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Artabotrine: A Novel Bioactive Alkaloid from *Artabotrys zeylanicus*

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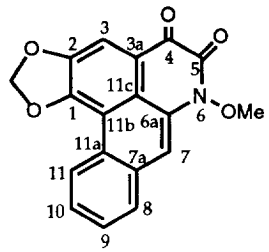
Abstract: The structure of artabotrine, an unprecedented bioactive *N*-methoxylated alkaloid from *Artabotrys zeylanicus*, has been deduced as *N*-methoxynorcepharadione A (**1**) from spectral data and single crystal X-ray analysis. The known oxoaporphine alkaloid, atherospermidine (**2**), was also isolated and shown to be active in a mechanism-based yeast bioassay.

In our continuing efforts to uncover anticancer active natural products with novel structural features[1] we have investigated the bioactive constituents in an extract derived from *Artabotrys zeylanicus* Hook. f. & Thoms., a plant of the Annonaceae family endemic to Sri Lanka, which showed DNA-modifying activity in our mechanism-based yeast bioasssay.[2,3] Bioactivity-guided fractionation resulted in the isolation of a novel N-methoxylated 4,5-dioxoaporphine alkaloid which we have named artabotrine (**1**) and an oxoaporphine alkaloid, atherospermidine (**2**),[4] both of which showed significant activity in our bioassay for potential anticancer activity. In this paper we report the structure elucidation of **1** and biological activities of **1** and **2**. Although there is no reported work on *A. zeylanicus*, previous investigations of *Artabotrys* species have resulted in the isolation of aporphine,[5-7] noraporphine,[5] oxoaporphine,[5,6] protoberberine,[5] and berberine[8] alkaloids, as well as sesquiterpenoids,[9] and steroids.[10]

RESULTS AND DISCUSSION

Chromatographic fractionation of a bioactive $CHCl_3$ extract of the stem bark of *A. zeylanica* employing the yeast bioassay as a guide afforded **1** as an orange-yellow crystalline solid, m.p. 287-289°C, and **2** as a dark orange gum. Both compounds were suspected to be alkaloids as they gave positive response to iodoplatinate and Dragendorff TLC spray reagents. The major alkaloid **1**, $C_{18}H_{11}NO_4$, exhibited two carbonyl bands in its IR spectrum at 1691 and 1660 $cm.^{-1}$ The UV spectrum of **1** had maxima at 450, 430, 310, 300, 280, 235, and 220 nm, some of which were characteristic of a 4,5-dioxoaporphine or related structure.[11]

The ^{1}H NMR spectrum of the major alkaloid in addition to a methoxy (δ 4.21, s) and a methylenedioxy (δ 6.48, s) group, had a 1H singlet (δ 8.12), a 1H ddd (δ 7.81) and four 1H dddd [δ 7.69, 7.70, 7.95, and 8.98 (see Table and the footnote g) in the aromatic region, accounting for all 11 protons. This ^{1}H NMR pattern can only be accommodated if the methylenedioxy group is placed at the 1,2 position of the aporphine skeleton. The ^{13}C NMR spectrum (Table 1), analyzed with the aid of a DEPT spectrum, showed the presence of one methyl, one methylene, six methine, and ten quaternary carbons. The ketone and amide carbonyl carbons in dioxoaporphines usually resonate at δ 178 and 157 ppm, respectively[11] and in **1** these two signals occurred at δ 175.4 and 152.0 ppm. Although the spectroscopic data, including HMBC NMR (Fig. 1 and Table 1), supported the structure **1** for artabotrine, the presence of an unprecedented *N*-methoxy function could not be established unequivocally. An X-ray diffraction analysis of a single crystal was thus undertaken.



