetamide (DMA) under Ar at 100°C. Subsequently, the mixture was concentrated in vacuo. The residue was purified by HPLC (Intakt Unison US-C18) eluted with 1% CH_3CN to give compound **10** (6 mg, 40%).

*Fluoroacetamidyl di-N-acetylchitobiose (**11**)*

Glycosylamine derived from 29 mg (0.07 mmol) of chitobiose was treated with sodium fluoroacetic acid (22 mg, 0.22 mmol) and DMT-MM (68 mg, 0.25 mmol) as described for **10** to give 23 mg of **11** (71%); ^{1}H NMR (400 MHz, D_2O): δ 4.99 (d, 1H, *J*=9.76 Hz), 4.85–4.8 (m, 1H), 4.78–4.69 (m, 1H), 4.45 (d, 1H, *J*=8.54 Hz), 3.8–3.3 (m, 12H), 1.92 (s, 3H), 1.85 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.56, 175.24, 172.26, 172.06, 102.01, 80.21(d, *J*=181.91 Hz),79.37, 78.72, 76.93, 76.51, 74.03, 73.19, 70.29, 61.12, 60.49, 56.18, 54.27, 22.69, 22.50; MALDI-TOF MS: calcd for $C_{18}H_{30}FN_3O_{11}$ $[M+Na]^+$ 506.18, found 506.08.

Method B: Iodoacetamidyl di-*N*-acetylchitobiose (**2**) (25 mg, 0.04 mmol) was treated with CsF (190 mg, 1.25 mmol) in DMA under Ar at 100°C as described for **10** to give **11** (12 mg, 50%).

*Fluoroacetamidyl tetra-N-acetylchitotetraose (**12**)*

Glycosylamine derived from 27 mg (0.03 mmol) of chitotetraose was treated with sodium fluoroacetic acid (52 mg, 0.52 mmol) and DMT-MM (60 mg, 0.22 mmol) as described for **10** to give 2 mg of **12** (7%); ^{1}H NMR (400 MHz, D_2O): δ 5.0 (d, 1H, *J*=9.51 Hz), 4.82–4.67 (m, 3H), 4.46–4.43 (m, 3H), 3.81–3.32 (m, 28H), 1.93–1.86 (m, 13H); ^{13}C NMR (100 MHz, D_2O): δ 175.37, 175.04, 172.09, 171.90, 102.10, 101.90, 101.84, 80.31 (d, *J*=179.92 Hz), 79.82, 79.61, 79.26, 78.83, 77.06, 76.62, 75.26, 75.22, 74.14, 73.24, 72.83, 72.77, 70.41, 61.28, 60.69, 60.61, 56.33, 55.81, 55.77, 54.48, 22.97, 22.77; MALDI-TOF MS: calcd for $C_{34}H_{56}FN_5O_{21}$ $[M+Na]^+$ 912.33, found 912.86.

Method B: Iodoacetamidyl tetra-*N*-acetylchitotetraose (**3**) (25 mg, 0.03 mmol) was treated with CsF (77 mg, 0.51 mmol) in DMA under Ar at 100°C as described for **10** to give **12** (11 mg, 33%).

*3-Chloropropionamidyl di-N-acetylchitobiose (**13**)*

Glycosylamine derived from 25 mg (0.06 mmol) of chitobiose was treated with 3-chrolopropionyl chloride (29 μL, 0.29 mmol) and $NaHCO_3$ (36 mg, 0.43 mmol) as described for **1** to give 20 mg of **13** (67%); ^{1}H NMR (400 MHz, D_2O): δ 4.95 (d, 1H, *J*=9.51 Hz), 4.44 (m, 1H), 3.78–3.29 (m, 14H), 2.60–2.56 (m, 2H), 1.91 (s,3H), 1.84 (s, 3H); ^{13}C NMR (100 MHz, D_2O): δ 175.20, 175.04, 174.28, 102.03, 79.45, 78.89, 77.00, 76.63, 74.15, 73.47,

70.42, 61.30, 60.64, 56.35, 54.57, 40.89, 39.43, 22.98; MALDI-TOF MS: calcd for $C_{19}H_{32}ClN_3O_{11}$ $[M+Na]^+$ 536.16, found 536.17.

