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A facile synthesis of a complex type N-glycan thiazoline as an effective inhibitor against the antibody-deactivating endo- β -N-acetylglucosaminidases

Chao Li, Shilei Zhu, Xin Tong, Chong Ou, and Lai-Xi Wang*

Department of Chemistry and Biochemistry, University of Maryland, 8051 Regents Drive, College Park, Maryland 20742, USA

Abstract

Endo- β -N-acetylglucosaminidases are a class of endoglycosidases that deglycosylate N-glycans from glycoproteins. We describe here a facile synthesis of a complex type N-glycan thiazoline as a new mechanism-based inhibitor for this class of enzymes. The synthesis started with the readily available sialoglycopeptide (SGP) and its conversion into the glycan thiazoline through several enzymatic and chemical reactions. The synthetic glycan thiazoline showed potent inhibitory activity against several endoglycosidases including the two antibody-deactivating enzymes, Endo-S and Endo-S2, from human pathogen *Streptococcus pyogenes*, which would be useful as tools for structural and functional studies of these enzymes.

Keywords

Oligosaccharide thiazoline; Enzyme inhibitor; Complex type N-glycan; endo- β -N-Acetylglucosaminidase; Antibody; Enzymatic deglycosylation

INTRODUCTION

Endo- β -N-acetylglucosaminidases (EC 3.2.1.96) (ENGases) are an important class of endoglycosidases that deglycosylate N-glycoproteins by hydrolyzing the β 1,4-glycosidic bond in the *N,N*-diacetylchitobiose core of the N-glycans. These enzymes have been found in various organisms ranging from bacteria, fungi and plant to mammals, and have shown remarkably different substrate specificity in protein deglycosylation. For example, the bacterial enzyme Endo-A from *Arthrobacter protophormiae* is specific for hydrolyzing high-mannose and hybrid type glycoforms [1,2]; the fungus enzyme Endo-M from *Mucor hiemalis* can hydrolyze both high-mannose and complex type N-glycans [3]; and the bacterial enzyme Endo-F3 is highly selective for core-fucosylated complex type N-glycans [4,5]. It has been demonstrated that the ENGase in the cytosol plays an important role in the processing of N-glycans and N-glycoproteins [6,7]. On the other hand, the bacterial enzymes Endo-S and Endo-S2 from *Streptococcus pyogenes* have been shown to be specific for deglycosylating human serum antibodies to reduce their effector functions [8–10], implying

*Corresponding Author's: wang518@umd.edu.

a functional role of these enzymes and also the potential applications of the enzymes for the treatment of autoimmune diseases [11–13]. In addition to the hydrolytic activity, some of the ENGases have also shown transglycosylation activities, and the corresponding glycosynthase mutants generated have found wide applications for the chemoenzymatic synthesis of oligosaccharides, large glycopeptides and homogeneous glycoproteins including therapeutic antibodies [14–19].

Given the biological significance of this class of endoglycosidases, substrate analogs and mechanism-based inhibitors are highly desirable for further understanding of the structures, substrate specificity, and functions of these enzymes. We have previously reported the synthesis of several oligosaccharide thiazolines as a new class of mechanism-based inhibitors for several endoglycosidases, including Endo-A and Endo-M [20]. The design was based on the fact that the sugar thiazoline, in which the anomeric oxygen in the corresponding sugar oxazolinium ion intermediate generated by the substrate-assisted mechanism is replaced by a sulfur atom, could serve as a mechanism-based inhibitors of related enzymes [21–23]. The synthetic high-mannose type N-glycan thiazoline showed μM inhibitory activity against Endo-A and a human endoglycosidase that are specific for high-mannose type N-glycans. Nevertheless, the most effective inhibitor showed only moderate inhibitory activity against Endo-M [20]. So far there are no effective inhibitors described for those endoglycosidases, such as Endo-F3, Endo-S, and Endo-S2 that are selective for complex type glycoforms. We report in this paper the synthesis of a complex type N-glycan thiazoline that is the thioglycosidic analog of the corresponding glycan oxazoline (Figure 1). We selected the non-sialylated N-glycan thiazoline as the synthetic target, as those complex type glycan-active enzymes such as Endo-S, Endo-S2, and Endo-M can work on both sialylated and non-sialylated glycoforms equally efficiently, and the sialylation does not seem to affect the substrate recognition [14–19]. Our inhibition assay indicated that the synthetic complex type glycan thiazoline acted as an effective inhibitor against the antibody-deactivating endoglycosidases, Endo-S and Endo-S2. It also showed much higher inhibitory activity against Endo-M than the previously reported high-mannose type N-glycan thiazoline.