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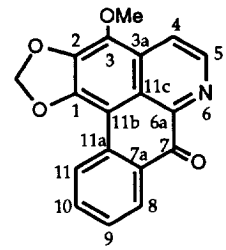

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Table 1. ^{1}H and ^{13}C NMR Spectral Data for 1 and 2.[a]

	1			2	
Atom	**δ_H[b]**	**δ_C[c]**	**HMBC**	**δ_H[b]**	**δ_C[c]**
1	-	151.6 (s)[e]	H-3	-	149.5 (s)
2	-	148.1 (s)	-	-	153.5 (s)
3	8.12 (s)[e]	109.3 (d)[d]	-	-	147.5 (s)
3a	-	122.9 (s)	-	-	145.1 (s)
4	-	175.4 (s)	H-3	8.10 (d, *J* = 8)	119.2 (d)[d]
5	-	152.1 (s)	H-3	8.84 (d, *J* = 8)	144.4 (d)[d]
6a	-	131.4 (s)	-	-	157.0 (s)
7	7.81 (ddd, J = 0.8, 0.6, 0.2)[e,g]	112.4 (d)[d]	H-8	-	182.5 (s)
7a	-	129.8 (s)	H-9	-	133.2 (s)
8	7.95 (dddd, *J* = 8.8, 1.6, 0.9, 0.8)[g]	128.9 (d)[d]	H-7, H-10	7.48 (t, *J* = 7)	127.6 (d)[d]
9	7.70 (dddd, *J* = 8.8, 8.0, 2.2, 0.2)[g]	127.9 (d)[d,e,f]	H-11	8.52 (dt, *J* = 7.5, 2)	128.7 (d)[d,e]
10	7.69 (dddd, *J* = 8.1, 8.0, 1.6, 0.2)[g]	126.8 (d)[d,e,f]	H-8	8.52 (dt, *J* = 7.5, 2)	126.6 (d)[d,e]
11	8.98 (dddd, *J* = 8.1, 2.2, 0.9, 0.6)[g]	128.5 (d)[d]	H-9	7.66 (dt, *J* = 7.5, 2)	133.8 (d)[d]
11a	-	125.9 (s)	H-8, H-10	-	136.2 (s)
11b	-	115.2 (s)	H-11	-	130.7 (s)
11c	-	118.9 (s)e	H-3, H-7	-	122.8 (s)
OCH_2O	6.48 (s)	103.2 (t)[d]	-	6.28 (s)	102.2 (t)[d]
OCH_3	4.21 (s)	63.3 (q)[d]	-	4.25 (s)	60.1 (q)[d]

[a]Recorded in $CDCl_3$ at 399.95 MHz (^{1}H) and 100.57 MHz (^{13}C).
[b]Assignment by comparison with related compounds.
[c]Multiplicities determined by DEPT experiments.
[d]Assignment based on HETCOR.
[e]Assignment based on HMBC.
[f]Values in the same column may be interchanged.
[g]Overlapping signals; Coupling pattern and constants determined by simulation experiments employing Varian VNMR software based on the FORTRAN program LAME (also known as LAOCOON) with magnetic equivalence added.

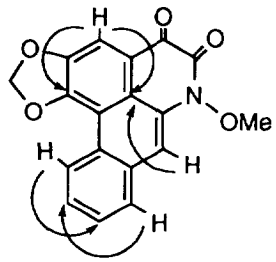


Fig. 1. Significant HMBC correlations observed for 1 (for full correlations, see Table 1)

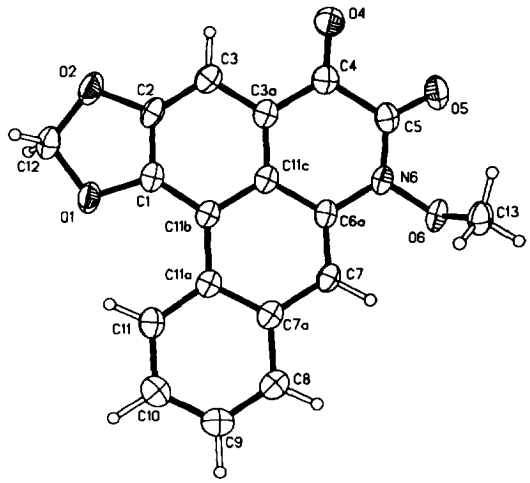
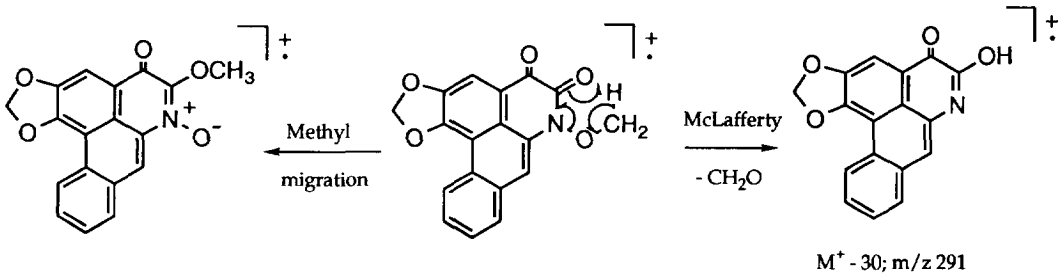


