Synthesis of Nitrogen Analogues of Salacinol and Their Evaluation as Glycosidase Inhibitors

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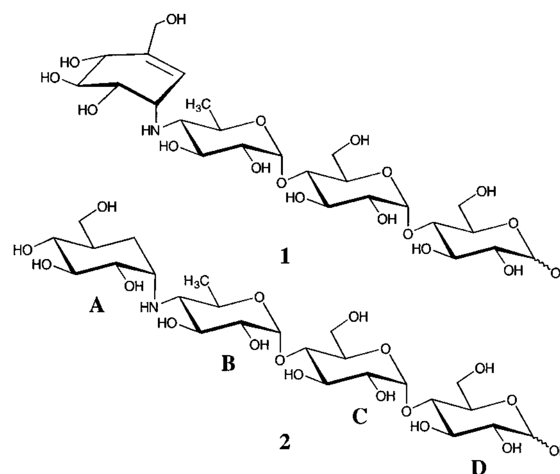
Received February 12, 2001

Abstract: The syntheses of two nitrogen analogues (**11** and **12**) of the naturally occurring sulfonium ion, salacinol (**7**) are described. The latter compound is one of the active principles in the aqueous extracts of *Salacia reticulata* that are traditionally used in Sri Lanka and India for the treatment of diabetes. The synthetic strategy relies on the nucleophilic attack of a 1,4-dideoxy-1,4-imino-D- or L-arabinitol at the least hindered carbon of 2,4-*O*-benzylidene D- or L-erythritol-1,3-cyclic sulfate. The nitrogen analogues bear a permanent positive charge and serve as mimics of the sulfonium ion. We reasoned that these ammonium derivatives should function in a manner similar to that of known glycosidase inhibitors of the alkaloid class such as castanospermine (**4**) and deoxynojirimycin (**5**). Enzyme inhibition assays indicate that salacinol (**7**) is a weak ($K_i = 1.7$ mM) inhibitor of glucoamylase, whereas compounds **11** and **12** inhibit glucoamylase with K_i values in the range ∼10-fold higher. The nitrogen analogues **11** and **12** showed no significant inhibitory effect of either barley α-amylase (AMY1) or porcine pancreatic α-amylase (PPA) at concentrations of 5 mM. In contrast, salacinol (**7**) inhibited AMY1 and PPA in the micromolar range, with K_i values of 15 ± 1 and 10 ± 2 μM, respectively.

Introduction

The controlled inhibition of glycosidase enzymes plays important roles in the biochemical processing of biopolymers containing carbohydrates.[1,2] The intrinsic low affinities of carbohydrate−protein interactions seem to have led Nature to select non-carbohydrate mimics as natural inhibitors of this important class of enzymes. Thus, for example, Nature uses protein inhibitors to inhibit amylase enzyme activity,[1] and carbohydrate mimics of the alkaloid class, such as polyhydroxylated piperidines, pyrrolidines, indolizidines, pyrrolizidines, and nortropanes, are widespread in plants and microorganisms and have been shown to possess glycosidase inhibitory activity.[2] The naturally occurring glycosidase inhibitor acarbose (**1**),[3] which contains a nitrogen atom in one of the linkages between sugar and pseudosugar units gives the highest known carbohydrate affinity for a binding protein and is currently used for the oral treatment of diabetes[4,5] (Chart 1).

Chart 1



It was generally believed that the carbohydrate mimics containing nitrogen are protonated in the enzyme active site and act as glycosidase inhibitors because of their ability to mimic the shape or charge of the presumed transition state for enzymatic glycoside hydrolysis.[6] Considerable synthetic effort has therefore led to a variety of nitrogen-containing analogues, some of which have shown inhibitory activity.[6] However,

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(2) For leading references: Elbein, A. D.; Molyneux, R. J. *Comprehensive Natural Products Chemistry*; Pinto, B. M., Ed.; Barton, D. H. R., Nakanishi, K., Meth-Cohn, O., Ser. Eds.; Elsevier: UK, 1999; Vol. 3, Chapter 7; Asano, N,; Nash, R. J.; Molyneux, R. J.; Fleet, G. W. J. *Tetrahedron: Asymmetry* **2000**, *11*, 1645−1680; McCarter, J. D.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885−892. Ly, H. D.; Withers, S. G. *Annu. Rev. Biochem.* **1999**, *68*, 487−522.