*Chloroacetamidyl cellobiose (***14***)*

Glycosylamine derived from cellobiose (56 mg, 0.16 mmol) was treated with chloroacetyl chloride (26 μL, 0.33 mmol) and $NaHCO_3$ (87 mg, 1.04 mmol) as described for **1** to give 27 mg of **14** (38%); ^1H NMR (400 MHz, D_2O): δ 5.20 (d, 1H, *J*=9.6 Hz), 4.68 (d, 1H, *J*=8.4 Hz), 4.38 (s, 2H), 4.11–3.85 (m, 7H), 3.69–3.46 (m, 5H); ^{13}C NMR (100 MHz, D_2O): δ 171.06, 102.85, 79.84, 78.34, 76.93, 76.42, 75.88, 75.33, 73.57, 71.89, 69.88, 61.04, 60.21; MALDI-TOF MS: calcd for $C_{14}H_{24}ClN_1O_{11}$ $[M+Na]^+$ 440.09, found 440.10.

*Chloroacetamidyl cellotetraose (***15***)*

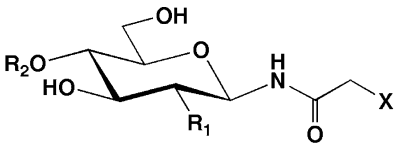
Glycosylamine derived from cellotetraose (50 mg, 0.07 mmol) was treated with chloroacetyl chloride (19 μL, 0.22 mmol) and $NaHCO_3$ (39 mg, 0.46 mmol) as described for **1** to give 28 mg of **14** (57%); ^1H NMR (400 MHz, D_2O): δ 5.03 (d, 1H, *J*=9.2 Hz), 4.49–4.55 (m, 3H), 4.21 (s, 2H), 3.99–3.89 (m, 4H), 3.84–3.81 (m, 3H), 3.75–3.62 (m, 10H), 3.43–3.29 (m, 4H); ^{13}C NMR (100 MHz, D_2O): δ 171.04, 102.89, 102.67, 102.63, 79.84, 78.77, 78.67, 78.22, 76.93, 76.38, 75.87, 75.24, 74.44, 74.39, 73.55, 73.33, 71.88, 69.86, 61.01, 60.31, 60.16; MALDI-TOF MS: calcd for $C_{26}H_{44}ClN_1O_{21}$ $[M+Na]^+$ 764.20, found 764.35.

Preparation of the extract of E. coli expressing yeast Png1

A 50 mL overnight culture of *E. coli* bearing pET28b-*PNG1-(His)$_6$* was cultured with LB media containing kanamycin (50 μg/mL) and chloramphenicol (30 μg/mL). It was transferred to fresh 450 mL LB media containing the same antibiotics, and further cultured at 37°C for 30 min. Subsequently, 5 mL of 0.1 M isopropylthiogalactoside was added to induce protein expression and cells were cultured for 3 h. After cells were collected by centrifugation, the pellet was resuspended in 25 mL of Tris-HCl buffer (pH 8.0) containing one tablet of complete protease inhibitor cocktail (Roche Applied Science, Germany) and 0.15 M NaCl, followed by addition of 500 μL of lysozyme (10 mg/mL in 20 mM Tris-HCl, pH 8.0). After 30 min incubation on ice, 2.5 mL of 10% Triton X-100 (final 1%), and β-mercaptoethanol (final conc. 10 mM) were added and further incubated for 20 min on ice. Sonication was performed using a UD-201 (Tomy Seiko Corp, Tokyo, Japan), sets to 30% maximum power 2 times for 10 sec with at least a 1 min interval between sonication treatments. After clearing the solution by centrifugation at 12,000 × *g* (30 min at 4°C), the resulting supernatant was filtrated with a syringe driven 0.45 μm filter device (Whatman Inc., Clifton, NJ).

