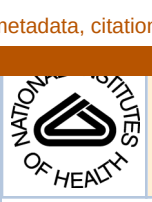

Published in final edited form as:

Nat Prod Commun. 2012 June ; 7(6): 725–727.

Cytotoxic and Anti-HIV Phenanthroindolizidine Alkaloids from *Cryptocarya chinensis*

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Abstract

Bioassay-guided fractionation of the cytotoxic ethanol extract of *Cryptocarya chinensis* has led to the isolation of 11 compounds, including two phenanthroindolizidine alkaloids [(−)-antofine (**1**) and dehydroantofine (**2**)], five pavine alkaloids (**3–7**), and four proaporphine alkaloids (**8–11**). The structures of the isolated compounds were determined by means of NMR spectroscopic methods, and supported by HRMS and optical rotation data. Compounds **1** and **2** showed cytotoxic activity against four cancer cell lines, L1210, P388, A549, and HCT-8, with **1** being the most potent against A549 and HCT-8 with EC_{50} values of 0.002 and 0.001 μg/mL, respectively. In addition, **2** is first reported to exhibit significant anti-HIV activity.

Keywords

Cryptocarya chinensis; Phenanthroindolizidine alkaloid; Pavine alkaloids; Proaporphine alkaloids

Since the first isolation of tylophorine in 1935 from the perennial climbing plant *Tylophora indica* [1], the number of phenanthroindolizidine alkaloids reported has grown considerably, presently encompassing close to 100 structurally related phenanthroindolizidines and phenanthroquinolizidines, together with their *seco*-derivatives and *N*-oxides [2]. These alkaloids exhibit various biological effects, including antitumor, antiamoebic, antibacterial, antiviral, anti-inflammatory, antimicrobial, antioxidant, and antifungal activities [2]. In folk medicine, phenanthroindolizidine alkaloids have been used as emetic, expectorant, and antipyretic agents [3], These alkaloids have been isolated principally from the Asclepiadaceae family, most importantly from the genera *Tylophora, Vincetoxicum, Pergularia,* and *Cynanchum,* but also from *Hypoestes* spp. (Acanthaceae), *Cryptocarya* spp. (Lauraceae), and *Ficus* spp. (Moraceae) [4].

The genus *Cryptocarya,* which includes about 350 species, is a rich source of secondary metabolites, such as flavonoids, pyrones, lignans, terpenoids, pavine, and proaporphine alkaloids [5]. *C. chinensis* (Hance) Hemsl. (Lauraceae) is an evergreen tree widely distributed in low-altitude forests in Taiwan and southern China [6]. Few phenanthroindolizidine alkaloids have been reported previously for this species [7]. Herein,

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we report the isolation of (−)-antofine (**1**) as a potent cytotoxic principle and dehydroantofine (**2**) as an anti-HIV agent. In addition, other types of alkaloids (**3–11**) were also isolated from this plant.

From the basic extract of *C. chinensis* wood, bioassay-guided fractionation and isolation led to the identification of the two active alkaloids (**1** and **2**). Comparison of ^{1}H and ^{13}C NMR spectra and optical rotation data with those from the literature suggested **1** to be (−)-antofine [8]. This alkaloid was previously isolated from *C. phellostemmon* and assigned the absolute configuration 13a*R* from its CD spectrum [7]. Thus, while phenanthroindolizidine alkaloids with the *S* configuration at C-13a exhibit a positive optical rotation measured at the sodium D line and a positive Cotton effect around 260 nm, the opposite is observed for alkaloids with the 13a*R* configuration [9]. The CD spectrum of **1** was in agreement with this rule. Thus, alkaloid **1** was unambiguously identified as (−)-(*R*)-13aα-antofine[8, 10].

Compound **2**, obtained as a yellow crystalline solid, has the molecular formula $C_{23}H_{27}NO_5$, as determined from HR-FAB MS data. The strong absorption bands in the UV spectrum at 322 nm and 283 nm were similar to those of dehydro derivatives of phenanthroindolizidine alkaloids [11], Examination of coupling patterns in the ^{1}H NMR spectrum and connectivities in the COSY and NOESY spectra of **1** and **2** allowed identification of the latter as a 9, 14-didehydro derivative of **1**. This assignment was supported by the appearance of two strongly deshielded proton singlets at δ 9.68 and 8.51 assignable to H-9 and H-14 of ring D, which showed NOESY correlations with H-8 and H-1, respectively. From all the data, the structure of **2** was postulated as dehydroantofine. To the best of our knowledge, this compound was previously isolated from *C. phyllostemon* and identified on the basis of its ^{1}H NMR spectrum alone [7]. Hence, we provide here the detailed spectroscopic data for the first time (Table 1).