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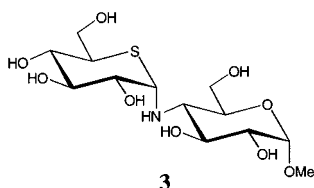
10.1021/ja0103750 CCC: $20.00 © 2001 American Chemical Society
Published on Web 06/08/2001

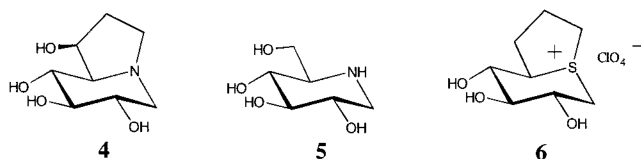
detailed kinetic analysis indicated that although **1** provides some resemblance to a transition-state analogue, it is likely that electrostatic stabilization contributes significantly to its high affinity for glucoamylase.[7] That electrostatic stabilization is important is supported by the observations that the substrate analogues, dihydroglucoacarbose (GAC) (**2**)[8] and the maltoside heteroanalogue (**3**)[9] are also competitive inhibitors of glucoamylase, with low K_i values. The analogue **3** is envisaged to mimic rings A and B of GAC. In contrast, it was shown that castanospermine (**4**) and deoxynojirimycin (**5**) are good competitive inhibitors of the β-glucosidase from *Agrobacterium sp.* but do not function as transition state analogues, despite their high affinity binding.[7] We contend that it is the electrostatic stabilization that leads to the high affinity binding (Chart 2).

Chart 2



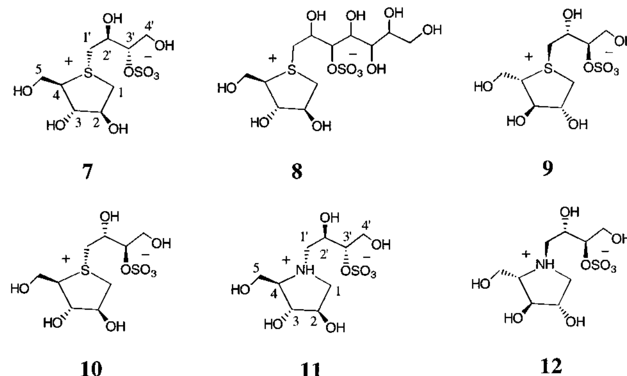
We have recently reported the synthesis of a sulfonium-ion mimic (**6**) of castanospermine (**4**).[10] We reasoned that the interaction of a permanent positive charge with active-site carboxylate residues would make a dominant contribution to the interaction energy (Chart 3).

Chart 3



The concept was validated by the recent isolation[11,12] of naturally occurring glucosidase inhibitors from *Salacia reticulata*, a plant from Sri Lanka ("Kothalahimbutu" in Singhalese) known for its antidiabetic properties. The compounds, salacinol (**7**) and kotalanol (**8**), contain a thiosugar sulfonium ion with an internal sulfate providing the counterion. In Type II diabetes, insulin secretion may be normal but the entry into cells of glucose (normally mediated by insulin) is compromised, and levels of glucose in the blood are high.[13] Inhibition of pancreatic α-amylase, which hydrolyzes starch into smaller oligosaccharides, and α-glucosidases, which break down these oligosaccharides further to glucose in the intestinal membrane are therefore the targets of other glucosidase inhibitors, for example, acarbose (**1**).[4,5] Such enzyme inhibition results in delayed glucose absorption into the blood and a smoothing or lowering of postprandial hyperglycemia, resulting in improved glycemic control. Salacinol (**7**) and kotalanol (**8**) may potentially have fewer long-term side effects than other existing oral antidiabetic agents. Recent animal studies have shown that the oral ingestion of an extract from a *S. reticulata* trunk at a dose of 5000 mg/kg had no serious acute toxicity or mutagenicity in rats[14] (Chart 4).