Purification of PNGase

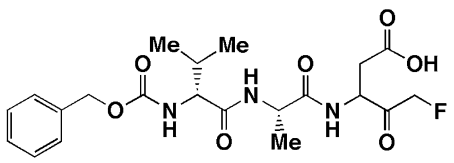
The extract was applied onto a 5 mL Ni-NTA agarose column and gently agitated, to keep the resin suspended in lysate solution for 1 h. The resin was washed with 50 mL of buffer A (20 mM sodium-phosphate buffer (pH 7.2), 150 mM NaCl) and 20 mL of 1% buffer B (20 mM sodium phosphate buffer (pH 7.2) 150 mM NaCl with 500 mM imidazole). Elution was performed with 20 mL 100% buffer B. Fractions were collected every 4 mL, and dithiothreitol and EDTA were added to a final concentration of 5 mM and 1 mM, respectively, and were monitored by SDS-PAGE. Fractions containing purified PNGase (eluted at 100% buffer B solution) were collected, ultrafiltrated with Amicon Ultra-



Glc = Glucose

GlcNAc = N-AcetylGlucosamine

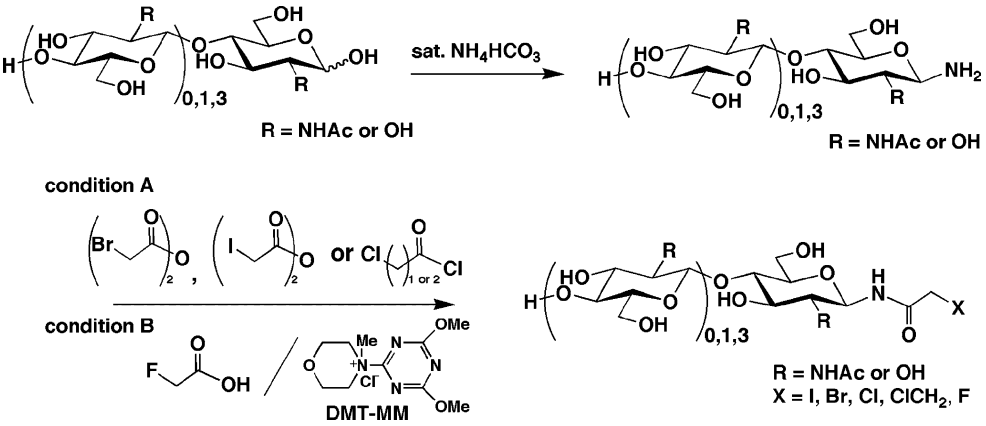
	X	R_1	R_2
1	I	NHAc	H
2	I	NHAc	GlcNAc
3	I	NHAc	$GlcNAc_3$
4	Br	NHAc	H
5	Br	NHAc	GlcNAc
6	Br	NHAc	$GlcNAc_3$
7	Cl	NHAc	H
8	Cl	NHAc	GlcNAc
9	Cl	NHAc	$GlcNAc_3$
10	F	NHAc	H
11	F	NHAc	GlcNAc
12	F	NHAc	$GlcNAc_3$
13	CH_2Cl	NHAc	GlcNAc
14	Cl	OH	Glc
15	Cl	OH	Glc_3



Z-VAD-fmk

Fig. 2 Structure of haloacetamidyl oligosaccharides

Scheme 1 Synthesis of haloacetamidyl oligosaccharides



15 (Mol. Cutoff, 10K; Millipore Corp., Billerica, MA) in 20 mM sodium-phosphate buffer (pH 7.2), 150 mM NaCl and 5 mM. Protein content of the final enzyme preparation was determined by CBB staining using bovine serum albumin as a standard, prior to enzyme assays.