RESULTS AND DISCUSSION

Synthesis of the complex type N-glycan thiazoline (2)

The synthesis of the designed complex type N-glycan thiazoline (**2**) started from the sialoglycopeptide (SGP) (**3**), which was isolated from hen egg yolks according to a previously published procedure with modifications [24]. Thus, SGP was treated with Endo-M that is able to hydrolyze complex type bi-antennary N-glycan to give the free sialylated N-glycan (**4**) in 93% yield (Scheme 1). The sialic acid moieties on **4** were specifically removed by an α -2,3,6,8-neuraminidase to obtain the asialo N-glycan (**5**) in an excellent yield. Then compound **5** was acetylated with $\text{Ac}_2\text{O}/\text{Py}$ to give the peracetylated derivative (**6**). Treatment of the α/β -mixture of **6** ($\alpha:\beta$ ca. 2:1) with Lawesson's reagent [2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide] [25] in toluene at 80 °C gave the thioacetamide α -isomer as the major product as well as the desired thiazoline derivative (**7**). This result was consistent with previous reports that while Lawesson's reagent could

efficiently convert acetamide into thioacetamide derivative, the Lawesson's reagent only converted the more active β -anomer into the corresponding thiazoline and the less active α -anomer would be still in the thioacetamide form [20,21,26]. Without further separation of the thioacetamide α -isomer and **7**, the mixtures were treated with combined Lewis acids (TMS-Cl and $\text{BF}_3 \cdot \text{Et}_2\text{O}$) to transform the thioacetamide α -isomer to the desired thiazoline derivative **7** in 78% yield in two steps. It should be noted that in contrast to the truncated and high-mannose N-glycans that contain only the reducing end GlcNAc moiety, the complex type N-glycan derivative (**6**) carries two additional internal GlcNAc moieties that would be simultaneously converted to the thioacetamide moieties upon treatment with the Lawesson's reagent. Thus, thiazoline derivative **7** contained two thioacetamide moieties that should be changed back to the natural N-acetyl groups. Following a recently reported procedure [27], reaction of **7** with silver acetate successfully transformed the thioacetamide derivative into the corresponding thiazoline diacetylimide derivative (**8**) in 72% yield. Finally, de-O-acetylation and mono-de-N-acetylation of **8** was achieved by treatment of **8** with MeONa in MeOH to give the desired complex type glycan thiazoline (**2**) in 71% yield (Scheme 1).

Assays of the inhibitory activities

The inhibitory activities of the synthetic glycan thiazoline (**2**) were examined against an array of ENGases. To probe the inhibitory activity against Endo-S and Endo-S2, a homogenous sialyl complex type glycoform of antibody rituximab (S2G2F-rituximab) was used as the substrate (Scheme 2A), which was prepared following our previously reported chemoenzymatic method [28]. The enzyme-released free glycans in the presence or absence of the inhibitor were quantified using Dionex HPAEC-PAD analysis [28]. It was found that glycan thiazoline **2** was a potent inhibitor against both Endo-S and Endo-S2 with the IC_{50} of 1.4 μM and 0.57 μM , respectively (Figure 2A). To examine the inhibitory activity against Endo-M, sialoglycopeptide SGP (**3**) was used as the substrate and the released N-glycans were measured by HPAEC-PAD analysis (Scheme 2B). It was observed that glycan thiazoline **2** was also effective in inhibiting Endo-M's activity, with an IC_{50} of 0.27 μM (Figure 2B). When the IC_{50} value was taken as an indicator for inhibition potency, compound **2** would be more active against Endo-M than Endo-S and Endo-S2 by about 5 times and 2 times, respectively. Moreover, in comparison with the high-mannose type $\text{Man}_9\text{GlcNAc}$ -thiazoline, which was previously reported with an IC_{50} of 6 μM against Endo-M [20], the synthetic complex type N-glycan thiazoline (**2**, IC_{50} of 0.27 μM) was 22 fold more active in inhibiting Endo-M than the previously reported high-mannose type glycan thiazoline [20].