Chart 4

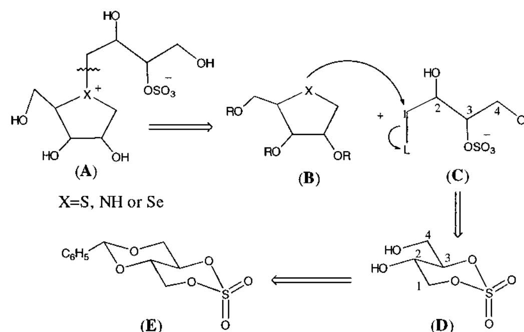


The synthesis of salacinol (**7**) and its stereoisomers (**9**, **10**) and conclusive proof of the structure of the natural product have recently been reported.[15−17] We now report the synthesis of the hitherto unknown nitrogen congeners (**11**, **12**) as potential glycosidase inhibitors.[16] We reasoned that the latter ammonium derivatives, bearing a permanent positive charge, should function in a manner similar to that of castanospermine (**4**) and deoxynojirimycin (**5**).

Results and Discussion

Retrosynthetic analysis indicated that salacinol (**7**) or its analogues (**A**) could be obtained by alkylation of anhydroalditol derivatives at the ring heteroatom (Scheme 1).[16,17] The alkylating agent could either be an open-chain electrophile (**C**) or a cyclic sulfate derivative such as **D** or **E**, whereby selective attack of the heteroatom at the least hindered primary center should afford the desired sulfonium, ammonium, or selenonium ions. We have found that the opening of benzylidene-protected cyclic sulfates by the amines proceeded smoothly to give compound **11** and its enantiomer **12**.[16]

Scheme 1



The iminoarabinitols **13** and **14** were synthesized from D-glucose[18] and D-xylose,[19] respectively. The 2,4-*O*-benzylidene-L (**15**)- and -D (**16**)- erythritol-1,3-cyclic sulfates were synthesized from L- and D-glucose, respectively,[17] in a manner similar

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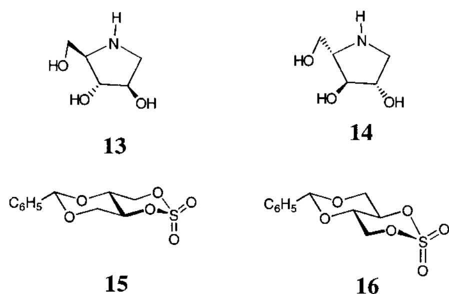
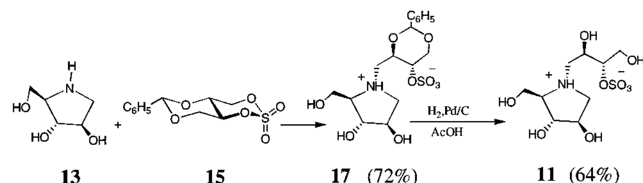
Chart 5



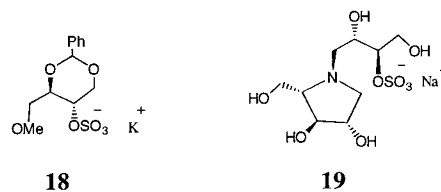
Scheme 2



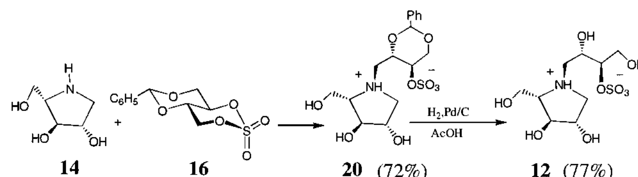
to that described for the corresponding 2,4-*O*-ethylidene derivative[20] (Chart 5).

