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Probing the active-site requirements of human intestinal N-terminal maltase-glucoamylase: Synthesis and enzyme inhibitory activities of a six-membered ring nitrogen analogue of kotalanol and its de-O-sulfonated derivative

Sankar Mohan [a], Lyann Sim [b], David R. Rose [b,c], B. Mario Pinto [a,*]

[a] Department of Chemistry, Simon Fraser University, Burnaby, British Columbia, Canada V5A 1S6
[b] Department of Medical Biophysics, University of Toronto and Division of Molecular and Structural Biology, Ontario Cancer Institute, Toronto, ON, Canada M5G 2M9
[c] Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

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ABSTRACT

In order to probe the active-site requirements of the human N-terminal subunit of maltase-glucoamylase (ntMGAM), one of the clinically relevant intestinal enzymes targeted for the treatment of type-2 diabetes, the syntheses of two new inhibitors are described. The target compounds are structural hybrids of kotalanol, a naturally occurring glucosidase inhibitor with a unique five-membered ring sulfonium-sulfate inner salt structure, and miglitol, a six-membered ring antidiabetic drug that is currently in clinical use. The compounds comprise the six-membered ring of miglitol and the side chain of kotalanol or its de-O-sulfonated derivative. Inhibition studies of these hybrid molecules with human ntMGAM indicated that they are inhibitors of this enzyme with comparable K_i values to that of miglitol (kotalanol analogue: 2.3 ± 0.6 μM; corresponding de-O-sulfonated analogue: 1.4 ± 0.5 μM; miglitol: 1.0 ± 0.1 μM). However, they are less active compared to kotalanol (K_i = 0.19 ± 0.03 μM). These results suggest that the $^3T_2$ enzyme-bound conformation of the five-membered thiocyclitol moiety of the kotalanol class of compounds more closely resembles the $^4H_3$ conformation of the proposed transition state for the formation of an enzyme–substrate covalent intermediate in the glycosidase hydrolase family 31 (GH31)-catalyzed reaction.

1. Introduction

The plant extracts from *Salacia reticulata*, known as kothalahimbutu in Singhalese, have been widely used in the ancient Ayurvedic system of medicine for treating type-2 diabetes.[1–6] *S. reticulata* is a large woody climbing plant widely found in Sri Lanka and southern parts of India. Typically, water stored overnight in a mug made from the roots of *S. reticulata* was given to patients as an herbal remedy for type-2 diabetes. Extracts from the other plants in the *Salacia* genus such as *Salacia chinensis*, *Salacia oblonga*, and *Salacia prinoides* have also been used in this herbal remedy.[5,6] Attempts to identify the source of the antidiabetic property possessed by these aqueous extracts have yielded a novel class of sulfonium-ion glucosidase inhibitors **1**–**5** (Fig. 1).[6–10] The absolute stereostructures, as shown in Figure 1, were eventually established through total synthesis.[11–17] Very recently, we have established the stereostructure of kotalanol **4** and de-O-sulfonated kotalanol **5**, the two most active components isolated from *S. reticulata* extracts, by total synthesis.[16] Four of these compounds **2**–**5** strongly inhibited the action of the human N-terminal subunit of maltase-glucoamylase (ntMGAM),[18,19] a

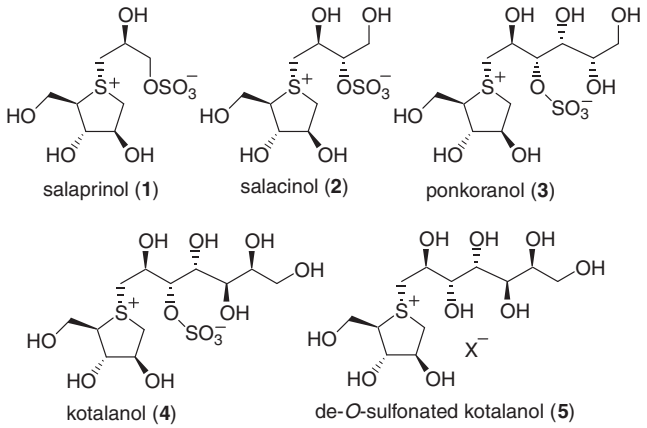

Figure 1. Sulfonium-ion glucosidase inhibitors isolated from *Salacia* species.

