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Caminoside A, an Antimicrobial Glycolipid Isolated from the Marine Sponge *Caminus sphaeroconia*[⊥]

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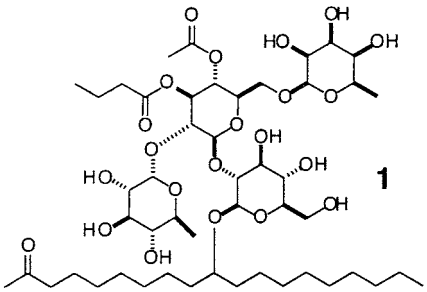
ABSTRACT



Extracts of the marine sponge *Caminus sphaeroconia* showed potent activity in a screen for bacterial type III secretion inhibitors. Bioassay guided fractionation of the extract led to the isolation of the novel antimicrobial glycolipid caminoside A (1). The structure of caminoside A was elucidated by analysis of spectroscopic data and chemical degradation.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea, causing significant mortality in children in developing countries due to dehydration, malnutrition, and other complications of the disease.[1] EPEC is very closely related to enterohemorragic *E. coli* 0157:H7 (EHEC),[2] which has been found in ground beef (hamburger disease), unpasteurized milk, bottled juices, and sewage-contaminated water. EHEC causes a bloody diarrhea that can lead to kidney failure and death in young children and the elderly. The first step in causing disease by EPEC and EHEC is intimate attachment of the bacteria to host cell surfaces.[2] Remarkably, these pathogenic *E. coli* insert their own receptors into the host cells to which they attach.[3] Delivery of this bacterial protein Tir (Translocated Intimin Receptor) requires the *E. coli* secreted proteins (Esps) and a type III secretory apparatus, which translocates secreted proteins across both bacterial membranes, out of EPEC and EHEC, into the host epithelial cells.

Because the type III secretory system is essential for EPEC and EHEC pathogenicity and is not found in nonpathogenic *E. coli*, it has been widely hypothesized that selective inhibitors of the type III secretion system should specifically attenuate pathogenic EPEC and EHEC without affecting the commensal *E. coli* flora.[4] In addition, by targeting a system that is specific for a virulence mechanism, there would be little or no selective pressure for viability, potentially reducing the development of resistance to these hypothetical

[⊥] Dedicated to Professor D. J. Faulkner on the occasion of his 60th birthday.

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novel antimicrobial agents. Despite the promise of this approach, there are no known chemical agents that selectively inhibit the type III secretion system, in large part because there has been no effective assay to screen for this activity.

We have used a high throughput assay developed in our laboratories to screen marine invertebrate extracts for their ability to inhibit the secretion of Esps by EPEC, without affecting growth or general secretion by the bacterium.[4d] Crude extracts of the marine sponge *Caminus sphaeroconia* Sollas showed strong activity in the assay. Bioassay guided fractionation of the extract led to the isolation of a complex family of novel glycolipids. The structure of the major component of the mixture, caminoside A (**1**), is described below.

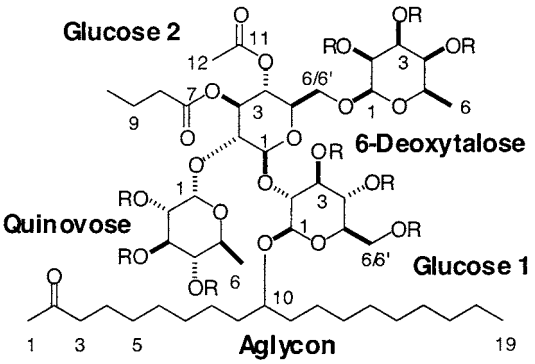

Figure 1. Structures of caminoside A (**1**) (R = H) and caminoside A peracetate (**2**) (R = Ac).

