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Nigricanosides A and B, Antimitotic Glycolipids Isolated from the Green Alga *Avrainvillea nigricans* Collected in Dominica

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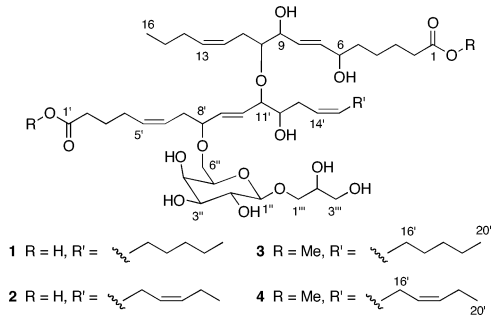
The widespread clinical use of the antimitotic plant natural products paclitaxel, vinblastine, vincristine, and the synthetic paclitaxel analogue Taxotere for the treatment of human cancer suggests that other compounds capable of causing mitotic arrest should be good candidates for development into new anticancer drugs.[1] Antimitotic natural products and synthetic analogues that have followed in the footsteps of paclitaxel and the Vinca alkaloids and entered anticancer clinical trials include the combretastatins, discodermolide, dolastatin-10, HTI-286, E7389, epothilone B, and cryptophycin 52.[1]

Most known antimitotic natural products were initially discovered because they exhibited potent in vitro cytotoxicity against either murine or human cancer cell lines and it was only later, upon detailed investigation of their mechanisms of action, that they were found to arrest cells in mitosis and target tubulin. A cell-based assay[2] developed in one of our laboratories provides a rapid and sensitive method to directly detect the presence of antimitotic agents in crude natural-product extracts.[3]



One of the extracts found to be highly active in the cell-based screen of our marine natural-product extract library came from the green alga *Avrainvillea nigricans* collected in Dominica. Assay-guided fractionation of the extract from our first collection of this alga failed to produce sufficient quantities of the active components for chemical analysis but it did show that the antimitotic compounds were extremely potent and present in only trace quantities. Repeated collection and pooling of the algal biomass during the intervening 8 years culminated in the isolation of sufficient quantities of the methyl esters of nigricanosides A (**1**) and B (**2**), two novel antimitotic glycolipids, for structure elucidation via cryoprobe-assisted NMR analysis. The details of the isolation, structure elucidation, and biological activities of the nigricanosides are presented below.

Specimens of *A. nigricans* (28 kg wet wt) were harvested by hand using SCUBA from reef flats near Portsmouth, Dominica, and extracted exhaustively with MeOH. The MeOH-soluble material was partitioned between H_2O and EtOAc, and the EtOAc-soluble portion was subsequently partitioned between hexanes/4:1 MeOH/H_2O and then CH_2Cl_2/2:1MeOH/H_2O. The concentrated residue from the antimitotic CH_2Cl_2 extract was methylated with trimethylsilyldiazomethane to facilitate its further fractionation. Methylated material was subjected, in sequence, to assay-guided normal phase flash, Sephadex LH-20, and reversed-phase flash column chromatographies. A final reversed-phase HPLC separation of antimitotic material gave pure samples of nigricanoside A dimethyl ester (**3**) (800 μg: 3×10^{-6}% wet wt) and nigricanoside B dimethyl ester (**4**) (400 μg: 1.5×10^{-6}% wet wt).

Nigricanoside A dimethyl ester (**3**) was obtained as an optically active ($[\alpha]_D$ −42) clear oil, that gave a $[M + Na]^+$ ion in the HRESIMS at *m/z* 923.5323 consistent with a molecular formula of $C_{47}H_{80}O_{16}$ (calcd for $C_{47}H_{80}O_{16}Na$, 923.5344) requiring eight sites of unsaturation. The resonances in the ^{1}H and ^{13}C NMR spectra of **3** acquired in 25:2 C_6D_6/DMSO-d_6 at 600 MHz with a cryoprobe were well dispersed. Forty-five resonances were observed in the 1D ^{13}C NMR spectrum between δ 14 and 137 ppm. Two additional carbonyl resonances at δ 173.4 (C-1) and 173.6 (C-1′), that were not observed in the 1D ^{13}C spectrum owing to the small sample size, were clearly identified from the HMBC data, thereby accounting for the forty-seven carbons indicated by the HRMS analysis. Ten olefinic methine resonances [δ 136.3 (C-7), 134.7 (C-9′), 131.3 (C-10′), 131.3 (C-13), 131.3 (C-15′), 130.5 (C-5′), 129.3 (C-8), 127.6 (C-14′), 127.5 (C-12), 127.0 (C-6′)] found in the ^{13}C NMR spectrum could be assigned to five olefins, which together with the two carbonyls accounted for seven of the eight required sites of unsaturation. The remaining site of unsaturation had to be present as a ring. HSQC data showed that only seventy-two of the eighty hydrogen atoms in **3** were attached to carbon (4 × Me, 19 × CH_2, 22 × CH). Consequently, there had to be eight hydroxyls, which were observed in ^{1}H NMR spectrum at δ 5.26 (OH-2″), 5.06 (OH-9), 4.79 (12′), 4.79 (OH-2″), 4.68 (OH-3″), 4.55 (OH-6), 4.39 (OH-3″), and 4.30 (OH-4″).