* Corresponding author. Tel.: +1 778 782 4152; fax: +1 778 782 4860.
E-mail address: bpinto@sfu.ca (B.M. Pinto).

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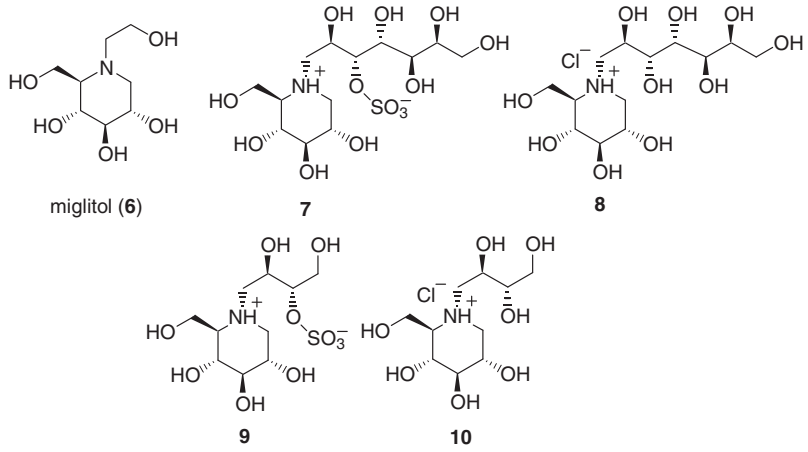


Figure 2. Miglitol and related analogues.

family 31 glycoside hydrolase (GH31)[20] and one of the clinically relevant intestinal glucosidases targeted for the treatment of type-2 diabetes.

Both kotalanol (**4**) and miglitol **6**[21] (Fig. 2), an antidiabetic drug that is currently in clinical use, are inhibitors of ntMGAM, with K_i values of 0.19 μM and 1.0 μM, respectively.[19] The active-site of ntMGAM consists of a −1 and a +1 sugar-binding site.[20] Recent crystals structures of ntMGAM in complex with kotalanol (**4**) and miglitol (**6**) indicated that kotalanol utilizes both subsites for binding, whereas miglitol binds only in the −1 subsite (Fig. 3).[19] While the five-membered thiocyclitol moiety with the permanent positive charge, the head group of kotalanol (**4**), makes hydrogen bonding and electrostatic contacts in the −1 subsite, the polyhydroxylated side chain makes several hydrogen bonding interactions in the +1 subsite (Fig. 3B). In the case of miglitol (**6**), the interactions in the −1 subsite are very similar to those observed with kotalanol, mainly hydrogen bonding interactions between the hydroxyl groups present in the six-membered iminocyclitol moiety, the head group of miglitol (Fig. 3A). But, unlike kotalanol, the *N*-hydroxyethyl side chain of miglitol does not make any interactions in the +1 subsite of ntMGAM, presumably accounting for the lesser potency of miglitol (**6**) relative to kotalanol (**4**). In addition, it was also suggested that the ring conformations adopted by the thiocyclitol and iminocyclitol moieties mimick two different points in the reaction trajectory of