CONCLUSION

A facile synthesis of a complex type N-glycan thiazoline was achieved starting from the sialoglycopeptide that is readily obtainable on a large-scale from chicken egg yolks. The synthetic glycan thiazoline represents a new mechanism-based inhibitor particularly effective for those endoglycosidases that are selective for hydrolyzing complex type N-glycoproteins including antibodies. This inhibitor may be useful as a transition state analog to form complexes with endoglycosidases for X-ray crystal structure studies of the enzymes. On the other hand, given the potential role of Endo-S and Endo-S2 in infection of the human

pathogen *Streptococcus pyogenes*, the synthetic inhibitor may be further tested for inhibiting bacterial infection and pathogenesis by *Streptococcus pyogenes*.

EXPERIMENTAL

Materials and methods.

All chemicals, reagents and solvents were purchased from Sigma–Aldrich and TCI and were used as is, unless specially noted. All enzymes were overexpressed in *E. coli* system and purified according to previously published reports. Monoclonal antibody rituximab was purchased from Premium Health Services Inc. (Columbia, MD) and the sialylated homogeneous glycoform of rituximab was prepared using the previously reported method [28]. High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) was performed on a Dionex ICS-5000 chromatography system (Fischer Scientific) equipped with an electrochemical detector (ED50) and an anion exchange column (PA200, 4 × 250 mm). The mobile phase (flow rate, 0.5 ml/min) was composed of 100 mM NaOH (eluent A) and 250 mM NaOAc in 100 mM NaOH (eluent B). The gradient used was as follows: 0 min, 100% eluent A; 20 min, 80% eluent A, 20% eluent B. The LC-ESI-MS was performed on an Exactive™ Plus Orbitrap Mass Spectrometer (Thermo Scientific) equipped with a C18 column (2.1 × 10 mm, 1.5 μm).

Preparation of the Sialo biantennary N-glycopeptide (3).

The extraction and purification procedures of SGP glycopeptide (3) from hen eggs following the previously published method [24]. Crude SGP was purified by preparative HPLC to provide pure glycopeptide (3) as a white powder (146 mg from 90 eggs). ESI-MS: Calcd. for C₁₁₂H₁₈₉N₁₅O₇₀, M = 2865.8. Found: 2866.7 [M+H]⁺.

Synthesis of the sialyl biantennary N-glycan (4).

A solution of 3 (50 mg, 50 mg/mL) in a PBS buffer (pH 7.4, 100 mM) was incubated with wild type Endo-M enzyme (0.2 mg/mL final) at 37 °C overnight. The hydrolysis was monitored by Dionex HPAEC-PAD analysis. The crude glycan was purified by preparative HPLC to give the product (4) as a white powder (33 mg, 93%). ¹H NMR (D₂O, 400 MHz): δ 5.25 (d, *J* = 2.4 Hz, 1 H), 5.14 (d, *J* = 1.2 Hz, 1 H), 4.98 (d, *J* = 1.4 Hz, 0.37H, H-1α), 4.61 (d, *J* = 8.2 Hz, 0.63 H, H-1β), 4.49 (d, *J* = 8.6 Hz, 1 H), 4.29 (m, 1 H), 4.22 (m, 1 H), 4.17 (m, 1 H), 4.05–3.88 (m, 18 H), 3.86–3.70 (m, 25 H), 3.66–3.48 (m, 12 H), 2.25 (m, 4 H), 2.11 (m, 15 H); ¹³C NMR (D₂O, 100 MHz): δ 176.7, 173.5, 173.4, 173.1, 172.8, 114.9, 112.8, 111.2, 106.5, 105.1, 104.8, 104.4, 103.7, 102.6, 100.9, 99.4, 88.3, 87.8, 86.4, 86.1, 85.5, 85.2, 83.4, 82.2, 81.3, 78.8, 78.3, 77.9, 77.3, 74.2, 74.0, 72.5, 69.1, 68.2, 61.0, 56.5, 43.9, 26.1, 23.4, 23.0, 22.8; ESI-MS: Calcd for C₇₆H₁₂₅N₅O₅₇, M = 2020.8; Found: 2021.6 [M+H]⁺.