HMBC correlations observed between methyl resonances at δ 3.41 (MeO-1) and 3.37 (MeO-1′) and carbonyl resonances at δ 173.4 (C-1) and 173.6 (C-1′), respectively, showed that the carbonyls were part of methyl esters. COSY and HSQC data identified the twelve and fourteen carbon linear fragments and the two and three carbon fragments of dimethyl ester **3** shown in Figure 1A. HMBC correlations confirmed the structures of the twelve and fourteen carbon fragments and, as shown in Figure 1B, elaborated the twelve carbon linear fragment into a C-16 oxylipin methyl ester having $\Delta^{7,8}$ and $\Delta^{13,14}$ olefins and hydroxyls at C-6 and C-9. Similarly, the HMBC data showed that the fourteen carbon linear fragment was part of a C-20 oxylipin methyl ester having $\Delta^{5',6'}$ and $\Delta^{9',10'}$ olefins and a hydroxyl at C-12′. HMBC correlations

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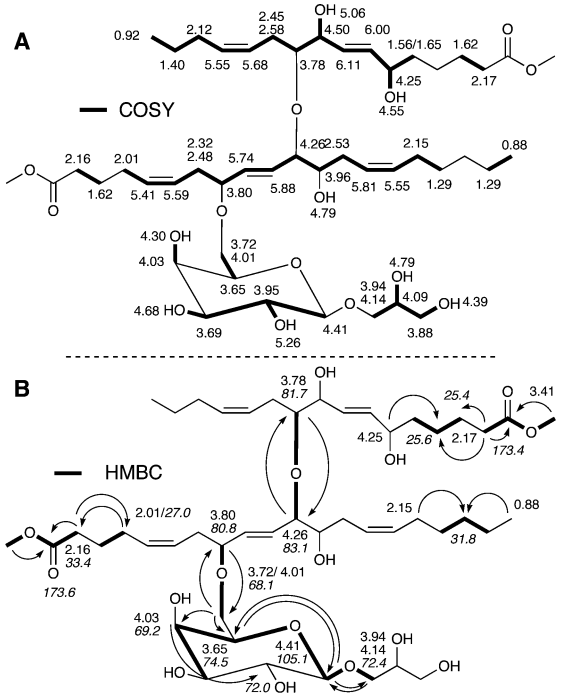

Figure 1. (A) Fragments of nigricanoside A dimethyl ester **3** (in bold) identified from HSQC and COSY data. (B) Chemical bonds (in bold) identified by the selected HMBC correlations shown for nigricanoside A dimethyl ester **3**.

observed between δ 3.78 (H-10) and 83.1 (C-11′) and between δ 4.26 (H-11′) and 81.7 (C-10) revealed that there was a C-10 to C-11′ ether connecting the C-16 and C-20 oxylipin chains (Figure 1B).

HMBC correlations shown in Figure 1B demonstrated that the remaining two and three carbon fragments identified from the COSY data were part of a hexapyranose joined to C-1 of a glycerol residue through a glycosidic linkage. The hexapyranose ring satisfied the last site of unsaturation required by the molecular formula of **3**. A final set of HMBC correlations observed between δ 3.80 (H-8′) and 68.1 (C-6″) and between δ 3.72/4.01 (H-6″) and 80.8 (C-8′) showed that there was an ether bond connecting C-8′ of the C-20 oxylipin and C-6″ of the hexapyranose, completing the constitution of **3** (Figure 1B).