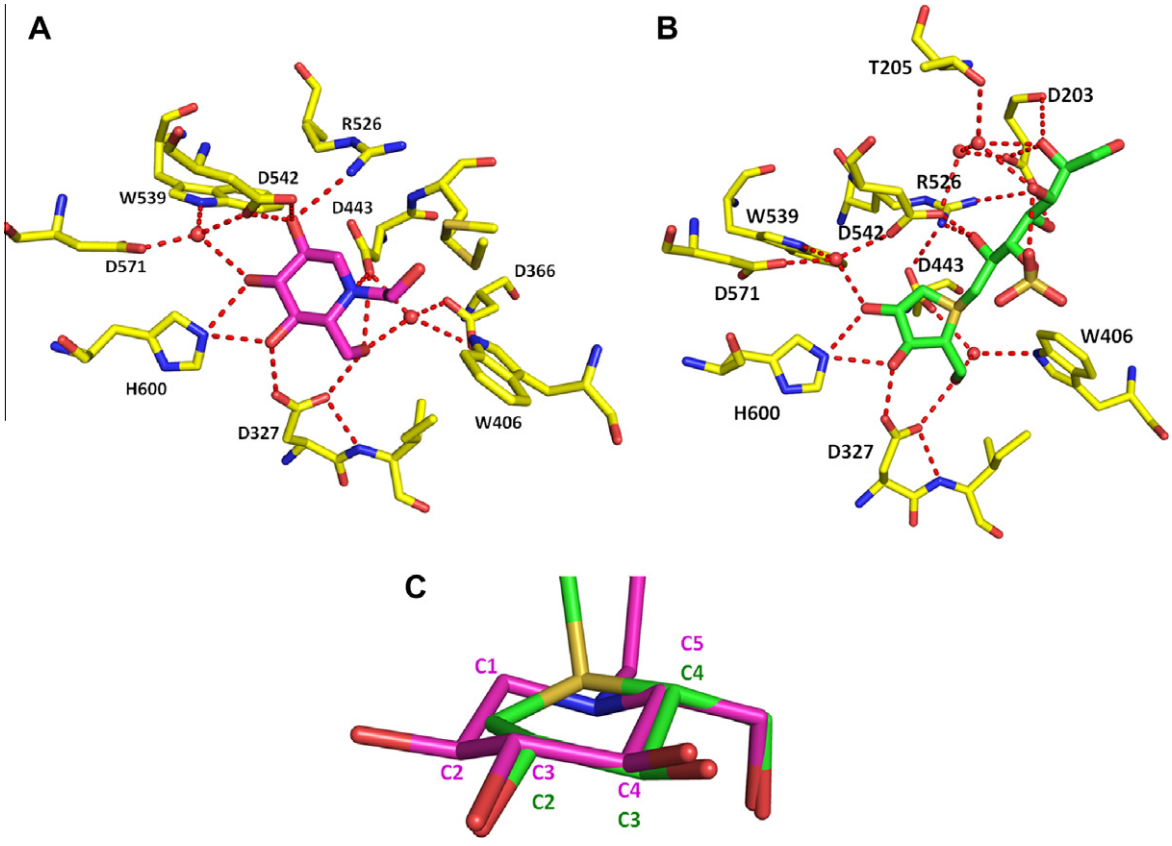


Figure 3. Comparison of the interactions of (A) miglitol (**6**) and (B) kotalanol (**4**) with the active-site residues of ntMGAM. (C) Comparison of the enzyme-bound conformation of the head group of miglitol to that of kotalanol in the −1 subsite. Ring carbon atoms of miglitol are numbered in pink and those of kotalanol are in green. Reproduced from the respective PDB files (3L4 W, 3L4 V).

the enzyme catalyzed reaction.[19] For the GH31 family, the proposed reaction mechanism follows a $^4C_1$–$^4H_3$–$^1S_3$ conformational itinerary for the formation of an enzyme–substrate covalent intermediate.[22] The thiocyclitol moiety of kotalanol (**4**) adopts a $^3T_2$ (carbohydrate numbering) conformation which closely resembles the proposed $^4H_3$ conformation of the oxacarbenium-ion like transition state, whereas the iminocyclitol moiety of miglitol (**6**) adopts a $^4C_1$ (carbohydrate numbering) conformation that resembles the substrate-binding conformation (Fig. 3C).[19]