Specimens of *C. sphaeroconia* were collected by hand using SCUBA from the upper walls of Toucari Caves (−10 m) in Dominica. Freshly collected sponge was frozen on site and transported to Vancouver over dry ice. Thawed sponge was cut into small pieces and extracted repeatedly with MeOH. The combined MeOH extracts were concentrated *in vacuo* and the resulting residue was chromatographed on Sepahdex LH 20 (eluent: MeOH) to give a series of contiguous fractions containing bioactive glycolipids that were combined and concentrated *in vacuo*. Further fractionation by multiple cycles of Sephadex LH 20 chromatography (eluent: EtOAc/MeOH/H_2O 20:5:2) gave an inseparable mixture of caminosides that had identical polysaccharide portions but differed in their lipid aglycon components. Acetylation of this mixture and purification of the products by gradient flash silica gel chromatography (CH_2Cl_2 to CH_2Cl_2/MeOH 9:1) and RPHPLC gave pure caminoside A peracetate (**2**) as an optically active ($[\alpha]^{25}_D$ −27° (*c* 0.024, MeOH)) colorless glass.

Caminoside A peracetate (**2**) gave a $[M + Na]^+$ ion at *m*/*z* 1427.6470 in the HRESMS appropriate for a molecular formula of $C_{67}H_{104}O_{31}Na$ (calcd 1427.6459) that required 16 sites of unsaturation. The ^{1}H NMR spectrum obtained for **2** at 800 MHz in C_6D_6 (Supporting Information) showed a series of deshielded methine resonances between δ 3.4 and 5.9 characteristic of protons on sp^3 carbons attached to oxygen atoms, a series of methyl resonances between δ 1.55 and 2.1, assigned to acetate methyls, and a series of aliphatic methylene and methyl proton resonances between δ 0.8 and 2.4, suggestive of a linear hydrocarbon fragment. These proton NMR features indicated that caminoside A (**1**) was a glycolipid.

^{13}C/DEPT and HSQC NMR data recorded for **2** identified four ketal methine carbon resonances at δ 96.2 (Qui-C-1: ^{1}H δ 5.46), 100.1. (Glu2-C-1: ^{1}H δ 4.88), 100.2 (Deoxytal-C-1: ^{1}H δ 4.77), and 100.9 (Glu1-C-1: ^{1}H δ 4.64), which were assigned to sugar anomeric carbons. The anomeric proton resonances provided entry points for making complete ^{13}C and ^{1}H NMR assignments for each of the four mono-

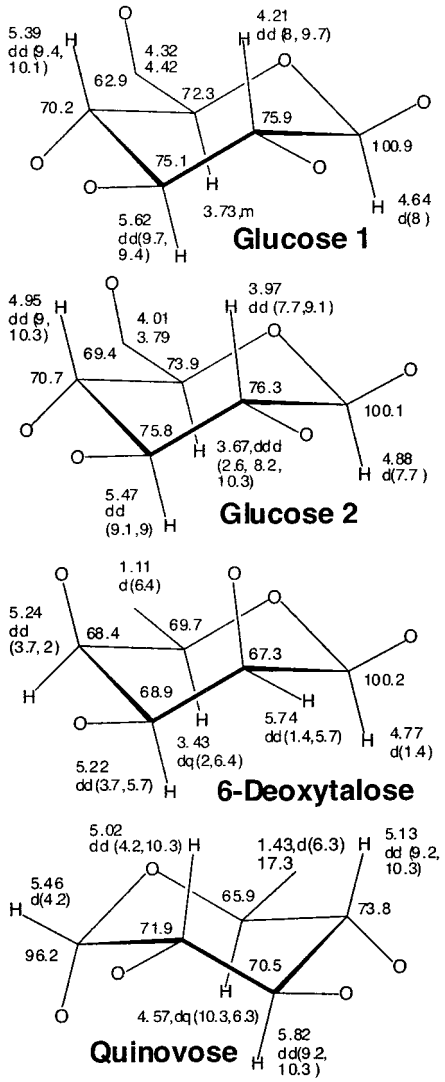

Figure 2. ^{1}H and ^{13}C NMR assignments for the monosaccharide residues of caminoside A peracetate (**2**).

