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MOLLUSCICIDAL ACRIDONE ALKALOIDS FROM *ANGOSTURA PANICULATA*: ISOLATION, STRUCTURES, AND SYNTHESIS

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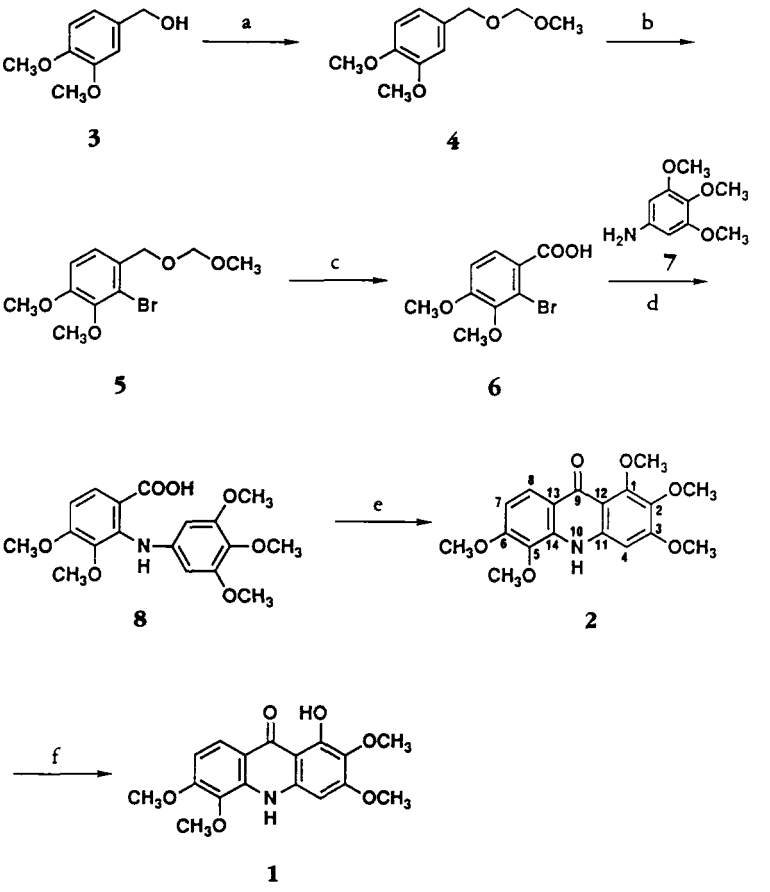
ABSTRACT.—Two novel acridone alkaloids, cuspanine [**1**] and cusculine [**2**], were isolated from the CH_2Cl_2 extract of the leaves of *Angostura paniculata* (Rutaceae). Their structures were established as 1-hydroxy-2,3,5,6-tetramethoxy-9-acridone for **1** and 1,2,3,5,6-pentamethoxy-9-acridone for **2** by means of spectroscopic studies, in particular nmr. These structural assignments were confirmed by synthesis, using a direct metallation method as a key reaction. Both alkaloids exhibited moderate molluscicidal activity against an aquatic snail, *Biomphalaria glabrata*, and cytotoxicity against several types of carcinoma cell lines.

We have recently described the isolation of various alkloids from Brazilian Rutaceous plants: two molluscicidal quinoline alkaloids from the leaves of *Galipea bracteata* (1), and two insect-growth-inhibitory 4-quinolinone alkaloids from the leaves of *Esenbeckia leiocarpa* (2). In our continuing search for biologically active phytochemicals, two novel acridone alkaloids have been isolated from the leaves of another Brazilian Rutaceous plant, *Angostura paniculata* (Engler) Elias. These alkaloids exhibited moderate molluscicidal and cytotoxic activities and were designated cuspanine [**1**] and cusculine [**2**]. Their structures were established based largely on nmr studies. However, the structures established are rather uncommon among the known acridone alkaloids. Therefore, in order to confirm the structures, their syntheses were carried out. This paper reports the isolation, structure determination, and syntheses of **1** and **2**.