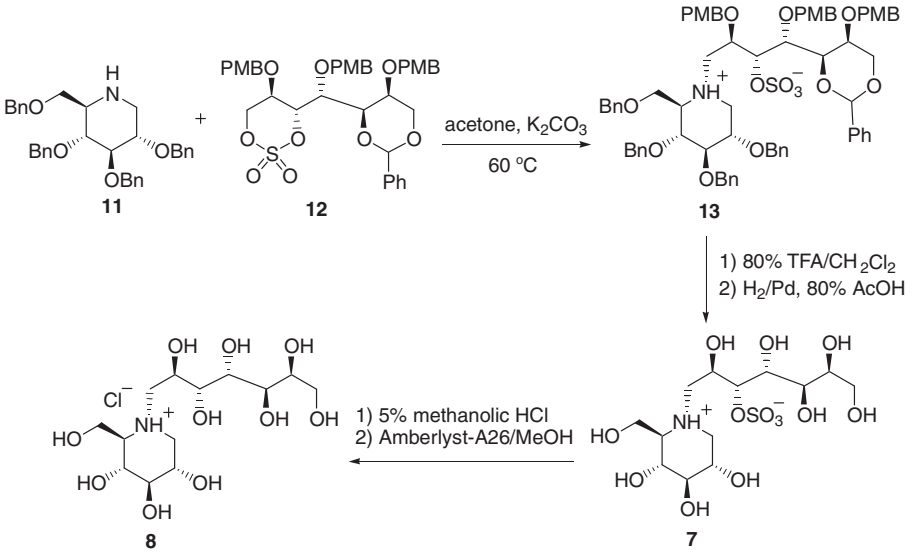
In order to probe this hypothesis further, it was of interest to synthesize an inhibitor **7** in which the *N*-hydroxyethyl substituent of miglitol was replaced with the polyhydroxylated side chain of kotalanol, as shown in Figure 2. The resulting hybrid molecule **7** was expected to show binding interactions in the +1 subsite as seen in the case of kotalanol (**4**). The corresponding de-O-sulfonated compound **8** (Fig. 2) is also of interest, as our recent structure–activity relationship (SAR) studies of kotalanol analogues consistently show that the inhibitory activities against ntMGAM improve significantly upon de-O-sulfonation.[23] For example, de-O-sulfonated kotalanol (**5**) (K_i = 0.03 μM) is a ~sevenfold better inhibitor of ntMGAM than kotalanol **4** (K_i = 0.19 μM) itself.[19] In addition, the target compounds **7** and **8**, in conjunction with the recent crystallographic studies,[19] could also be used to probe the relative importance of transition state mimicry by the kotalanol class of compounds. We recently reported our first attempt to probe the active-site requirements of ntMGAM using a 3′-*O*-methyl-substituted ponkoranol analogue.[24] We now report the synthesis of compounds **7** and **8** and their evaluation as glucosidase inhibitors against ntMGAM. It is noteworthy that the miglitol analogue **9** containing the 4-carbon polyhydroxylated chain present in salacinol (**2**) and the corresponding de-O-sulfonated derivative **10**, synthesized by us[25] and others,[26] were found to be as active as salacinol (**2**) against rat intestinal α-glucosidases (Fig. 2).[26] In comparison with the inhibitory activity of miglitol (K_i = 1.0 ± 0.1 μM),[19] the target compounds **7** and **8** did not show any significant improvement.

2. Results and discussion

The required benzyl-protected deoxynojirimycin derivative **11** was prepared using literature methods.[27] The other coupling partner, the heptitol-derived cyclic sulfate **12**, was prepared from D-perseitol, as reported earlier.[16] The coupling reaction of **11** with the cyclic sulfate **12** was performed in acetone in the presence of K_2CO_3, as shown in Scheme 1. The coupled product **13** was found to be unstable, as indicated by thin layer chromatography (TLC), probably due to partial removal of the *para*-methoxybenzyl (PMB) protecting groups. Hence, without any further characterization, compound **13** was deprotected. Treatment with 80% trifluoroacetic acid (TFA) followed by hydrogenolysis using 10% Pd/C and H_2 at 100 psi in aqueous acetic acid gave the target compound **7** in 62% yield (two steps).

The structure of compound **7** was confirmed by 1D and 2D NMR analyses. At neutral pH, the ^{1}H NMR spectrum of **7** in D_2O was extremely broad, however all the resonances were sharpened when the NMR sample was made basic (pH >8) by the addition of small amount of solid K_2CO_3. The broadening of the resonances at neutral pH and sharpening at basic pH indicate that the product **7** exists as an equilibrium mixture of ammonium salt and the corresponding tertiary amine. In the ^{1}H NMR spectrum, in addition to the desired compound resonances, one also observed a singlet at 1.8 ppm resulting from KOAc contamination; a similar observation was noted in our previous studies.[25] Compound **7** was then converted into the corresponding de-O-sulfonated derivative **8** using 5% methanolic HCl, followed by treatment with Amberlyst A-26 (chloride resin) in methanol (Scheme 1). Analysis of the ^{1}H NMR coupling constants revealed that the six-membered iminocyclitol moiety in both compounds, **7** and **8**, exists in a $^4C_1$ (Fig. 4) conformation in solution. It is important to note that this is also the bioactive conformation of miglitol in the ntMGAM active-site.[19]