Compound **17** was synthesized by alkylation of 1,4-dideoxy-1,4-imino-D-arabinitol (**13**) with the benzylidene-protected cyclic sulfate (**15**) (1.2 equiv), in dry methanol containing K_2CO_3 at 60−75 °C to give the protected compound **17** in 72% yield (Scheme 2). A side product **18** was also formed in 16% yield which was assigned to be the product of methanolysis of the cyclic sulfate (**15**). Deprotection of the coupled product **17** by hydrogenolysis over a Pd/C catalyst gave compound **11** in 64% yield. Evidence of the tertiary ammonium structure was obtained by high-resolution FAB mass spectrometery, run in the positive ion mode. If the compound had been a tertiary amine with H^+ or Na^+ associated with the sulfate anion, only the H^+ or Na^+ ions would be observed; the negative sulfate ion would be grounded out on the source wall and would not leave the source. Since the M + H peaks are observed, the tertiary ammonium cation must be present with the internal sulfate counterion.[21] The presence of a tertiary ammonium structure was also confirmed by ^{1}H NMR spectroscopy. In compounds **11** and **12** the protons that are on carbons which are α to the ring nitrogen are all deshielded and broadened at neutral pH. For compound **12**, altering the pH to ~12 with sodium hydroxide resulted in upfield shifts and sharper signals for these proton resonances. We attribute these shifts to the formation of the tertiary amine sulfate **19** at high pH. The stereochemistry at the stereogenic nitrogen center in **11** was established by means of a NOESY experiment (Chart 6). A correlation between H-1′ and H-4, confirmed the *trans* relationship between the erythritol side chain and the C-2 and C-4 substituents on the anhydroarabinitol moiety, which is identical to the stereochemistry at the stereogenic sulfur atom in salacinol (**7**); no correlation between H-1′

Chart 6



Scheme 3



and H-3 was observed. We have chosen to name compound **11** ghavamiol.

The enantiomer of ghavamiol (**12**) was similarly obtained by the reaction of compound **14** with the cyclic sulfate **16** to produce the ammonium salt **20** in 72% yield. Deprotection as before, produced compound **12** in 77% yield. The ^{1}H and ^{13}C NMR spectra for the enantiomer **12** were essentially identical to those of ghavamiol (**11**) except for small changes in chemical shifts due to concentration effects. Proof of structure and stereochemistry were obtained as described above (Scheme 3).

Enzyme Inhibition Assays. Compounds **11** and **12** were tested for their inhibition of three glycosidase enzymes, namely glucoamylase G2,[22,23] porcine pancreatic α-amylase, and barley α-amylase.[24] The effects were compared to those of salacinol (**7**). Glucoamylase G2 was weakly inhibited by salacinol (**7**) (K_i = 1.7 mM). In comparison, ghavamiol (**11**) and its enantiomer (**12**) showed very weak inhibition, with K_i values in the range ~10-fold higher than for **7**. We therefore estimate that compounds **11** and **12** cannot have K_i values that are less than 10mM. The nitrogen analogues **11** and **12** showed no significant inhibitory effect of either barley α-amylase (AMY1) or porcine pancreatic α-amylase (PPA) at concentrations of 5 mM. In contrast, salacinol (**7**) inhibited AMY1 and PPA in the micromolar range, with K_i values of 15 ± 1 and 10 ± 2 μM, respectively. It would appear then that the nitrogen analogues **11** and **12** and salacinol (**7**) show discrimination or selectivity for certain glycosidase enzymes, and further testing against a wider panel of enzymes that includes human small intestinal maltase-glucoamylase[25] and human pancreatic α-amylase[26] is planned to map the enzyme selectivity profiles of these compounds.

Experimental Section

Synthesis. Optical rotations were measured at 23 °C. ^{1}H and ^{13}C NMR spectra were recorded at 400.13 and 100.6 MHz. All assignments were confirmed with the aid of two-dimensional ^{1}H,^{1}H (COSYDFTP) or ^{1}H,^{13}C (INVBTP) experiments using standard Bruker pulse programs. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra were obtained for samples dispersed in a 2,5-dihydroxybenzoic acid matrix using a PerSeptive Biosystems Voyager-DE

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(21) The M + H peaks in the positive mode are generated by the addition of the H^+ neutralizing the sulfate ion, thereby making the molecule positively charged (from the nitrogen cation).

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instrument. Column chromatography was performed with Merck Silica gel 60 (230−400 mesh). High-resolution mass spectra were liquid secondary ionization fast atom bombardment (LSIMS (FAB)), run on a Kratos Concept H double focusing mass spectrometer at 10000 RP, using *meta*-NO_2-benzyl alcohol as matrix or, in the case of compounds **18** and **20**, negative LSIMS with glycerine as matrix and PEG-sulfate as the mass reference.