Fig. 2. X-ray structure of Artabotrine (1)



Scheme 1. MS fragmentations of 1 producing an N-oxide and a fragment ion at *m/z* 291

The crystal and molecular structures of artabotrine (**1**) were determined from three-dimensional X-ray diffraction data collected at reduced temperature. An ORTEP diagram is shown in Fig. 2, and the final atomic coordinates are given in Table 2. As no X-ray crystallographic data for 4,5-dioxoaporphines are available in the literature, comparisons were made with isatin-type structures. The isatin-type structure of **1** was supported by several lines of evidence; e.g. the C(4)-O(4) and the C(5)-O(5) distances of 1.218 and 1.215 Å, respectively, are equivalent within the error of the experiment. These would be expected to differ in the case of a molecule in which one of the sites represented a nitrogen-oxide moiety. For comparison also, a typical amine *N*-oxide bond distance would be in the range of 1.26 to 1.29 Å.[12] Further, the C(4)-C(5) bond distance of 1.529 Å, while larger than the 1.48 Å value expected for a normal Csp^2-Csp^2 single bond, is consistent with values reported for *cis*-diketone structures, and with the value reported for the structure of isatin.[13] Similarly, the C(5)-N distance of 1.359 Å compares well with the 1.352 Å value reported for isatin and *N*-methyl isatin[14] as does the N-C(6a) distance of 1.409 Å, as compared to 1.402 Å and 1.407 Å for isatin and *N*-methyl isatin,

respectively. Examples of molecules which incorporate the *N*-methoxy moiety are rare in the Cambridge Structural Database. However, one example showed an N-O distance of 1.410 Å.[15] In that case the N is attached to one sp^2 and one sp^3 carbon, and some difference from the 1.395 Å N-O distance observed in **1** would be expected. A second example was encountered in the recent literature where the N-O distance for methoxyimino N was reported as 1.411 Å.[16]

With the X-ray crystal structure in hand, we attempted to interpret the MS data for artabotrine (**1**). The HREIMS of **1** showed a significant peak at *m/z* 291 ($C_{18}H_{11}NO_5$) due to the loss of a molecule of formaldehyde from the molecular ion, presumably as a result of a McLafferty-type fragmentation of the molecular ion. A weak peak at *m/z* 305 ($C_{18}H_{11}NO_4$) is due to loss of oxygen from the molecular ion, presumably by loss from an *N*-oxide formed by migration of a methyl group (Scheme 1). These fragmentations further confirmed the proposed structure **1** for artabotrine.

Table 2. Atomic Coordinates (x10^4) and Equivalent Isotropic Displacement Coefficients (Å^2 x 10^3) for Artabotrine (1).[a]

Atom	x/a	y/b	z/c	U(eq)	Atom	x/a	y/b	z/c	U(eq)
O-1	2725(1)	-147(1)	11939(1)	38(1)	C-6a	2338(2)	91(1)	8958(1)	25(1)
O-2	1141(1)	1729(1)	12081(1)	42(1)	C-7	3296(2)	-981(1)	8853(1)	28(1)
O-4	-341(2)	3493(1)	9162(1)	48(1)	C-7a	3979(2)	-1716(1)	9534(1)	27(1)
O-5	190(1)	2466(1)	7671(1)	43(1)	C-8	5010(2)	-2802(1)	9407(1)	32(1)
O-6	1889(1)	249(1)	7517(1)	34(1)	C-9	5699(2)	-3503(2)	10054(1)	34(1)
C-1	2244(2)	340(1)	11189(1)	28(1)	C-10	5346(2)	-3160(2)	10846(1)	35(1)
C-2	1309(2)	1464(2)	11271(1)	30(1)	C-11	4333(2)	-2113(2)	10985(1)	32(1)
C-3	678(2)	2177(2)	10620(1)	31(1)	C-11a	3644(2)	-1344(1)	10335(1)	26(1)
C-3a	1028(2)	1704(1)	9846(1)	28(1)	C-11b	2636(2)	-181(1)	10439(1)	25(1)
C-4	416(2)	2457(2)	9134(1)	32(1)	C-11c	1976(2)	559(1)	9753(1)	25(1)
C-5	733(2)	1917(2)	8290(1)	31(1)	C-12	1989(2)	689(2)	12527(1)	36(1)
N-6	1663(1)	803(1)	8280(1)	29(1)	C-13	3256(2)	855(2)	7154(1)	38(1)

