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Novel *seco*-Dibenzopyrrocoline Alkaloid from *Cryptocarya oubatchensis*

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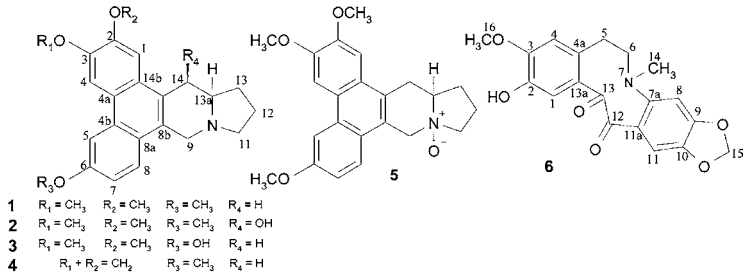
ABSTRACT



A novel *seco*-dibenzopyrrocoline alkaloid, named oubatchensine 6, and five phenanthroindolizidines (1–5) were isolated from *Cryptocarya oubatchensis*, and their structures were elucidated. Displacement centrifugal partition chromatography was used to purify compounds 1 and 6. Structure determination of the latter was carried out by mass spectrometry, NMR spectroscopy, quantum chemistry, and computer-assisted structure determination. Cytotoxic activity against KB cells was then investigated.

The biological screening of several extracts from Neocaledonian flora allowed the selection of *Cryptocarya oubatchensis* (Lauraceae) as a source of alkaloids with extremely promising in vitro cytotoxic activity against human carcinoma cell lines. The Lauraceae family, mainly found in western Asia, produces well-known aromatic evergreen trees or shrubs such as laurel, cinnamon, cassia, camphor, and avocado or deciduous plants such as sassafras. About 40 alkaloids have already been described in the *Cryptocarya* genus. Most of them are antitumoral, bactericidal, antimicrobial, fungicidal, insecticidal, or antioxidant agents.[1−3] *C. oubatchensis* has been previously investigated, and three quaternary dibenzopyrrocoline alkaloids were identified.[4] In the present study, compounds **1**−**5** were isolated from the leaves and compounds **1** and **6** were isolated from the bark of *C. oubatchensis*. The latter were isolated by pH-zone refining centrifugal partition chromatography (CPC),[5] a displacement mode that is specific to both acidic and basic analytes. CPC is a support-free liquid−liquid chromatographic technique that provides important benefits for natural compound purification, such as no sample loss on solid support and high selectivity and recovery.[6,7] Structure determination of **6** was supported by computer-assisted struc-

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ture determination,[8] and NMR chemical shifts were calculated by quantum chemistry tools.

The pulverized dried leaves and barks (respectively, 0.54 and 1.2 kg) were defatted with heptane and then submitted to typical alkaloid extraction.[9] The alkaloid extract from leaves (1.76 g) was successively submitted to normal-phase (alumina) and reversed-phase (C18 bounded silica) or gel permeation (Sephadex LH20) chromatographic solid supports leading to the isolation of 33 mg of **1** (0.006%), 10.3 mg of **2** (0.002%), 17.4 mg of **3** (0.003%), 10 mg of **4** (0.002%), and 6.5 mg of **5** (0.001%). From the alkaloid bark extract (1.9 g), the purification of **1** and **6** was performed by pH-zone refining centrifugal partition chromatography (CPC). In general, CPC displacement chromatography is performed by dissolving a displacer in the mobile phase and a retainer in the stationary one. By adding an acid or a base in the stationary phase as retainer, Ito[10] first introduced the pH-zone refining mode. For the first time in CPC, isotachic trapezoidal blocks of analytes separated by steep boundaries, so-called shock layers, were observed. This protocol is restricted to solutes that are ionizable and show a dramatic difference in solubility between their neutral and ionized forms. Alkaloids are thus good candidates for pH-zone refining CPC purification.

Experimental conditions were as follows. The solvent system was a mixture of methyl-*tert*-butylether/acetonitrile/water (4:1:5, v/v). Triethylamine (1.5 mM) was added to the aqueous mobile phase, and methanesulfonic acid (2 mM) was added to the organic stationary one. The column was first filled with the stationary phase. The alkaloids of the crude extract (1.9 g) were introduced into the column in their acidic form by adding methanesulfonic acid (pH 3). The basic mobile phase was pumped through in the ascending mode, and the alkaloids were progressively displaced in the organic phase by order of decreasing K_a. The flow rate was 8 mL/min, and the rotation speed was 1300 rpm, resulting in 48 bar back pressure and 72% stationary-phase retention. The effluent was monitored by UV absorbance at 220 nm. Purification by pH-zone refining gave two pure compounds. Compound **6** was displaced first (21.8 mg, 0.002%) followed by compound **1** (68 mg, 0.006%).

The structures of (−)-13aα-antofine **1**,[11a,b] (−)-14β-hydroxy-13aα-antofine **2**,[12] (−)-13aα-6-*O*-desmethyl-antofine **3**,[13a,b] ficuseptine C **4**,[14] and (−)-10β,13aα-antofine *N*-oxide **5** were found by comparison with their published spectroscopic properties.