Enzyme Inhibition Assays. The glucoamylase G2 form from *Aspergillus niger* was purified from a commercial enzyme (Novo Nordisk, Bagsvaerd, Denmark) as described.[22,23] The initial rates of glucoamylase G2-catalyzed hydrolysis of maltose was tested with 1 mM maltose as substrate in 0.1 M sodium acetate pH 4.5 at 45 °C using an enzyme concentration of 7.0×10^{-8} M and five inhibitor concentrations in the range of 1 μm to 5 mM. The effect of the inhibition on rates of substrate hydrolysis were compared for the different compounds. The glucose released was analyzed in aliquots removed at appropriate time intervals using a glucose oxidase assay adapted to microtiter plate reading and using a total reaction volume for the enzyme reaction mixtures of 150 or 300 μL.[27] The K_i values were calculated assuming competitive inhibition from $1/v = (1/V_{max}) + [(K_m)/(V_{max}[S]K_i)] \times [I]$, where v is the rate measured in the presence or absence of inhibitor, [I] and [S] the concentrations of inhibitor and substrate, K_m 1.6 mM, and k_{cat} 11.3 s^{-1} were determined before using ENZFITTER, as described.[27,28]

Recombinant barley α-amylase isozyme 1 (AMY1) was produced and purified as described.[24] An aliquot of the porcine pancreatic α-amylase (PPA) crystalline suspension (in ammonium sulfate) was dialyzed extensively against the assay buffer without BSA. The enzyme concentration was determined by aid of amino acid analysis as determined using an LKB model Alpha Plus amino acid analyzer. Porcine pancreatic α-amylase (PPA) and bovine serum albumin (BSA) were purchased from Sigma. Amylose EX-1 (DP17; average degree of polymerization 17) was purchased from Hayashibara Chemical Laboratories (Okayama, Japan). The inhibition of AMY1 (3×10^{-9} M) and PPA (9×10^{-9} M) activity toward DP17 amylose was measured at 37 °C in 20 mM sodium acetate, pH 5.5, 5 mM $CaCl_2$, 0.005% BSA (for AMY1) and 20 mM sodium phosphate, pH 6.9, 10 mM NaCl, 0.1 mM $CaCl_2$, 0.005% BSA (for PPA). Six different final inhibitor concentrations were used in the range of 1 μM to 5 mM. The inhibitor was preincubated with enzyme for 5 min at 37 °C before addition of substrate. Initial rates were determined by measuring reducing sugar by the copper-bicinchoninate method as described.[24,29] The K_i values were calculated assuming competitive inhibition, as described above for the case of glucoamylase, and a K_m of 0.57 mg/mL and k_{cat} of 165 s^{-1} for AMY1 and 1 mg/mL and 1200 s^{-1} for PPA, as determined in the substrate concentration range 0.03−10 mg/mL using ENZFITTER.[28] For the K_i determinations, [S] = 0.7 mg/mL amylose DP 17 for AMY1 and [S] = 2.5 mg/mL amylose DP 17 for PPA.