Synthesis of the asialo biantennary N-glycan (5).

The sialyl N-glycan (4) (50 mg, 50 mg/mL) was dissolved in buffer (pH 5.5, 5 mM CaCl₂, 50 mM NaOAc). Then α2–3,6,8-neuraminidase (40 μL, 2 U/μL) was added and the reaction mixture was incubated at 37 °C overnight. The reaction was monitored by LC-MS. After the completion of enzymatic hydrolysis, the crude product was purified on a Sephadex G-15

size-exclusion column to obtain the asialo N-glycan (**5**) as a white powder (32.4 mg, 91%). ^1H NMR (D_2O , 400 MHz): δ 5.21 (d, J = 2.4 Hz, 1 H), 5.11 (d, J = 1.2 Hz, 1 H), 4.92 (d, J = 1.2 Hz, 0.33 H, H-1 α), 4.57 (d, J = 7.6 Hz, 0.67 H, H-1 β), 4.45 (d, J = 8.0 Hz, 1 H), 4.25 (m, 1 H), 4.19 (m, 1 H), 4.11 (m, 1 H), 3.99–3.83 (m, 18 H), 3.80–3.65 (m, 21 H), 3.62–3.43 (m, 9 H), 2.04 (m, 9 H); ^{13}C NMR (D_2O , 100 MHz): δ 174.3, 174.0, 173.6, 114.4, 112.7, 111.5, 106.2, 105.3, 104.7, 103.2, 102.5, 100.2, 99.3, 88.6, 86.7, 86.2, 85.8, 83.7, 82.1, 79.3, 78.9, 77.5, 74.4, 72.8, 69.2, 61.3, 56.8, 23.6, 23.2, 22.7. ESI-MS: Calcd for $\text{C}_{54}\text{H}_{91}\text{N}_3\text{O}_{41}$, M = 1437.5; Found: 1438.5 $[\text{M}+\text{H}]^+$, 1460.5 $[\text{M}+\text{Na}]^+$.

Synthesis of the peracetylated asialo biantennary N-glycan (**6**).

A solution of the sialo N-glycan (**5**, 50 mg, 0.035 mmol) in pyridine (5 mL) and acetic anhydride (5 mL) was stirred at 40 °C for 12 h when the TLC indicate the completion of acetylation. The mixture was concentrated by vacuum evaporation. The residue was applied to a Sephadex LH-20 (GE Healthcare) size-exclusion column and was eluted with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1, v/v) to obtain the peracetylated N-glycan derivative (**6**) as a white powder (71 mg, 85%) after lyophilization. ^1H NMR (CDCl_3 , 400 MHz): δ 6.58 (d, J = 9.2 Hz, 1 H), 6.07 (m, 0.64 H, H-1 α), 5.67 (m, 1 H), 5.61 (m, 0.36 H, H-1 β), 5.59 (m, 2 H), 5.33–5.24 (m, 9 H), 5.13–4.86 (m, 10 H), 4.74 (m, 3 H), 4.49–4.85 (m, 5 H), 4.72–3.94 (m, 14 H), 3.89–3.27 (m, 7 H), 2.13–1.94 (m, 78 H); ^{13}C NMR (CDCl_3 , 100 MHz): δ 172.5, 172.4, 172.2, 172.0, 171.4, 171.3, 170.9, 170.6, 170.1, 169.8, 169.5, 169.3, 113.7, 112.4, 112.0, 111.8, 111.5, 102.2, 102.1, 101.7, 101.5, 100.4, 100.2, 99.7, 99.2, 89.4, 88.6, 88.2, 88.0, 87.9, 87.5, 87.4, 86.2, 85.7, 84.8, 81.3, 80.8, 77.5, 77.2, 75.7, 71.6, 65.3, 64.9, 62.5, 60.8, 24.7, 20.6. ESI-MS: Calcd for $\text{C}_{100}\text{H}_{137}\text{N}_3\text{O}_{64}$, M = 2404.8; Found: 2405.7 $[\text{M}+\text{H}]^+$, 2427.7 $[\text{M}+\text{Na}]^+$.