RESULTS AND DISCUSSION

After repeated and various column chromatographies, the CH_2Cl_2 extract of the leaves of *A. paniculata* yielded two new acridone alkaloids, cuspanine [**1**] and cusculine [**2**]. In conjunction with both ^1H- and ^{13}C-nmr and eims data, the molecular formula $C_{17}H_{17}NO_6$ was assigned to cuspanine [**1**]. In addition, together with the uv and ir data, an acridone-type structure could be assigned to cuspanine. This was apparent because keto group signals, characteristic of acridone at δ 181.14 (s) and 1635 cm^{-1}, were observed. The presence of a free phenolic group was deduced from the uv spectrum by using $AlCl_3$ as the shift reagent. Thus, when a few drops of $AlCl_3$ solution was added to cuspanine in MeOH, a typical bathochromic shift was observed. However, the region of 3000–3600 cm^{-1} in the ir spectrum showed only weak and broad absorption, because the phenolic group forms a stable 6-membered ring intramolecular hydrogen bond with the keto group. The ^1H-nmr spectrum of **1** showed two aromatic proton signals at δ 6.92 and 8.06 ($J = 8.0$ Hz for each) which can be attributed to H-7 and H-8, respectively (3). This indicated the tetrasubstituted nature of ring C. The remaining signal at δ 6.23 (s) could be attributed to the aromatic proton at either H-2 or H-4. This was confirmed by ^1H nmr using nuclear Overhauser enhancement spectroscopy (NOESY). In the NOESY spectrum of cuspanine, an nOe was observed between the aromatic proton H-4 at δ 6.23 and the amine proton at δ 8.44. This result, itself, was conclusive for the assignment of the remaining proton to the 4 position in ring C. This



SCHEME 1. Syntheses of **1** and **2**. Reagents and conditions: (a) $MeOCH_2Cl$/$EtN(iPr)_2/CH_2Cl_2$; (b) *n*-BuLi/THF/0°; $Br_2CHCHBr_2$, (c) 47% HBr/EtOH/75°; Jones' reagent; (d) **7**/DMF/-K_2CO_3/Cu/140–150°; (e) PPA/xylene/140–150°; (f) concHCl/EtOH then $HClO_4$/EtOH reflux.

conclusion also established the position of the remaining MeO group at the 2 position of ring A. Therefore, the structure **1** was proposed for cuspanine.

Cusculine [**2**] is very closely related to cuspanine [**1**]. The only difference observed in the ^{1}H-nmr spectrum was an additional peak for an MeO group, suggesting the presence of five MeO groups in the molecule. In addition to this observation, the bathochromic shift observed in the uv spectrum of **1** was not detected with **2**, indicating that an MeO group was substituted for the phenolic group of **1**. This was further supported by the ^{13}C-nmr spectrum which showed three signals in lower field at δ 61.89, 61.53, and 60.94, indicating the presence of three hindered MeO groups (4) and leading to structure **2** for cusculine.

Cuspanine [**2**], 1,2,3,5,6-pentamethoxy-9-acridone, was synthesized from 2-bromo-3,4-dimethoxybenzoic acid [**6**]. The first method that was tried for the preparation of **6** was the bromination of isovanillin in refluxing $CHCl_3$, followed by methylation (Me_2SO_4) and oxidation (Jones' reagent). However, the bromination gave a poor yield (18%), so the following alternate approach, using a directed metallation method, was adopted.

The methoxymethyl ether (MOM ether) [**4**], prepared from 3,4-dimethoxybenzyl alcohol [**3**] and methoxymethyl chloride, was lithiated in Et_2O with *n*-butyllithium at 0°. The resulting yellow precipitates were treated with 1,1,2,2-tetrabromoethane (5) at −78°, and the mixture was gradually warmed to room temperature to give a bromide **5** with a 66% yield. Hydrolysis of the MOM ether of **5** with HBr in warm EtOH, followed by oxidation with Jones' reagent, gave **6** in a 69% yield. Ullmann condensation (6) of **6** and 3,4,5-trimethoxyaniline [**7**] in the presence of freshly prepared copper powder and K_2CO_3 in DMF gave **8** with a 56% yield appearing as orange plates. Treatment of **8** with polyphosphoric acid and xylene (7) at 150° gave **2** in the form of yellow prisms with a yield of 55%. Selective hydrolysis of the 1-MeO group of **2** was achieved by refluxing in MeOH with concentrated HCl and 70% $HClO_4$ to afford **1** (Scheme 1). The physical data for both **1** and **2** were identical with those of the natural products. The structure of **1** was unambiguously determined by a single crystal X-ray analysis (Figure 1).