Finally, we comment on the glucosidase inhibitory activities of compounds **7** and **8** against ntMGAM using maltose as a substrate. Both compounds, **7** and **8**, inhibited ntMGAM activity with similar K_i values, 2.3 ± 0.6 μM and 1.4 ± 0.5 μM, respectively. In comparison with the inhibitory activity of miglitol (K_i = 1.0 ± 0.1 μM),[19] the target compounds did not show any significant improvement, suggesting that the newly appended polyhydroxylated side chain, contrary to our expectation, does not contribute appreciably to binding in the +1 subsite of the ntMGAM active-site. Supporting evidence can be realized from the fact that de-O-sulfonation (**8**) also did not have any effect on the inhibitory activity, in contrast to the results obtained to date with kotalanol and related analogues.[19,23] On the other hand, the decrease in inhibitory activities of **7** and **8** compared to kotalanol (K_i = 0.19 ± 0.03 μM)[19] suggests the importance of the five-membered ring thiocyclitol moiety with a permanent positive charge found in the kotalanol class of compounds. These results corroborate our recent conclusion from



Scheme 1. Synthesis of target compounds **7** and **8**.

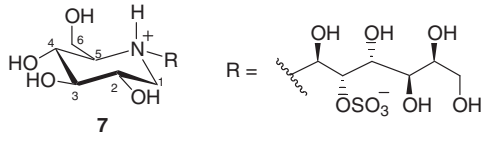


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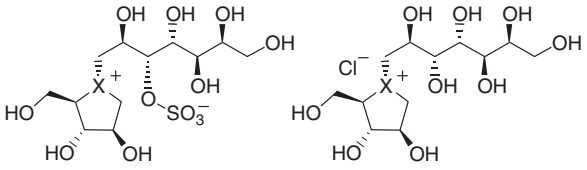
Figure 4. Conformation of iminocyclitol moiety in solution.



4, X = S; K_i = 0.19 μM
14, X = NH; K_i = 90 μM
15, X = Se; K_i = 0.08 μM

5, X = S; K_i = 0.03 μM
16, X = NH; K_i = 0.06 μM
17, X = Se; K_i = 0.02 μM

Figure 5. Kotalanol and de-O-sulfonated kotalanol and their nitrogen and selenium analogues.

X-ray crystallographic studies that the $^3T_2$ enzyme-bound conformation of the five-membered thiocyclitol moiety of the kotalanol class of compounds more closely resembles the $^4H_3$ conformation of the proposed transition state for the formation of an enzyme–substrate covalent intermediate in the glycosidase hydrolase family 31 (GH31)-catalyzed reaction (see Fig. 3C).[19]

The effect of heteroatom on inhibitory activities in the five-membered ring series also deserves comment. We note that the five-membered ring nitrogen analogue (**14**, Fig. 5)[23]of kotalanol showed less inhibitory activity (K_i = 90 μM against ntMGAM) than the corresponding sulfur (kotalanol, **4**) or selenium (**15**, Fig. 5)[23] congeners, and even less compared to the six-membered ring nitrogen analogue (**7**). The shorter C–N bond length compared to C-S and C-Se bond lengths must position **14** unfavorably in the active-site. Removal of the sulfate moiety allows repositioning of the de-O-sulfonated nitrogen analogue (**16**, Fig. 5), as with the sulfur (**5**) and selenium (**17**) congeners (Fig. 5) to allow better electrostatic contacts with the positively charged heteroatom and the catalytic aspartate residue, as well as more intimate contacts with the polyhydroxylated chain,[19] and gives a K_i value that is of similar magnitude to those for **5** and **17.**[23] We contend, therefore, that the conformation of all of the five-membered ring compounds resembles the proposed $^4H_3$ transition state for the formation of an enzyme–substrate covalent intermediate in the GH31-catalyzed reaction. This is apparently not the case for the six-membered ring ground-state mimics (miglitol, **6**[19] and the nitrogen analogues **7** and **8** reported here), or the analogous six-membered ring, sulfur and selenium congeners which show higher K_i values.[18]