Synthesis of the peracetylated thiazoline derivative (**7**).

To a solution of the peracetylated N-glycan (**6**, 50 mg, 0.021 mmol) in toluene (5 mL) was added the Lawesson's reagent (45.8 mg, 0.11 mmol). The resulting mixture was stirred at 80 °C for 5 h. The reaction was monitored by TLC. After completion, the solvent was removed by vacuum evaporation and the residue was subject to purification on a Sephadex LH-20 column. The column was eluted with $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ (1:1, v/v) to afford a mixture of the thioacetamide and the desired product (**7**). Without further purification, the mixture (46 mg) was dissolved in anhydrous dichloroethane (2 mL) and then TMS-Cl (50 μL , 0.396 mmol), $\text{BF}_3\cdot\text{Et}_2\text{O}$ (52 μL , 0.41 mmol) and 2,4,6-collidine (53 μL , 0.4 mmol) were added. The reaction mixture was stirred at room temperature for 3 h. After diluted with CH_2Cl_2 , the organic phase was separated and washed with aqueous NaHCO_3 and brine. The organic layer was dried with anhydrous Na_2SO_4 and filtered. The filtrate was concentrated by vacuum evaporation and the residue was subject to flash silica gel chromatography to afford the peracetylated thiazoline derivative (**7**) as a white powder (38 mg, 78%). ^1H NMR (CDCl_3 , 400 MHz): δ 6.62 (m, 1 H), 6.21 (m, 1 H), 5.70 (m, 2 H), 5.69 (m, 2 H), 5.59–4.80 (m, 14 H), 4.76–4.48 (m, 4 H), 4.49–4.05 (m, 9 H), 4.02–3.74 (m, 7 H), 3.70–3.53 (m, 4 H), 2.54 (m, 6 H), 2.17–1.98 (m, 72 H); ^{13}C NMR (CDCl_3 , 100 MHz): δ 179.1, 179.0, 169.4, 169.1, 168.5, 168.3, 168.0, 167.7, 167.5, 167.4, 160.6, 114.6, 114.0, 113.3, 112.1, 111.9, 102.6, 102.3, 102.2, 101.5, 101.9, 100.3, 99.8, 88.6, 87.3, 87.4, 87.1, 86.4, 86.2, 86.6, 85.2,

84.9, 83.7, 80.9, 79.5, 76.2, 76.1, 74.4, 70.8, 68.3, 64.6, 63.5, 61.1, 58.4, 34.3, 21.6; ESI-MS: Calcd for $C_{98}H_{133}N_3O_{59}S_3$, $M = 2392.7$. Found: 2393.6 $[M+H]^+$.

Synthesis of the peracetylated thiazoline diacetylimide derivative (8).

The peracetylated thiazoline derivative (7) (20 mg, 8 μ mol) was dissolved in CH_2Cl_2 (5 mL) and then AgOAc (20 mg, 0.12 mmol) was added. The resulting mixture was stirred at room temperature with exclusion of light for 5 h. The mixture was then filtered and concentrated. The residue was subject to flash silica gel chromatography to give the peracetylated thiazoline diacetylimide (8) as a white powder (14.7 mg, 72%). 1H NMR ($CDCl_3$, 400 MHz): δ 6.21 (m, 1 H), 6.17 (m, 2 H), 5.91 (m, 1 H), 5.72 (m, 3 H), 5.48–4.92 (m, 8 H), 4.66 (m, 1 H), 4.58–4.42 (m, 2 H), 4.39–3.95 (m, 9 H), 3.91–3.44 (m, 16 H), 2.35 (m, 12 H), 2.17–1.98 (m, 69 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 198.8, 198.6, 170.8, 170.5, 169.9, 169.4, 169.2, 168.8, 168.3, 168.1, 112.3, 111.7, 111.2, 110.6, 110.4, 101.9, 101.4, 101.1, 100.2, 99.7, 99.3, 99.2, 87.6, 86.2, 86.1, 85.6, 85.3, 83.1, 85.0, 84.9, 84.6, 83.5, 78.4, 75.6, 75.2, 75.0, 74.7, 69.8, 67.5, 63.1, 63.0, 60.8, 25.5, 20.7; ESI-MS: Calcd for $C_{102}H_{137}N_3O_{63}S$, $M = 2444.7$. Found: 2445.6 $[M+H]^+$.