1′-((1,4-Dideoxy-1,4-imino-D-arabinitol)-4-*N*-ammonium)-2′,4′-*O*-benzylidene-1′-deoxy-L-erythritol-3′-sulfate (17) and Potassium 2,4-*O*-benzylidene-1-*O*-methyl-L-erythritol-3-sulfate (18). A mixture of 1,4-dideoxy-1,4-imino-D-arabinitol (**13**) (100 mg, 0.7 mmol) and 2,4-*O*-benzylidene-L-erythritol-1,3-cyclic sulfate (**15**) (235 mg, 1.2 equiv) were dissolved in dry MeOH (0.5 mL), and anhydrous K_2CO_3 (15 mg) was added. The mixture was stirred in a sealed tube in an oil-bath (75 °C) overnight. The solvent was removed under reduced pressure, and column chromatography [CH_2Cl_2/MeOH, 4.5:1] of the crude product gave **17** (219 mg, 72%) and **18** (40 mg, 16%) as amorphous solids. **17**: $[\alpha]_D$ +36.8° (*c* 0.4, MeOH), ^{1}H NMR (CD_3OD): δ 7.53−7.30 (5H, m, Ar), 5.61 (1H, s, C*H*Ph), 4.53 (1H, dd, $J_{4'ax,4'eq} = 11.0$, $J_{3',4'eq} = 5.2$ Hz, H-4′eq), 4.28 (1H, brt, $J_{1'b,2'} = J_{2'b,3'} = 9.8$ Hz, H-2′), 4.20 (1H, ddd, $J_{3',4'ax} = 9.7$ Hz, H-3′), 4.14 (1H, brs, H-2), 4.03 (1H, brd, $J_{1'a,1'b} = 10.7$ Hz, H-1′), 3.94 (1H, brs, H-3), 3.92 (1H, dd, $J_{4,5a} = 5.1$ Hz, H-5a), 3.86 (1H, dd, $J_{4,5b} = 7.2$, $J_{5a,5b} = 12.3$ Hz, H-5b), 3.81 (1H, dd, H-4′ax), 3.62 (1H, brd, $J_{1a,1b} = 11$ Hz, H-1a), 3.47 (1H, brd, H-1b), 3.43 (1H, brs, H-4), 3.33 (1H, brd, H-1′b); ^{13}C NMR (CD_3OD): δ 138.66 (C_{ipso}), 130.15 (C_{para}), 129.23 (2C) and 127.40 (2C) (C_{ortho} and C_{meta}), 102.34 (*C*HPh), 77.81 (C-4), 77.52 (C-3), 77.40 (C-2′), 76.19 (C-2), 70.27 (C-4′), 68.92 (C-3′), 62.68 (C-1), 60.41 (C-5), 58.61 (C-1′); MALDI-TOF MS: *m/e* 428 (M^+ + Na),406 (M^+ + H); HRMS. Calcd for $C_{16}H_{23}O_9SN$ (M + H): 406.1179. Found: 406.1192. **18**: $[\alpha]_D$ +31.1° (*c* 0.8, MeOH), ^{1}H NMR (CD_3OD): δ 7.49−7.30 (5H, m, Ar), 5.55 (1H, s, C*H*Ph), 4.55 (1H, dd, $J_{4eq,4ax} = 10.6$, $J_{3,4eq} = 5.4$ Hz, H-4eq), 4.29 (1H, ddd, $J_{2,3} = 9.7$, $J_{3,4ax} = 10.6$ Hz, H-3), 3.90 (1H, ddd, $J_{1a,2} = 1.8$, $J_{1b,2} = 6.5$ Hz, H-2), 3.86 (1H, dd, $J_{1a,1b} = 11.1$ Hz, H-1a), 3.76 (1H, dd, H-4ax), 3.61 (1H, dd, H-1b), 3.39 (3H, s, OC*H*$_3$); ^{13}C NMR (CD_3OD): δ 139.21 (C_{ipso}), 129.87 (C_{para}), 129.03 (2C) and 127.44 (2C) (C_{ortho} and C_{meta}), 102.42 (*C*HPh), 80.61 (C-3), 72.92 (C-1), 70.38 (C-2), 67.96 (C-4), 59.56 (O*C*H$_3$); MALDI-TOF negative ion MS: *m/e* 303 (M − K); HRMS. Calcd For $C_{12}H_{15}O_7S$ (M − K): 303.0538. Found: 303.0543.

1′-((1,4-Dideoxy-1,4-imino-L-arabinitol)-4-*N*-ammonium)-2′,4′-*O*-benzylidene-1′-deoxy-D-erythritol-3-sulfate (20). A mixture of 1,4-Dideoxy-1,4-imino-L-arabinitol (**14**) (80 mg, 0.6 mmol) and 2,4-*O*-benzylidene-D-erythritol-1,3-cyclic sulfate (**16**) (190 mg, 1.2 equiv) was dissolved in dry MeOH (0.5 mL), and anhydrous K_2CO_3 (10 mg) was added. The mixture was stirred in a sealed tube in an oil-bath (75 °C) overnight. The solvent was removed under reduced pressure, and column chromatography [CH_2Cl_2/MeOH, 5:1] of the crude product gave an amorphous solid (175 mg, 72%). $[\alpha]_D$ −32.5° (c 2.4, MeOH); MALDI-TOF MS: *m/e* 428 (M^+ + Na),406 (M^+ + H); HRMS. Calcd for $C_{16}H_{23}O_9SN$ (M − H): 404.1015. Found: 404.1007.

General Procedure for the Deprotection of the Protected Ammonium Sulfates. The protected compound (200 mg, 0.5 mmol) was dissolved in AcOH/H_2O, 4:1 (5 mL) and stirred with Pd−C (30 mg) under H_2 (52 psi). After 60 h the reaction mixture was filtered through a pad of Celite, which was subsequently washed with MeOH. The combined filtrates were concentrated, and the residue was purified by column chromatography.