Inhibition assay for PNGase

Required concentration for 50% inhibition (IC_{50}) of PNGase activity was determined using varying concentrations of haloacetamidyl derivatives. No preincubation was performed and reaction was initiated by adding PNGase to the reaction mixture. PNGase activity was measured using RNase B (Sigma-Aldrich Corp., St. Louis, MO, USA) as a substrate as described previously [13]. Typically, the reaction mixture of 30 μL included purified Png1 (0.3 μM), RNase B (0.2 mg/mL), 200 mM Mes-NaOH (pH 6.7) and 10 mM dithiothreitol. The reaction mixture was incubated for 30 min at 37°C and subsequently quenched by adding 2 × sample buffer, followed by SDS-PAGE, and quantitated using Multi Gauge ver. 2.2.

Carbohydrate-probe binding assay and lectin blotting

To assess the binding of **6** to Png1, 10 μL of the enzyme fraction (140 μg/mL) (final 3.5 μM) was added to 10 μL of 200 mM Mes-NaOH buffer (pH 6.7) containing 10 mM dithiothreitol and 10 μL of bromoacetamidyl chitotetraose **6** (final concentration of 1 mM). The reaction was performed for 1 h at 37°C and was stopped by adding 30 μL of 2× SDS-PAGE sample buffer. Ten μL of each sample were applied to SDS-PAGE. Lectin blotting was carried out essentially as described previously for Western blotting [10]. For lectin staining horseradish peroxidase-labeled WGA (Honen Corp., Tokyo, Japan) was used with a dilution of 1:5000. Sample detection was carried out using ECL-plus. The gel was visualized using LAS-1000 (Fujifilm) and the bands detected were quantitated using Multi Gauge ver 2.2.

Table 1 Results of inhibition assay of haloacetamidyl oligosaccharides

Compound	IC50(μM)
1	<1,000
2	0.1
3	0.6
4	<1,000
5	0.1
6	0.2
7	<1,000
8	4
9	18
10	<1,000
11	<1,000
12	<1,000
13	<1,000
14	<1,000
15	<1,000
Z-VAD-fmk	494

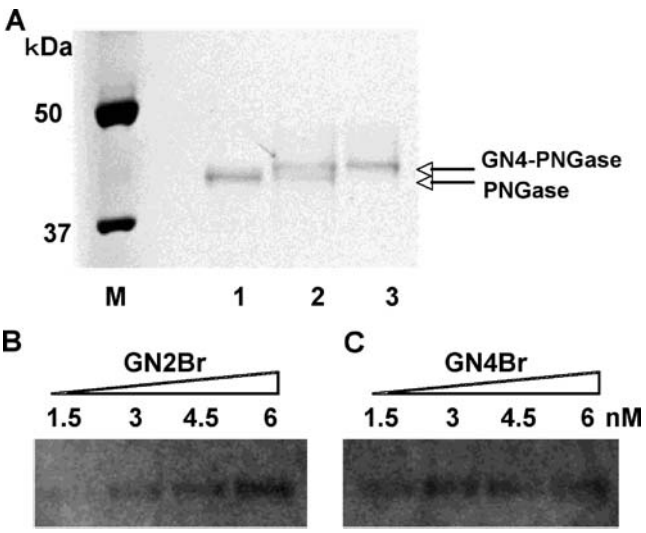


Fig. 3 Determination of glycosylated PNGase by SDS-PAGE (A) and lectin blot analysis (B)