Synthesis of the asialo biantennary N-glycan thiazoline (2).

The peracetylated thiazoline diacetylimide (8, 15 mg, 6.1 μ mol) was dissolved in MeOH (2 mL) containing MeONa (0.27 mg, 5 μ mol). The reaction mixture was stirred at room temperature for 2 h. After the completion of deacetylation, cationic resin (Dowex 50w-x8, 10 mg) was added to neutralize the reaction mixture. Then the mixture was filtered and the filtrate was concentrated. The residue was applied to a Sephadex G-15 size-exclusion column which was eluted with water to give the complex type N-glycan thiazoline (2) as a white powder (6.2 mg, 71%) after lyophilization. 1H NMR (D_2O , 400 MHz): δ 5.18 (d, $J = 3.6$ Hz, 1 H), 5.05 (d, $J = 1.8$ Hz, 1 H), 4.90 (d, $J = 1.4$ Hz, 1 H), 4.52 (d, $J = 8.4$ Hz, 1 H), 4.41 (d, $J = 8.6$ Hz, 1 H), 4.22 (m, 1 H), 4.16 (m, 1 H), 4.04 (m, 1 H), 3.90–3.82 (m, 18 H), 3.78–3.61 (m, 21 H), 3.57–3.38 (m, 9 H), 2.02 (m, 9 H); ^{13}C NMR (D_2O , 100 MHz): δ 174.8, 174.4, 173.9, 113.7, 112.5, 111.3, 105.8, 105.2, 104.5, 103.1, 102.3, 100.6, 99.2, 88.4, 87.8, 86.1, 83.9, 83.6, 81.7, 79.0, 78.9, 77.2, 74.3, 72.1, 68.6, 60.1, 56.3, 23.5, 22.6, 21.1; ESI-MS: Calcd for $C_{54}H_{89}N_3O_{39}S$, $M = 1436.35$. Found: 1437.14 $[M+H]^+$.

Enzyme Inhibition assays with the synthetic glycan thiazoline.

The inhibition assays against Endo-S and Endo-S2 were performed using a synthetic homogeneous glycoform (S2G2F-glycoform) of rituximab as the substrate. A solution of S2G2F-rituximab (10 μ M) in a PBS buffer (100 μ L, 100 mM, pH 7.4) was incubated with the respective enzyme (5 μ M) in the presence of serial dilutions (0.02 to 50 μ M) of the N-glycan thiazoline (2). Reactions without any inhibitor were carried out as a control. The mixture was incubated at 30 $^{\circ}C$ for 10 min and the reaction was quenched by 0.1% TFA solution. The enzyme-released free N-glycans were quantitated by HPAEC-PAD analysis. The inhibition assay against Endo-M was performed using SGP glycopeptide (3) as the substrate and followed the same procedure as those with enzymes Endo-S and Endo-S2.

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N-glycan oxazoline (**1**)

N-glycan thiazoline (2)

Figure 1. Structures of the complex type N-glycan oxazoline (**1**) and the corresponding complex type N-glycan thiazoline (**2**).

Figure 2.

Inhibition effect of CT-thiazoline (**2**) on various endo-glycosidases. A) Inhibition of Endo-S and Endo-S2 by **2** using S2G2F-rituximab as substrate; B) Inhibition of Endo-M by **2** using SCT-Fmoc as substrate.

Scheme 1.
Synthesis of the complex type N-glycan thiazoline (**2**).

Scheme 2.

Reactions for assessing the inhibiting activities of the complex type N-glycan thiazoline (**2**) against endoglycosidase. A) the reaction for measuring the inhibiting activity against the antibody-specific endoglycosidases Endo-S and Endo-S2; B) the reaction for measuring the inhibiting activity against Endo-M.