3. Experimental section

3.1. General methods

Optical rotations were measured at 23 °C and reported in deg dm^{-1} g^{-1} cm^3. ^{1}H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively. All assignments were confirmed with the aid of two-dimensional ^{1}H, ^{1}H (COSY) and/or ^{1}H, ^{13}C (HSQC) experiments using standard pulse programs. Processing of the spectra was performed with MestRec and/or MestReNova software. Analytical thin layer chromatography (TLC) was performed on aluminum plates precoated with Silica Gel 60F-254 as the adsorbent. The developed plates were air-dried, exposed to UV light and/or sprayed with a solution containing 1% $Ce(SO_4)_2$ and 1.5% molybdic acid in 10% aqueous H_2SO_4, and heated. Column chromatography was performed with Silica Gel 60 (230–400 mesh). High resolution mass spectra were obtained by the electrospray ionization method, using an Agilent 6210 TOF LC/MS high resolution magnetic sector mass spectrometer.

3.2. Enzyme kinetics

Activity of recombinant N-terminal domain of maltase-glucoamylase (ntMGAM) was determined using the glucose oxidase assay[18] to follow the production of glucose from maltose upon addition of the enzyme (0.8 nM). A no-inhibitor control and five different inhibitor concentrations were used in combination with 7 different maltose concentrations (ranging from 1.5 to 24 mM). A reaction time of 60 min at 37 °C was employed. Reactions were linear within this time frame. The weights of compounds **7** and **8** were adjusted for the presence of KOAc. Values of K_i and standard deviations were determined by the program GraFit 4.0.14 (Erithacus Software)[18] which employs nonlinear fitting of the data for each inhibitor concentration to the Michaelis–Menten equation. Competitive inhibition was confirmed by examination of the Lineweaver–Burk plot. K_i values were determined by the equation $K_i = [I]/[(K_{mobs}/K_m) + 1]$, where K_{mobs} is the K_m in the presence of inhibitor.

3.3. 7′-[(1,5-Dideoxy-1,5-imino-D-glucitol)-5-*N*-ammonium]-7′-deoxy-D-perseitol-5′-sulfate (7)

The cyclic sulfate **12**[16] (403.5 mg, 0.56 mmol) and the benzyl-protected deoxynojirimycin derivative **11**[27] (266 mg, 0.51 mmol) were dissolved in acetone (2.5 mL), and anhydrous K_2CO_3 (20 mg) was added. The mixture was stirred in a sealed tube in an oil bath at 60 °C for 5 days. The solvent was removed under reduced pressure, and the product was purified through a short silica column with EtOAc/MeOH (95:5) as eluent to yield the protected ammonium salt (**13**, 202 mg, 73% yield, based on the recovered 150 mg of unreacted starting material **11**). However, the coupled product **13** was found to be unstable, probably due to partial deprotection of the PMB protecting groups, as indicated by thin layer chromatography (TLC). Hence, without any further characterization, the protected compound **13** (200 mg, 0.16 mmol) was stirred in a mixture of 80% trifluoroacetic acid (3 mL) and CH_2Cl_2 (1 mL) at room temperature for 3 h. When starting material **13** had been fully consumed, all of the volatile components were removed under reduced pressure, the crude material was dissolved in 80% acetic acid (20 mL), and the solution was stirred with 10% Pd/C catalyst (400 mg) under H_2 at 100 psi for 4 days. The catalyst was then removed by filtration and all of the volatile components were removed under reduced pressure. The crude compound was purified by column chromatography (EtOAc/MeOH/H_2O 6:3:1) to give the desired compound **7** as a pale-yellow foam (65 mg, containing 8 wt % of KOAc by ^{1}H NMR, 85% yield after correcting for acetate content). $[\alpha]_D^{23}$ = +4.1 (*c* 0.25, H_2O). ^{1}H NMR (D_2O, 600 MHz, pH >8 by adding K_2CO_3): δ 4.59 (1H, br d, $J_{3',2'}$ = 6.0 Hz, H-3′), 4.28 (1H, ddd, $J_{2',1'a}$ = 3.6, $J_{2',1'b}$ = 9.0 Hz, H-2′), 4.04 (1H, dd, $J_{6a,6b}$ = 12.6, $J_{6a,5}$ = 3.0 Hz, H-6a), 4.0 (1H, br d, $J_{4',5'}$ = 10.2 Hz, H-4′), 3.99 (1H, br dd, H-6′), 3.86 (1H, dd, $J_{6b,5}$ = 3.6 Hz, H-6b), 3.78 (1H, dd, $J_{5',6'}$ = 1.2 Hz, H-5′), 3.70 (2H, m, H-7′a and H-7′b), 3.62 (1H, ddd, $J_{2,1a}$ = 4.8, $J_{2,1b}$ = 10.8, $J_{2,3}$ = 9.0 Hz, H-2), 3.39 (1H, dd, $J_{4,3}$ = 9.0, $J_{4,5}$ = 10.2 Hz, H-4), 3.31 (1H, dd, H-3), 3.24 (1H, dd, $J_{1'a,1'b}$ = 15.0, H-1′a), 3.17 (1H, dd, $J_{1a,1b}$ = 12.0, H-1a), 2.78 (1H, dd, H-1′b), 2.50 (1H, ddd, H-5), 2.43 (1H, dd, H-1b). ^{13}C NMR (D_2O, 150 MHz, pH >8 by adding K_2CO_3): δ 167.8 K_2CO_3, 79.2 (C-3′), 78.2 (C-3), 70.0 (C-4), 69.8 (C-6′), 68.9 (C-5′), 68.8 (C-2′), 68.6 (C-4′), 68.5 (C-2), 65.9 (C-5), 63.3 (C-7′), 57.9 (C-6), 56.1 (C-1), 53.2 (C-1′). HRMS calcd for $C_{13}H_{27}NO_{13}SNa$ (M+Na): 460.1095. Found: 460.1092.