Results and discussion

Syntheses of haloacetamidyl oligosaccharide derivatives

Based on the nature of PNGase, we designed oligosaccharide based inhibitor having simple structure as shown in Fig. 2. These compounds consist of sugar residue for the PNGase binding and a thiol-reactive haloacetamidyl group for covalent modification of the catalytic cystein residue, and were synthesized as depicted in Scheme 1. Thus, commercially available oligosaccharides were first converted to the corresponding glycosylamine under Kochetokov's conditions [14] using saturated NH_4HCO_3, which was acylated with corresponding haloacetic acid derivative in H_2O/dioxane (1/1) [15]. In case of iodo and bromoacetamidyl group, the desired target compounds (**1~6**) were obtained in reasonable yields, employing the acid anhydride. For chloroacetamide derivatives, chloroacetyl chloride was more suitable, cleanly giving desired products **7~9**. For the preparation of fluoro derivatives, we used fluoroacetic acid in combination with DMT-MM [16] to obtain **10** and **11** in 58% and 71% yield, respectively. Fluoroacetamidyl chitotetraose (**12**) was prepared from **3** through displacement reaction with CsF in 33% yield. Synthesis of 3-chloropropionamidyl chitobiose (**13**) was achieved using 3-chloropropionyl chloride in 67% yield.

Inhibition of PNGase by haloacetamidyl oligosaccharides

To examine the activity of synthetic compounds, the concentration required for 50% inhibition (IC_{50}) was determined [13] (see Table 1). Iodo-, bromo-, and chloroacetamidyl chitobiose (**2**, **5**, and **8**) as well as chitotetraose derivatives (**3**, **6**, and **9**) were found to be very potent; judging from IC_{50} values, activity of these compounds was more than a 1,000-fold more effective than Z-VAD-fmk. In contrast, monosaccharide derivatives (**1**, **4**, and **7**), cellooligosaccharide derivatives (**14** and **15**), and fluoroacetamidyl derivative (**10**, **11**, and **12**), exhibited no inhibition. Additionally, chloropropionyl derivative **13** was also devoid of the activity. These results clearly indicate the importance of haloacetamidyl group and chitobiose structure.

In order to verify the mode of inhibition, we incubated PNGase with bromoacetamidyl chitotetraose (**6**) and subjected it to SDS-page analysis [10]. A distinct shift in the elution position of PNGase was observed (see Fig. 3a), indicating the covalent attachment of chitotetraose derivative **6** to PNGase. Subsequently, PNGase was subjected to lectin staining, in order to detect bound chitotetraose. Indeed, lectin (WGA) staining revealed the presence of glycosylated PNGase (see Fig. 3b). This data clearly indicates the covalent modification of PNGase by our inhibitors, being in good agreement with the proposed mode of action. The glycosylated PNGase was detected more clearly using chitotetraose derivative by lectin blotting analysis.

Discussion

In this study, we have established the synthesis of a series of haloacetamidyl oligosaccharide derivatives and evaluated their PNGase inhibiting activity. Structurally simple iodo, bromo, and chloroacetamidyl chitobiose and chitotetraose derivatives act as irreversible PNGase inhibitor via covalent modification of the catalytic triad. In contrast, fluoroacetamidyl derivatives exhibited no PNGase inhibitory activity, possibly due to the poor activity of fluoroamidyl group toward nucleophilic displacement. Furthermore, location of the electrophilic trap is crucial, indicated by the stark difference between chloroacetyl and chloropropionyl derivatives. Cellooligosaccharide derivatives, such as chloroacetamidyl cellobiose **14** and cellotetraose **15** were only poor inhibitors, emphasizing the importance of the *N*-acetyl group of chitobiose for PNGase recognition and inhibition. Our results clearly showed that the iodo, bromo, and chloroacetamidyl chitobiose and chitotetraose derivatives are more efficient inhibitors than Z-VAD-fmk *in vitro* experiments. However, the low permeability of free disaccharide to the cell membrane limited their use to *in vivo* application (data not shown). It has been known that introduction of the hydrophobic substituent to carbohydrate related compounds renders them more permeable to cell membranes [11, 17]. In order to employ them as a cell permeable inhibitor, synthesis of the *in vivo* inhibitor is currently under investigation.

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