1′-((1,4-Dideoxy-1,4-imino-D-arabinitol)-4-*N*-ammonium)-1′-deoxy-L-erythritol-3′-sulfate (11). Column chromatography [$CHCl_3$/MeOH/H_2O, 7:3:1] of the crude product gave an amorphous solid (64%). $[\alpha]_D$ +7.2° (c 2.6, MeOH); ^{1}H NMR (CD_3OD): δ 4.26−4.20 (2H, m, H-2′, H-3′), 4.15 (1H, m, $J_{2,3} = 6$ Hz, H-2), 3.98 (1H, br-s, $J_{3,4} = 4$ Hz, H-3), 3.94−3.87 (3H, m, $J_{3',4'a} = 4$ Hz, H-5a, H-5b, H-4′a), 3.81 (1H, m, $J_{4'a,4'b} = 12.0$, $J_{3',4'b} = 3.5$ Hz, H-4′b), 3.74−3.62 (2H, m, $J_{1a,1b} = 13$ Hz, $J_{1'a,1'b} = 14$ Hz, H-1′a, H-1a), 3.49−3.42 (1H, m, $J_{1b,2} = 7$ Hz, H-1b), 3.40−3.35 (1H, m, H-4), 3.15 (1H, m, $J_{1'b,2'} = 6$ Hz, H-1′b); ^{13}C NMR (CD_3OD): δ 81.17 (C-3′), 78.27 (C-3),77.86 (C-4), 76.19 (C-2), 68.07 (C-2′), 62.57 (C-1), 61.67(C-4′), 60.72 (C-1′, C-5); HRMS. Calcd for $C_9H_{18}O_9SN$ (M + H): 318.0859. Found: 318.0863.

1′-((1,4-Dideoxy-1,4-imino-L-arabinitol)-4-*N*-ammonium)-1′-deoxy-D-erythritol-3′-sulfate (12). Column chromatography [$CHCl_3$/MeOH/H_2O, 7:3:1] of the crude product gave an amorphous solid (77%). $[\alpha]_D$ −7.7° (c 0.76, MeOH); HRMS. Calcd for $C_9H_{18}O_9SN$ (M + H): 318.0859. Found: 318.0856.

1′-((1,4-Dideoxy-1,4-imino-L-arabinitol)-4-*N*-yl)-1′-deoxy-D-erythritol-3′-sulfate (19). A solution of **12** in CD_3OD was adjusted to pH 12 by the addition of aliquots of a concentrated solution of NaOH in CD_3OD. ^{1}H NMR (CD_3OD): δ 4.36 (1H, ddd, $J_{2',3'} = 5.8$, $J_{3',4'a} = 4.2$, $J_{3',4'b} = 4.8$ Hz, H-3′), 4.00 (1H, ddd, $J_{1'a,2'} = 5.9$, $J_{1'b,2'} = 6.9$ Hz, H-2′), 3.94−3.90 (2H, m, H-3, H-2), 3.90 (1H, dd, $J_{4'a,4'b} = 12$ Hz, H-4′a), 3.80 (1H, dd, H-4′b), 3.73 (1H, dd, $J_{5a,5b} = 11.4$, $J_{4,5a} = 3.7$ Hz, H-5a), 3.66 (1H, dd, $J_{4,5b} = 3.5$ Hz, H-5b), 3.15 (1H, dd, $J_{1a,1b} = 10.5$ Hz, H-1a), 3.10 (1H, dd, $J_{1'a,1'b} = 13.0$ Hz, H-1′a), 2.77 (1H, dd, $J_{1b,2} = 4.5$ Hz, H-1b), 2.52 (1H, dd, H-1′b), 2.43 (1H, ddd, $J_{3,4} = 3.8$ Hz, H-4).; ^{13}C NMR (CD_3OD): δ 82.28 (C-3′), 80.68 (C-3), 77.56 (C-2), 75.24 (C-4), 71.15 (C-2′), 62.16 (C-4′), 61.85 (2C, C-1 and C-5), 58.69 (C-1′).

Acknowledgment. We are grateful to Sidsel Ehlers for technical assistance with the glucoamylase assays and the Natural Sciences and Engineering Research Council of Canada for financial support.

JA0103750

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