(4) (a) Mecsas, J.; Strauss, E. J. *Emerging Infect. Dis.* **1996**, *2*, 271. (b) Hueck, C. J. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 379. (c) Cornelis, G. R.; Van Gijsegem, F. *Annu. Rev. Microbiol.* **2000**, *54*, 735. (d) Gauthier, A.; Finlay, B. *ASM News* **2002**, *68*, 383.

saccharides in the molecule via the COSY, TOCSY, HSQC, and HMBC data (Figure 2 and Table 1, Supporting Information). Two of the sugar spin systems, originating with the anomeric protons at δ 4.64 (Glu1-H-1) and 4.88 (Glu2-H-1), could be assigned to hexoses. Weak HMBC correlations observed between Glu1-H-1 (δ 4.64) and Glu1-C-5 (δ 72.3), and between Glu2-H-5 (δ 3.67) and Glu2-C-1 (δ 100.1), indicated that the hexoses existed in the pyranose forms. Analysis of the vicinal coupling constants for the H-1 to H-5 regions in both systems showed only large coupling constants consistent with all axial/axial coupling (Table 1, Supporting Information) demonstrating that both monosaccharides were glucose residues with β anomeric configurations.

The remaining two sugar spin systems, originating with the anomeric protons at δ 4.77 (Deoxytal-H-1) and 5.46 (Qui-H-1), could be assigned to 6-deoxy hexoses. Once again, HMBC correlations observed between Deoxytal-H-5 (δ 3.43) and Deoxytal-C-1 (δ 100.2), and between Qui-H-5 (δ 4.57) and Qui-C-1 (δ 96.2), showed that both of the deoxyhexoses were in the pyranose forms. Vicinal coupling constant analysis (Figure 2) showed that the deoxyhexose with the anomeric resonance at δ 5.46 was a quinovose with an α anomeric linkage. ROESY correlations observed between the H-5 resonance at δ 3.43 and both the H-3 (δ 5.22) and H-1 (δ 4.77) resonances in the remaining deoxyhexose spin system showed that H-1, H-3, and H-5 were all axial. The H-1/H-2, H-2/H-3, H-3/H-4, and H-4/H-5 vicinal coupling constants in this spin system were all small (1.4 to 5.7 Hz), consistent with a 6-deoxytalose residue having a β anomeric configuration.

Methanolysis of peracetate **2** with HCl and MeOH yielded, after purification, the aglycon **3** and a mixture of the methyl glycosides of glucose, quinovose, and 6-deoxytalose (Figure 3). The aglycon **3** gave a $[M + NH_4]^+$ ion at *m/z* 316 in the CIMS and a $[M - H_2O]^+$ ion at *m/z* 280 in the EIMS consistent with a molecular formula of $C_{19}H_{38}O_2$. The ^{13}C/APT and ^1H NMR data for **3** identified a methyl ketone (C_6D_6: δ ^1H 1.64, s, 3H; ^{13}C 207), a carbinol methine (δ ^1H 3.76; ^{13}C 81.7), an aliphatic methyl (δ ^1H 0.90, t, $J = 6$ Hz), and 15 aliphatic methylene carbons. These data were only consistent with a C-19 linear aliphatic chain having a methyl ketone at one terminus, the aliphatic methyl at the other terminus, and a secondary alcohol at some point in the interior of the chain. The base peak in the HREIMS spectrum of **3**, which appeared at *m/z* 171.1386 corresponding to a molecular formula of $C_{10}H_{19}O_2$ (calcd 171.1385), was assigned to an α cleavage of the bond adjacent to the secondary alcohol on the side remote from the methyl ketone terminus (Figure 3). This fragmentation located the hydoxyl functionality at C-10 in the aglycon **3**. Analysis of the NMR data obtained for the peracetate **2** (Table 1, Supporting Information) confirmed the presence of the aglycon **3** (methyl ketone: δ ^1H 1.69, s, 3H and ^{13}C 206.0; carbinol methine: ^1H 3.76, m, 1H and ^{13}C 81.7; terminal methyl: ^1H 0.95, t, $J = 7.2$ Hz, 3H and ^{13}C 14.3). HMBC correlations observed between the aglycon carbinol methine resonance at δ 3.76 (H-10) and the anomeric carbon resonance of one of the glucose residues (δ 100.9, Glu1-C-1), and between the Glu1