3.4. 7′-[(1,5-Dideoxy-1,5-imino-D-glucitol)-5-*N*-ammonium]-7′-deoxy-D-perseitol chloride (8)

Compound **7** (50 mg, 0.11 mmol) was stirred in 5% methanolic HCl (3 mL) at room temperature for 3.5 h. The solvent was evaporated and the residue was treated with Amberlyst A-26 resin (60 mg, chloride form) in MeOH (1 mL). After stirring for 2.5 h, the resin was removed by filtration and the solvent was evaporated to give compound **8** as a pale-yellow foam in quantitative yield (42 mg). $[\alpha]_D^{23}$ = +8.1 (*c* 0.5, H_2O). ^1H NMR (D_2O, 600 MHz, pH >8 by adding K_2CO_3): δ 4.05 (1H, dd, $J_{6a,6b}$ = 12.6, $J_{6a,5}$ = 3.0 Hz, H-6a), 4.01 (1H, ddd, $J_{2',1'a}$ = 4.8, $J_{2',1'b}$ = 7.2, $J_{2',3'}$ = 7.8 Hz, H-2′), 3.99 (1H, br dd, $J_{6',7'a}$ = $J_{6',7'b}$ = 6.0 Hz, H-6′), 3.89 (1H, d, $J_{4',5'}$ = 9.6 Hz, H-4′), 3.88 (1H, dd, $J_{6b,5}$ = 3.0 Hz, H-6b), 3.81 (1H, d, H-3′), 3.70 (2H, br d-like, H-7′a and H-7′b), 3.66 (1H, br d, H-5′), 3.60 (1H, ddd, $J_{2,1a}$ = 4.8, $J_{2,1b}$ = 10.8, $J_{2,3}$ = 9.0 Hz, H-2), 3.39 (1H, dd, $J_{4,3}$ = 9.0, $J_{4,5}$ = 9.6 Hz, H-4), 3.31 (1H, dd, H-3), 3.23 (1H, dd, $J_{1'a,1'b}$ = 14.4, H-1′a), 3.20 (1H, dd, $J_{1a,1b}$ = 12.0, H-1a), 2.68 (1H, dd, H-1′b), 2.45 (1H, ddd, H-5), 2.40 (1H, dd, H-1b). ^{13}C NMR (D_2O, 150 MHz, pH >8 by adding K_2CO_3): δ 164.9 K_2CO_3, 78.2 (C-3), 72.0 (C-3′), 70.2 (C-6′), 69.9 (C-4), 69.2 (C-5′), 68.4 (C-2, C-4′), 68.1 (C-2′), 66.1 (C-5), 63.3 (C-7′), 57.6 (C-6), 56.2 (C-1), 53.9 (C-1′). HRMS calcd for $C_{13}H_{28}NO_{10}^+$ (M^- Cl^-): 358.1713. Found: 358.1720.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.09.059.

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