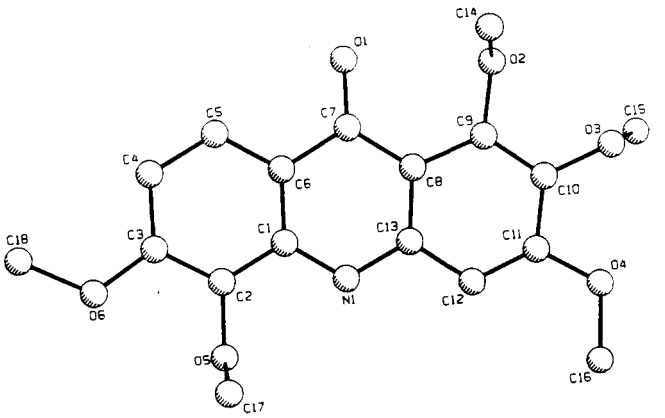


FIGURE 1. Molecular structure of 1,2,3,5,6-pentamethoxy-9-acridone [**2**] from X-ray crystallography.

Both alkaloids exhibited moderate molluscicidal activity against the aquatic snail *Biomphalaria glabrata*. The LC_{50} of cuspanine [**1**] was 5 ppm and that of cusculine [**2**] was 20 ppm. There are only a few examples in the literature (8,9) where alkaloids exhibiting high toxicity to snails are observed. These two alkaloids also showed moderate cytotoxic activity against several carcinoma cell lines. The IC_{50}'s of **1** and **2** against HeLa epithelioid carcinoma cells were 2.5 and 8.5 μg/ml, respectively. In addition, they also inhibited the growth of BT-20 human breast carcinoma cells.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All the procedures were the same as previously described (2).

ISOLATION OF CONSTITUENTS.—The leaves of *A. paniculata* were collected in Minas Gerais, Brazil, in February 1988, and identified by Dr. José R. Pirani, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP. A voucher specimen was deposited in the Herbarium of this institute. The dried pulverized leaves were first extracted with CH_2Cl_2 followed by MeOH at ambient temperature. A subsequent molluscicidal bioassay using *B. glabrata* indicated the CH_2Cl_2 extract to be active. The CH_2Cl_2 extract (2.983 g) was chromatographed over Si gel, using CH_2Cl_2, EtOAc, and MeOH. This process yielded 11 fractions. The bioactive fraction, eluted with EtOAc, was rechromatographed over Si gel (Lichroprep), affording cuspanine [**1**] (60 mg). The fractions eluted with MeOH after the same procedure gave cusculine [**2**] (200 mg).

Cuspanine, 1-hydroxy-2,3,5,6-tetramethoxy-9-acridone [**1**].—Yellow crystals: mp 164–166° (from MeOH); uv λ max (MeOH) nm (log ε) 212 (3.84), 256 (4.83), 324 (3.78), 384 (3.60); ir λ max (MeOH + $AlCl_3$) nm 220, 250, 272, 340, 420; ir ν max (KBr) cm^{-1} 1635, 1620, 1570, 1520, 1290, 1210, 1030; ^1H nmr ($CDCl_3$, 500 MHz) δ ppm 8.44 (1H, br s, NH), 8.06 (1H, d, J = 8.0 Hz, H-8), 6.92 (1H, d, J = 8.0 Hz, H-7), 6.23 (1H, s, H-4), 4.02, 4.00, 3.95, 3.90 (each 3H, s, OMe); ^{13}C nmr ($CDCl_3$, 125 MHz) δ ppm 181.14 (s, C-9), 159.41 (s, C-3), 155.53 (s, C-1), 154.52 (s, C-6), 138.06 (s, C-11), 135.02 (s, C-5), 133.72 (s, C-14), 130.27 (s, C-2), 122.11 (d, C-8), 114.74 (s, C-13), 107.57 (d, C-7), 104.55 (s, C-12), 87.58 (d, C-4), 61.05 (q, OMe), 60.88 (q, OMe), 56.23 (q, OMe), 56.08 (q, OMe). [These assignments are bsed on comparison with those of the data previously reported (10).] Eims *m/z* (rel. int.) 331 (90), 316 (100), 301 (10), 272 (12).