[a]Equivalent isotropic U is defined as one third of the trace of the orthogonalized U_{ij} tensor.

Based on its spectral data (Table 1 and Experimental), the minor bioactive alkaloid was identified as atherospermidine (**2**).[4] The bioactivity profile for artabotrine (**1**) and atherospermidine (**2**) in our mechanism-based yeast mutant bioassays are summarized in Table 3. Both **1** and **2** showed significant and selective activities in RS 321 and RS 322 YK (rad 52Y) assays compared with the wild-type RS 188N (RAD^+) strain. Cytotoxic activities of **1** were determined in two cell lines. Artabotrine (**1**) was found to be active in both wild-type and camptothecin-resistant P-388 leukemia cell lines (Table 2).

Table 3. Bioactivity Data for Artabotrine (1), Atherospermidine (2) and Camptothecin.[a]

Compound	Organism or cell line				
	RS 322YK (rad52Y)	RS 321N	RS 188N (rad+)	P-388 (wild-type)	P-388 (camptothecin-resistant)
1	2.16	1.20	>200	1.59	1.12
2	16	27	>50	NT[b]	NT[b]
Camptothecin	0.6	-	100	0.012	> 20

[a]Results are expressed as IC_{12} (RS 322YK, RS 321N, RS 188N) (μg/ml) or IC_{50} (P-388, wild-type and camptothecin-resistant cell lines) (μM) values.

[b]Not tested.

EXPERIMENTAL

General Experimental Procedures. These are identical to those reported in ref. 2; mp was determined on a Kofler hot stage apparatus and is uncorr.; UV spectra were recorded in EtOH with a Shimadzu UV 160 and Beckman DU 50 spectrometers; the IR spectrum was for a KBr disc on a Shimadzu IR 408 spectrometer; ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian Unity 400 spectrometer at 399.95 and 100.57 MHz, respectively, with TMS as internal standard; ^{1}H-^{1}H COSY, DEPT, ^{1}H-^{13}C HETCOR and HMBC NMR experiments were performed on the same spectrometer, using standard Varian pulse sequences; in HMBC determinations a 9 Hz optimization was employed for the long-range coupling pathways; MS were taken on a VG 7070 E-HF mass spectrometer; flash and medium pressure liquid chromatography (MPLC) used Si gel (230-400 mesh); a pressure pump was employed for MPLC; TLC and PTLC used Si gel and Whatman $LKC_{18}F$ plates.

Plant Material. The plant material was collected at Welimada in the Uva province of Sri Lanka, and identified by Mr. D.A.S. Wijesundera of the Peradeniya Royal Botanic Gardens. Voucher specimens have been deposited in the herbaria of the Peradeniya Royal Botanic Gardens and at the Medical Research Institute of Sri Lanka.

Bioassays. Identical to those reported in ref. 2.

Extraction and Isolation of Alkaloids. The dried and powdered stem bark of *A. zeylanicus* (2.5 kg) was successively and exhaustively extracted with hot hexane, $CHCl_3$ and MeOH. Evaporation under *vacuo* afforded hexane (15.0 g), $CHCl_3$ (19.0 g), and MeOH (22.0 g) extracts. Only the $CHCl_3$ extract was found to be bioactive. A portion (18.0 g) of the $CHCl_3$ extract was subjected to flash chromatography over Si gel with solvent gradients ranging from CH_2Cl_2 to CH_2Cl_2 containing increasing amounts of MeOH. The bioactive fraction eluted with 2.5% MeOH in CH_2Cl_2 on further purification by MPLC on Si gel and elution with 0.5% MeOH in $CHCl_3$ yielded artabotrine (**1**) as an orange-yellow solid (21.0 mg, 8.9 x 10^{-4}%). The late fractions of the MPLC on evaporation gave a brown solid which was separated by prep. RP-TLC (MeOH-H_2O, 85:15) affording a further quantity (2.0 mg) of **1** and atherospermidine (**2**) (1.5 mg, 6.3 x 10^{-5} %).