H-7 (δ 6.00) and H-8 (δ 6.11) had a scalar coupling of 15.5 Hz, and H-9′ (δ 5.74) and H-10′ (δ 5.88) had a scalar coupling of 15.7 Hz, demonstrating that the $\Delta^{7,8}$ and $\Delta^{9',10'}$ olefins both had the E configuration. ROESY correlations observed between H-12 (δ 5.68) and H-13 (δ 5.55), between H-5′ (δ 5.41) and H-6′ (δ 5.59), and between H-14′ (δ 5.81) and H-15′ (δ 5.55) showed that the $\Delta^{12,13}$, $\Delta^{5',6'}$, and $\Delta^{14',15'}$ olefins all had Z configurations. H-1″ (δ 4.41) showed a 7.7 Hz coupling to H-2″ (δ 3.95) indicating a β glycosidic linkage to glycerol. ROESY correlations between H-1″ and both H-5″ (δ 3.65) and H-3″ (δ 3.69), and the absence of large couplings between H-4″ (δ 4.03) and either H-3″ or H-5″, identified the hexapyranose as galactose. Elucidation of the absolute configuration of dimethyl ester **3** is ongoing in our laboratory. The full details await the time-consuming isolation of additional compound.

Nigricanoside B dimethyl ester (**4**) was obtained as an optically active ($[\alpha]_D$ −34) clear oil, that gave a $[M + Na]^+$ ion in the HRESIMS at *m*/*z* 921.5186 consistent with a molecular formula of $C_{47}H_{78}O_{16}$ (calcd for $C_{47}H_{78}O_{16}Na$, 921.5188), requiring nine sites of unsaturation. Detailed analysis of the NMR data for **4** (Supporting Information) showed that it differed from **3** simply by having a Z $\Delta^{17',18'}$ olefin.

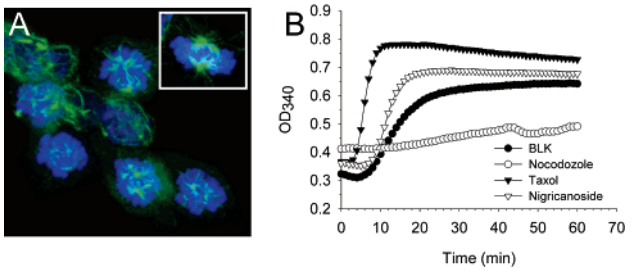

Figure 2. (A) Mitotic arrest phenotype elicited by nigricanoside dimethyl ester **3**. Cells were exposed to 10 nM **3** for 20 h, microtubules were visualized by immunostaining (green) and DNA with Hoechst 33342 (blue). The inset shows a control metaphase cell. (B) Effect of **3** on tubulin polymerization at 37 °C. All compounds are 10 μM. BLK = blank control.

Methylation of crude fractions during the isolation of **3** and **4** resulted in a change in chromatographic behavior and an apparent decrease in potency for the active components. Therefore, the natural products are probably the free dicarboxylic acids **1** and **2**. Nigricanoside A dimethyl ester **3** arrests human breast cancer MCF-7 cells in mitosis with an IC_{50} of 3 nM. Arrested cells have highly disorganized microtubule spindles, compared with the bipolar spindles of untreated metaphase cells (Figure 2). Ester **3** at 10 μM stimulates the polymerization of pure tubulin in vitro and it inhibits the proliferation of both MCF-7 and human colon cancer HCT-116 cells with an $IC_{50} \approx 3$ nM. Hydrogenation of the alkenes in **3** significantly reduces the antiproliferative activity against both cell lines ($IC_{50} \approx 300$ nM).

Monogalactosyldiacylglycerols are major membrane lipids in higher plants and blue green algae.[4] The normal structural architecture of these common lipids involves a glycosidic linkage between galactose and C-1 of glycerol and ester linkages between saturated or unsaturated fatty acids[5] and the other two alcohols on the glycerol residue. Although the nigricanosides contain the same components as the monogalactosyldiacylglycerols, the ether linkages connecting the oxylipins to each other and to the galactose residue are without precedent. Therefore, the nigricanosides are the first examples of a new class of ether-linked glycoglycerolipids. The potent antimitotic activity of the nigricanosides and their ability to promote tubulin polymerization is also without precedent among previously known glycoglycerolipids, and these biological activities make them potentially exciting anticancer drug leads.

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Supporting Information Available: Experimental details for the isolation and bioassays; tabulated NMR data and spectra for **3** and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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