Cusculine, 1,2,3,5,6-pentamethoxy-9-acridone [**2**].—Yellow crystals: mp 223–224° (from MeOH); uv λ max (MeOH) nm (log ε) 220 (3.87), 264 (4.87), 312 (3.58), 372 (3.8); ir ν max (KBr) cm^{-1} 1680, 1610, 1570, 1260; ^1H nmr ($CDCl_3$, 500 MHz) δ ppm 8.30 (1H, br s, NH), 8.10 (1H, d, J = 8.0 Hz, H-8), 6.86 (1H, d, J = 8.0 Hz, H-7), 6.48 (1H, s, H-4), 4.00, 3.98, 3.95, 3.92, 3.88 (each 3H, s, OMe); ^{13}C nmr ($CDCl_3$, 125 MHz) δ ppm 176.17 (s, C-9), 157.98 (s, C-3), 154.58 (s, C-1), 153.93 (s, C-6), 139.37 (s, C-14), 138.07 (s, C-11), 134.35 (s, C-5), 133.56 (s, C-2), 123.05 (d, C-8), 117.42 (s, C-13), 110.36 (s, C-12), 106.97 (d, C-7), 93.77 (d, C-4), 61.89 (q, OMe), 61.53 (q, OMe), 60.94 (q, OMe), 56.12 (q, OMe), 56.03 (q, OMe).

3,4-Dimethoxybenzyl methoxymethyl ether [**4**].—To a solution of 3,4-dimethoxybenzyl alcohol (1.714 g, 10.2 mmol) and ethyl diisopropyl amine (5 ml, 28.7 mmol) in 20 ml of CH_2Cl_2 was added chloromethyl methyl ether (1.5 ml, 19.7 mmol) at 0°. The mixture was stirred for 2 h at 25° and 2 h at 34° and quenched with ice-H_2O. The organic layer was washed successively with dilute HCl, aqueous $NaHCO_3$, and saturated aqueous NaCl, dried (over $MgSO_4$), and concentrated in vacuo. Vacuum distillation afforded 1.972 g (93%) of **4** as a colorless oil: bp 85–87°/0.09 mmHg; ir ν max (neat) cm^{-1} 1610, 1595, 1515, 1265, 1150, 1040, 920, 855, 810, 750; ^1H nmr ($CDCl_3$, 60 MHz) δ ppm 3.41 (3H, s, CH_2OCHH_3), 3.87 (3H, s, OMe), 3.89 (3H, s, OMe), 4.54 (2H, s, $PhCH_2O$), 4.69 (2H, s, OCH_2O), 6.88 (3H, br s, Ph).

2-Bromo-3,4-dimethoxybenzyl methoxymethyl ether [**5**].—To a solution of **4** (1.552 g, 7.3 mmol) in 12 ml of anhydrous Et_2O was added *n*-butyllithium (1.6 M *n*-hexane solution, 5 ml, 8.6 mmol) at 0°. The resulting yellow suspension was stirred for 1 h at 0°, after which the mixture was cooled to −72° and a solution of 1,1,2,2-tetrabromoethane (1 ml, 8.6 mmol) in 5 ml of THF was added. The reaction flask was gradually warmed to room temperature and stirred overnight. Following the standard workup the crude product was purified by chromatography [Si gel, elution with C_6H_6-EtOAc (95:5)] to afford **5** as a yellow oil (1.41 g, 66%): ir ν max (neat) cm^{-1} 1595, 1485, 1265, 1150, 1030, 920, 810, 680; ^1H nmr ($CDCl_3$, 60 MHz) δ ppm 3.43 (3H, s, CH_2OMe), 3.86 (6H, s, 2 × OMe), 4.62 (2H, s, $PhCH_2O$), 4.73 (2H, s, OCH_2O), 6.87 (1H, d, J = 8.6 Hz, H-6), 7.17 (1H, d, J = 8.6 Hz, H-5).