Oubatchensine **6** showed a molecular ion peak at *m*/*z* 356.1122 (M + H$^+$) in TOF ESI + HRMS that suggested the molecular formula $C_{19}H_{17}NO_6$ (Δ −3.4 ppm), thus accounting for 12 insaturations. The ^{1}H, *J*-modulated ^{13}C, HSQC, HMBC, COSY, and NMR spectra showed 17 protons (including a labile one) and 19 carbons, among which were two CH_3, three CH_2, four CH, and ten C groups.

The HSQC spectrum showed eight quaternary and four methine aromatic carbons, suggesting the presence of two aromatic rings. HMBC data in Figure 1 and Table 1 revealed

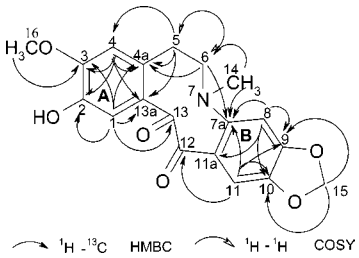

Figure 1. Key COSY and HMBC correlations for **6**.

the presence of two distinct correlation groups which lead to the individualization of the benzene ring resonances. Signals from H1, H4, H8, and H11 appeared as perfect singlets in the ^{1}H analysis and were therefore placed in the para position on each benzene ring. This information, along

Table 1. NMR Spectral Data of Oubatchensine (**6**)[a]

position	δ_C	DEPT	δ_H (mult.; *J* in Hz)	HMBC (H → C)
1	115.5	CH	7.27 (s)	2, 3, 4a, 13
2	145.1	C		
3	150.7	C		
4	112.1	CH	6.87 (s)	2, 3, 4a, 5, 13, 13a
4a	131.2	C		
5	31.1	CH_2	2.54 (m)	4, 4a, 6, 13a
6	59.9	CH_2	3.37 (m)	4a, 5, 7a, 14
			2.66 (m)	
7a	149.9	C		
8	103.9	CH	7.19 (s)	7a, 9, 10, 11a, 12
9	147.8	C		
10	155.5	C		
11	104.7	CH	7.24 (s)	7a, 9, 10, 11a, 12
11a	128.9	C		
12	195.7	C		
13	184.8	C		
13a	128.9	C		
14	41.1	CH_3	2.45 (s)	6, 7a
15	103.0	CH_2	6.16 (s)	9, 10
			6.18 (s)	
16	55.0	CH_3	3.93	3

[a] NMR experiments were performed in CD_3OD with TMS as an internal standard; ^{1}H, ^{13}C, COSY, HSQC, HMBC, and NOESY were acquired at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

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with the lack of relevant COSY correlations, led to the deduction that two 1,3,4,6-tetrasubstituted benzene rings were present in the molecule. Carbon C15 (δ 103.0) bound to two hydrogens (δ 6.16, 6.18) was identified as a methylenedioxy group being fused with the aromatic B ring, at positions 9 and 10 (see Figure 1 and Table 1).

The H16−C3 HMBC correlation indicated attachment of the methoxy group to position 3 of ring A. Positions 5 and 6 were linked together according to their COSY correlations. The C5−C6 fragment was connected to ring A as shown by the H5−C4, H5−C4a, and H5−C13a HMBC correlations. The chemical shifts of methyl group 14 ($\delta_H = 2.45$, s, and $\delta_C = 41.1$) were consistent with those of a *N*-methyl group. HMBC correlations of H14 with C6 and C7a allowed the connection of C6 to C7a through the nitrogen atom. This was confirmed by the H6−C7a HMBC correlation. Carbonyl groups C13 ($\delta_C = 184.4$) and C12 ($\delta_C = 195.7$) correlated with the aromatic protons in rings A and B, respectively. To keep the aromatic rings with two hydrogens in para positions, the carbonyl groups were then attached to C13a and C11a. This was supported by H1−C13 and H11−C12 correlations. The two carbonyl groups were connected together to complete the structure and to fulfill the insaturation number requirement.

The proposed structure was confirmed by means of the LSD structure elucidation program. The aim of the LSD program[8] is to find all possible molecular structures of an organic compound that are compatible with its NMR spectroscopic data. Structure building relies on connectivity data found in 2D NMR spectra (COSY, HSQC, HMBC) without any reference to a chemical shift database. Data in Table 1 was completed with the following constraints. Heteroatoms were only bonded to carbons. From their chemical shift value, C1, C4, C4a, C8, C11, C11a, and C13a were bonded to carbon atoms only. For the same reason, C2, C3, C6, C7a, C9, C10, C12, and C13 were bonded to a single heteroatom. Carbon 15, identified as an acetal, was bonded to two oxygen atoms. The presence of two six-membered aromatic rings was imposed, and one of them was fused with the methylene dioxy group at C15 to form a five-membered ring. At this stage, LSD produced 32 solutions. A quick inspection of the first ones showed that one or both carbonyl groups were placed within one or both aromatic rings. The solution set was then filtered to eliminate these cases and resulted in a set of eight (2^3) solutions. They corresponded to the assignments of inversion of positions 8 and 11, 9 and 10, and 2 and 3. The latter changes the structure itself. The long-range H16−C4 HMBC correlation favors the proposed structure. This point will be validated by theoretical value computations, as shown below.