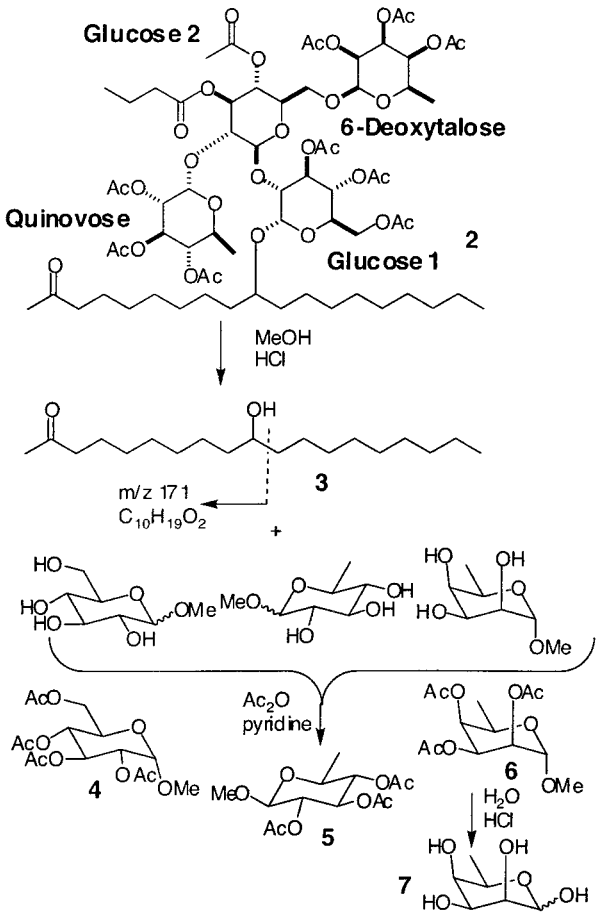

Figure 3. Chemical degradation of caminoside A peracetate (**2**).

anomeric proton resonance (δ 4.64) and the carbinol methine resonance at δ 81.7 (C-10) demonstrated their connection via an ether linkage.

The mixture of monosaccharide methyl glycosides obtained from the methanolysis reaction was peracetylated with Ac_2O/pyridine and the products were purified via HPLC to give α-1-methoxy-2,3,4,6-O-acetylglucose (**4**) and β-1-methoxy-2,3,4-O-acetyl-6-deoxyglucose (**5**) (Figure 3). Comparison of the NMR data and specific rotations for **4** and **5** with authentic material prepared from L-glucose and D-quinovose, using the reaction conditions described above for transformation of the natural product, confirmed the presence of these monosaccharides in caminoside A (**1**) and showed that the glucose residues had the D configuration and the quinovose residue had the L configuration. The NMR data obtained for 1-methoxy-2,3,4-*O*-acetyl-6-deoxytalose (**6**) were in complete agreement with the assigned structure. Authentic 6-deoxytalose was not available for comparison purposes, so **6** was hydrolyzed to the native sugar and its specific rotation and ^{13}C NMR data were compared with literature values,[5] confirming its identity and showing that it had the D configuration.