2-Bromo-3,4-dimethoxybenzoic acid [**6**].—To a solution of **5** (0.5274 g, 2.04 mmol) in 10 ml of EtOH at 75° was added 47% HBr (1 ml), and the solution was stirred for 20 min. The mixture was poured into 8 ml of ice-cold saturated $NaHCO_3$, and the aqueous layer was extracted four times with EtOAc. The standard workup procedure afforded 2-bromo-3,4-dimethoxybenzyl alcohol as an oil which crystallized on standing (0.4298 g). Mp 80–81.5° (from Et_2O/*n*-hexane); ir ν max (neat) cm^{-1} 3350, 1585, 1480, 1400, 1260, 1025, 910, 800; ^1H n;mr ($CDCl_3$, 60 MHz) δ ppm 2.02 (1H, s, OH), 3.86 (3H, s, OMe), 3.87 (3H, s, OMe), 4.68 (2H, s), 6.87 (1H, d, J = 8.6 Hz, H-6), 7.15 (1H, d, J = 8.6 Hz, H-5). *Anal.* found C 43.87, H 4.53; calcd for $C_9H_{11}O_3Br$, C 43.75, H 4.49.

To a solution of 2-bromo-3,4-dimethoxybenzyl alcohol (0.42 g) in 10 ml of Me_2CO, Jones' reagent was added dropwise until the orange color persisted. The mixture was stirred overnight at room temperature. The solvent was removed in vacuo, and the residue was dissolved in H_2O and extracted with EtOAc. The combined extracts were dried and concentrated in vacuo. The residue was recrystallized from EtOAc/Et_2O to afford colorless needles (0.386 g, 69%): mp 218–219°; ir ν max (Nujol) cm^{-1} 2800–2600, 1700; ^1H nmr ($CDCl_3$/DMSO-d_6, 60 MHz) δ ppm 3.85 (3H, s, OMe), 3.96 (3H, s, OMe), 6.92 (1H, d, J = 8.6 Hz, H-5), 7.78 (1H, d, J = 8.6 Hz, H-6), 9.98 ppm (1H, br s, COOH). *Anal.* found C 41.40, H 3.46; calcd for $C_9H_9O_4Br$, C 41.41, H 3.47.

2-(3′,4′,5′-Trimethoxyphenyl)amino-3,4-dimethoxybenzoic acid [**8**].—To a solution of **6** (1.074 g, 4.1 mmol) and 3,4,5-trimethoxyaniline (0.90 g, 4.9 mmol) in 5 ml of DMF was added anhydrous K_2CO_3 (0.7 g, 5.1 mmol) and freshly prepared Cu powder (11) (0.32 g, 5.0 mgatom). The mixture was heated at 140–150° for 2 h under argon atmosphere. After removal of the solvent in vacuo, the residue was acidified with dilute H_2SO_4 and extracted with $CHCl_3$. The solvent was removed in vacuo, and the residue was partitioned between 1 M aqueous NaOH and $CHCl_3$. Undissolved material was removed by filtration through a layer of Celite. The organic layer was extracted with 1 M aqueous NaOH. The combined aqueous layers