Despite the similar environment that characterized C12 (δ_C 195.7) and C13 (δ_C 184.8), they displayed a significant difference in their chemical shift values. To explain and confirm the proposed structure of oubatchensine (**6**), ^{1}H and ^{13}C NMR chemical shifts and spin−spin coupling constants were calculated with quantum chemistry tools using the GAUSSIAN 03 software package.[15] The geometry was optimized at the HF-DFT(B3LYP)[16−18] level using the 6-31G(d) basis set. Chemical shifts were subsequently predicted at the B3LYP/6-311++G(2d,p) level using the GIAO method,[19,20] relative to the absolute shielding constants of TMS obtained at the same level of theory. Three conformers were identified, and their existence was confirmed by frequency analysis. Boltzmann weighting according to the relative energy of conformers showed that the reported one (see Figure 2) was dominant. It should be noted

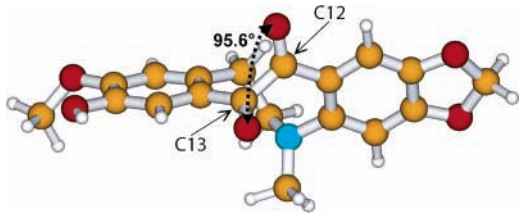

Figure 2. Lowest-energy conformer of **6**, showing the different environments of both the C12 and C13 carbonyl groups.

that in this favorable conformation, the dihedral angle involving the two carbonyl double bonds was 95.6°. The calculated results agreed favorably with our NMR experimental data.

In particular, the relative chemical shift values of C12 and C13 were well reproduced by our calculations (203.7 and 199.0, respectively). To keep the computational CPU time to reasonable limits, the HF-DFT(B3LYP)/6-31G(d) formalism was applied to qualitatively estimate the nuclear spin−spin coupling constants.[21−23] The connection of the methoxy group at C3 can be justified by the observation of the H16−C4 HMBC correlation and the predicted values of ^4J (H16−

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C4) and ^5J (H16−C1). Their order of magnitudes are, respectively, 0.2 and 0.01 Hz. The assignments of H1 and H4 are confirmed by the predicted values of ^3J (H1−C13) and ^4J (H4−C13). The intensity ratio of the observed HMBC correlations, I(H1−C13)/I(H4−13), is about 2. The HMBC experiment was optimized for the observation of 7 Hz coupling constants, and therefore, the correlation intensity is an increasing function of the coupling constant when it is less than 7 Hz. Our assignments are thus confirmed by the ratio of the calculated coupling constants: J(H1−C13)/J(H4−13) ≈ 3.

The bioactivity of compounds **1**−**6** was evaluated on human KB carcinoma cells (Table 2). Phenanthroindolizine analogues **1**−**3** exhibited very pronounced cytotoxicity toward the KB cancer cell line, with IC_{50} values ranging from 1 to 6.4 nM. Conversely, compounds **4**−**6** displayed low to null activity. As described in previous studies,[13b,24] the rigid *o*-phenanthrene structure is responsible for the high cytotoxicity of these secondary metabolites. Cytotoxicity significantly increases for **3** relative to **1**, due to the presence of the hydroxyl group at C6. The presence of the methylene dioxy group instead of methoxy groups at positions 2 and 3 significantly increases the IC_{50} values from **1** to **4**. The basic nitrogen atom in **1**−**4** also played an important role because the *N*-oxide derivative **5** only showed low activity. Oubatchensine **6**, which belongs to the *seco*-dibenzopyrrocoline group, was inactive against human KB carcinoma cells.

Table 2. Cytotoxicity of Compounds **1**−**6** Isolated from *Cryptocarya oubatchensis* toward Human KB Carcinoma Cells

	compound						
	1	**2**	**3**	**4**	**5**	**6**	docetaxel
IC_{50} values (nM)	4.3	6.4	1	inactive[a]	120	inactive[a]	0.2

[a] Inactive = IC_{50} value > 500 nM.

In conclusion, CPC pH-zone refining purification of an alkaloid extract from *Cryptocarya oubatchensis* led to the isolation of oubatchensine **6**, a molecule that, to our knowledge, belongs to a new *seco*-dibenzopyrrocoline natural compound class. Oubatchensine is structurally close to cryptowolinol, a dibenzopyrrocoline isolated from *C. phylostemon* and *C. oubachensis*.[4] Structure elucidation required both theoretical chemistry and artificial intelligence support. Biological assays confirmed that phenanthroindolizidine-type alkaloids are potent antitumor agents. Although some dibenzopyrrocolines are known to present interesting biological activities, this was not the case for *seco* compound **6** in our tests.

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Supporting Information Available: ^{1}H and ^{13}C NMR, high-resolution mass spectrum of oubatchensine (**6**), and the corresponding LSD data set. This material is available free of charge via the Internet at http://pubs.acs.org.

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