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HMBC data provided evidence for the nature of the linkages between the four monosaccharides. Correlations between the 6-deoxytalose anomeric proton resonance at δ 4.77 and the Glu2 C-6 resonance at δ 69.4 demonstrated that there was a 1,6-glycosidic linkage between the 6-deoxytalose and Glu2 residues. A strong HMBC correlation between the Glu2 H-2 resonance at δ 3.97 and the Qui anomeric carbon resonance at δ 96.2 established a 1,2 glycosidic bond between Qui and Glu2. Finally, a correlation between the Glu1 H-2 resonance at δ 4.21 and an anomeric resonance at δ 100.1 (Glu2-C-1) showed there was a 1,2-glycosidic link between Glu2 and Glu1. As described above, coupling constant data showed that glucose-1 and glucose-2 had β anomeric configurations and that quinovose had an α anomeric configuration. ROESY data showed that the 6-deoxytalose residue had the β anomeric configuration.

Comparison of the ESMS of the underivatized natural product mixture of caminosides, differing only in the aglycon fragment, injected in MeOH ($[M + Na]^+$ *m*/*z* 1049) and MeOD ($[M + Na]^+$ *m*/*z* 1058) showed that there were only 9 exchangeable protons, even though the identified tetrasaccharide fragment had 11 available hydroxyls. In addition, the NMR data for the underivatized natural product mixture contained resonances that could be assigned to acetyl (δ ^{1}H 1.96, s, 3H and ^{13}C 20.4, 169.2) and butyryl (δ ^{1}H 0.83, t, 3H; 1.47, m, 2H; 2.29, dt, 1H, 2.17, dt, 1H dt and ^{13}C 13.3, 17.7, 35.4, 172.0) residues, respectively. Therefore, caminoside A (**1**) had to already contain an acetate and a butyrate ester before derivatization with acetic anhydride to give the peracetate **2**.

With the structure of the tetrasaccharide fragment of caminoside A in hand from analysis of the NMR data for the peracetate **2**, it was possible to go back and assign ^{1}H and ^{13}C NMR resonances to the sugar portion of caminoside A (**1**) using the data for the mixture of natural products that differed only in the aglycon. Using these assignments for the underivatized compound, HMBC correlations were observed between the Glu2 H-4 resonance at δ 4.57 and the acetyl carbonyl resonance at δ 169.2 (Glu2-C-11), and between the Glu2 H-3 resonance at δ 5.16 and the butyryl carbonyl resonance at δ 172.0 (Glu2-C-7). This placed the butyrate ester at C-3 of Glu2 and the acetate ester at C-4 of Glu2, completing the structure of caminoside A and its peracetate as shown in **1** and **2**, respectively (Figure 1). The lack of dispersion in the methylene proton resonances near the C-10 region of the aglycon made it impossible to use Mosher-type methodology to determine the absolute configuration at C-10.

Sponges are emerging as a rich source of novel biologically active glycolipids.[6] Caminoside A (**1**) has several structural features not found in sponge glycolipids reported to date. It has a fully substituted glucose residue (Glu-2) embedded in the middle of the molecule, it contains a 6-deoxytalose residue, which is rare in nature, and the methyl ketone functionality in the lipid aglycon is without precedent in sponge metabolites.

Caminoside A (**1**) is the first natural product known to be active ($IC_{50} = 20$ μM) in a new bioassay designed to screen for type III secretion inhibitors that potentially represent novel agents to control pathogenic *E. coli* by thwarting their pathogenicity without actually killing the bacteria.[4d] The full significance of the biological activity displayed by caminoside A (**1**) in the new assay is currently under investigation in our laboratories. The type III secretion inhibitor screen was designed to select for compounds that did not display conventional antimicrobial activity against *E. coli*. To confirm that this was indeed the case for caminoside A (**1**), it was screened for traditional antimicrobial activity against a panel of human and plant pathogens. Caminoside A (**1**) showed reasonably potent in vitro inhibition of methicillin resistant *Staphylococcus aureus* (MIC: 12 μg/mL) and vancomycin resistant *Enterococcus* (MIC: 12 μg/mL). As expected, it did not inhibit the growth of gram negative pathogenic *E. coli* (MIC: >100 μg/mL).

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Supporting Information Available: Tables of NMR data, 1D and 2D NMR spectra for caminoside A (**1**) and the peracetate (**2**), and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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