were acidified with dilute HCl, extracted with $CHCl_3$, dried, and evaporated. The residue was recrystallized from EtOAc/*n*-hexane to afford orange plates (0.838 g, 56%): mp 162–164°, ir ν max (Nujol) cm^{-1} 1320, 2750–2500, 1650, 1595, 920, 840, 790, 740, ^{1}H nmr ($CDCl_3$, 60 MHz) δ ppm 3.50 (3H, s, OMe), 3.80 (9H, s, 3 × OMe), 3.93 (3H, s, OMe), 6.21 (2H, s, H-2′, H-6′), 6.68 (1H, d, J = 9 Hz, H-5), 7.90 (1H, d, J = 9 Hz, H-6), 8.27 (2H, br s, exchangeable with D_2O, NH and COOH). *Anal.* found C 58.35, H 5.76, N 3.54; calcd for $C_{18}H_{21}NO_7 \cdot \frac{1}{2}$MeOH, C 58.57, H 6.11, N 3.69.

1,2,3,5,6-Pentamethoxy-9-acridone [**2**].—To a mixture of freshly prepared polyphosphoric acid (from 1 g of P_2O_5 and 1 ml of H_3PO_4) and 10 ml of xylene, a solution of **8** (0.364 g, 1 mmol) in 2 ml of EtOAc was added. The mixture was mechanically stirred vigorously at 140–150° for 1 h. After the reaction flask was cooled to room temperature, H_2O was added, and the solution was made basic with 5% aqueous NaOH and extracted with $CHCl_3$. The organic layer was washed with saturated aqueous NaCl, dried, and evaporated. The residue was recrystallized from MeOH to afford yellow prisms (0.189 g, 55%), mp 241–243°. Other physical data were identical with those of the natural product. Crystal data for **2**: $C_{18}H_{19}O_6N$, M = 376.39, monoclinic, space group $P2_1/a$, a = 13.256 (6), b = 8.915 (3), c = 16.275 (3) Å, $\alpha = \gamma = 90$, β = 101.93 (2)°, V = 1881.9 (9) Å^3, Z = 4, F(000) = 796, $\mu_{(MoK\alpha)}$ = 0.95 cm^{-1}, D_c = 1.328 g/cm^3, graphite monochromated (Mo-Kα) radiation (λ = 0.7107 Å); 1719 reflections with $I > 3.00\sigma(I)$ used in refinement to R = 0.072. R_w = 0.098.[1]

1-Hydroxy-2,3,5,6-tetramethoxy-9-acridone [**1**].—To a solution of **2** (212 mg, 0.614 mmol) in 4 ml of MeOH was added 2 drops of concentrated HCl. The mixture was refluxed under argon atmosphere for 18 h, after which 1 drop of 70% $HClO_4$ was added and heating was continued for 4 h. The solvent was removed under reduced pressure, and the residue was extracted with $CHCl_3$. The extract was washed with aqueous $NaHCO_3$ and brine, dried, and evaporated. The residue was purified by chromatography [Si gel, elution with $CHCl_3$-MeOH (99:1)] to afford **1** (123 mg, 60%) as yellow needles, mp 161–163° (from MeOH). Further elution gave the recovered **2** (89 mg, 40%). Other physical data were identical with those of natural product.

CELL LINES AND MEDIA.—All cell lines used for the assay were purchased from American Type Culture Collection (Rockville, MD). They are HeLa ATCC CCL 219, epithelioid cerevix carcinoma from human, and BT-20 ATCC HTB 19, breast carcinoma from human. Both cell lines were maintained on minimum essential medium (Eagle) with non-essential amino acids supplemented with 10% (v/v) heat-inactivated fetal calf serum, and incubated in a humidified atomosphere of 5% CO_2-95% air at 37°. These human monolayer cultures were subcultured with a 0.25% trypsin/EDTA solution.

CYTOTOXICITY ASSAY.—The cytotoxicity assays were performed by a microculture tetrazolium (MYY) (Sigma) method (12). Briefly, cells were harvested and inoculated into 96-well microtitre plates at 4000–6000 cells/well, with various concentrations of the samples. After incubation, 50 µl MTT (3 mg/ml in PBS, pH 7.2) was added. The formazan dye ws solubilized by adding 100 µl DMSO to each well, followed by gentle shaking. The extinction coefficient was measured for each well using a Uniskan I Photometer Labsystems, at a wavelength of 620 nm.

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