Artabotrine (1). Orange-yellow rods, mp 287-289°; UV (EtOH) λ_{max} (log ε) 450 (3.90), 430 (3.91), 310 (4.03), 300 (3.98), 280 (3.76), 235 (4.35), 220 (4.32) nm; IR (KBr) 1691, 1660, 1506, 1490, 1408,1363, 1318, 1236, 1048, 1013, 943, 740 cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 1; HREIMS, *m/z* (rel. int. %) 321 (M^+, 43) $C_{18}H_{11}NO_5$, 305 (5) $C_{18}H_{11}NO_4$, 291 (35) $C_{17}H_9NO_4$, 278 (100) $C_{16}H_8NO_5$, 263 (47) $C_{15}H_8NO_3$; M^+ 321.0638. Calc. for $C_{18}H_{11}NO_5$, M 321.0637.

Atherospermidine (2). Dark orange gum; UV (EtOH) λ_{max} (log ε) 315 (3.90), 280 (4.50), 247 (4.40); for ^{1}H and ^{13}C NMR data, see Table 1.

X-Ray Structure Determination of Artabotrine (1). Data Collection: A suitable crystal was flash cooled in a stream of N_2 gas. Lattice parameters were determined from the setting angles of 25 reflections well distributed in reciprocal space measured on an Enraf Nonius CAD-4 diffractometer. Nearly a full sphere of intensity data were collected on the diffractometer using graphite monochromated Cu radiation and an ω-20 variable speed scan technique. Three orientation controls were monitored to assess any crystal movement during the experiment. The intensities of 3 standard reflections measured at the beginning, every 3 h and at the end of exposure time showed an overall increase of 4%. Data were corrected for this variation and for Lorentz and polarization effects. No corrections were made for absorption. Equivalent reflections were averaged.

Crystal Data: Empirical formula, $C_{18}H_{11}NO_5$; formula weight: 321.28 amu; crystal color and habit: orange yellow, rods; crystal dimensions: a, 8.322(1); b, 10.007(1); c, 16.452(4) Å; β= 93.567(7)°; volume: 1367.4 (4)Å^3; SP. grp. $P2_1/n$; density (calc): 1.561 mgm^{-3}; F(000) 664 electrons; linear absorption coefficient μ (MoKα): 0.971 mm.$^{-1}$

Solution and Refinement. The structure was solved by direct methods using the SHELXS program series and refined using the SHELXL-93 refinement program. Non-hydrogen atomic positions were initially refined with isotropic displacement factors and subsequently with anisotropic displacement parameters. The function minimized was $\Sigma w(F_o^2-F_c^2)$.[2] Weights, w, were eventually assigned to the data as $w=1/[\sigma^2(F_o^2)+(0.0552P)^2+0.212P]$ where $P=[MAX(F_o^2,0)+2F_c^2]/3$. Hydrogen atom parameters were refined in the final model. The full-matrix least-squares refinement (on F^2) converged ($\Delta/\sigma_{max}=0.07$) to values of the conventional crystallographic residuals R=0.042 for observed data [I> 2σ(I)] and R=0.045 (wR2=0.113) for all data. A final difference Fourier map was featureless with residual density between $\pm 0.21 eÅ^{-3}$ Values of the neutral atom scattering factors were taken from the International Tables for X-ray Crystallography. The position of the N atom was determined by examination of the thermal parameters and difference electron density peaks in the one ring. Significant positive residual electron density was observed when this position was treated as a C atom. The Hamilton R-factor ratio test for two models, one of which has this position assigned as a carbon atom (R=0.205) and the other of which has the position assigned as a nitrogen (R=0.149), is consistent with the hypothesis that the position assigned as N(6) is a nitrogen at a 99.995% probability level.

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