Cytotoxic activity of the isolated alkaloids (**1–11**) was assessed *in vitro* against human leukemia (L-1210), murine lymphocytic leukemia (P-388), human pulmonary cancer (A-549), and human colon cancer (HCT-8) cell lines. Compound **1** exhibited significant cell growth suppression against all four cell lines with EC_{50} values less than 0.1 μg/mL, whereas its dehydro analogue, **2**, showed only moderate activity. In prior structure-activity relationship (SAR) studies of various natural cytotoxic phenanthroindolizidine alkaloids, it was found that (i) a rigid phenanthrene structure is a prerequisite for significant cytotoxic activity, (ii) the unshared electron pair on nitrogen is important for high potency, and (iii) the cytotoxic potency is highly sensitive to the substitution types and patterns on the phenanthrene ring [12]. As expected, based on the prior SAR, the degree of unsaturation in the indolizidine played a crucial role in the cytotoxic effects of phenanthroindolizidines **1** and **2**. While the planarity and rigidity of the indolizidine in dehydro **2** would be increased by the presence of double bonds adjacent to the phenanthrene nucleus, the cytotoxic activity of **2** was substantially reduced relative to **1**.

Interestingly, in an in *vitro* anti-HIV assay, **2**, but not **1**, suppressed HIV-infected H9 cell growth significantly, with an EC_{50} value of 1.88 μg/mL (Table 2). This promising finding is the first example of a dehydrophenanthroindolizidine alkaloid exhibiting anti-HIV activity. Thus, this compound type might provide a useful lead for anti-AIDS drug development.

In addition, other types of alkaloids were also purified from the active fraction. These compounds were pavine alkaloids **3–7** [(−)-6-hydroxycrychine (**3**), (−)-12-hydroxyeschscholtzidine (**4**), (+)-eschscholtzidine (**5**), (+)-eschscholtzidine-*N*-oxide (**6**), and (−)-argemonine (**7**)], as well as proaporphine alkaloids **8–11** [cryprochine (**8**), isocryprochine (**9**), (+)-8,9-dihydrostepharine (**10**), and prooxocryptochine (**11**) [13].

In conclusion, the isolation of **1** and **2** from *C. chinensis* has been reported for the first time. Based on the cytotoxicity data of **1** and **2**, the degree of unsaturation of the indolizidine moiety played a crucial role in the cytotoxic potency, with **1** being significantly more potent than its dehydro analogue **2**. Most notably, this report is the first to show significant anti-HIV activity with a dehydrophenanthroindolizidine (**2**).

Experimental

General

Melting points, Yanagimoto MP-S3 micro melting point apparatus; Optical rotations, JASCO DIP-370 digital polarimeter; UV, Hitachi U-3210 spectrophotometer; IR, Shimadzu FT-IR Prestige-21 spectrophotometer; 1D and 2D NMR, Bruker AVANCE-300 spectrometers; FABMS, JEOL JMS-700 spectrometer; EIMS, VG-70-250S spectrometer; CC, silica gel (70–230 mesh, 230–400 mesh). TLC was conducted on precoated Kieselgel 60 F 254 plates (Merck) and the compounds were detected either by examining the plates under a UV lamp or by treating the plates with a 10% methanolic solution of *p*-anisaldehyde followed by heating at 110°C.

Plant materials

The wood of *C. chinensis* was collected from Kaohsiung Hsien in Taiwan in July 2004 and identified by Prof. C. S. Kuoh, Department of Life Science, National Cheng Rung University. A voucher specimen (Kuoh 040007) was deposited in the Herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and isolation

The plant material (10.0 kg) was powdered and extracted with 95% EtOH (10 × 25 L) at room temperature and filtered. The combined filtrate was concentrated and dissolved in 3% HOAc. The acidic solution was neutralized with NH_4OH (aq) and the alkaloids were extracted with $CHCl_3$. This alkaloidal layer was extracted with 2% NaOH, and then the organic layer was acidified with 2% H_2SO_4. The resulting acidic solution was neutralized with 10% NaOH and partitioned with diethyl ether. (−)-Antofine (**1,** 18 mg) was obtained directly from the diethyl ether condensate and recrystallized from acetone. After filtration of **1**, the remaining bioactive condensate (11 g) was chromatographed on silica gel and eluted with a gradient of benzene and acetone (19:1–1:1) to afford **2** (10.8 mg), **3** (16.1 mg), **4** (12.3 mg), **5** (5.1 mg), **6** (8.1 mg), **7** (13.2 mg), **8** (6.7 mg), **9** (38.5 mg), **10** (3.2 mg), and **11** (1.9 mg).

Dehydroantofine (2)

$C_{23}H_{26}NO_4$, yellow crystals.

MP: 280°C (dec).

UV (MeOH) λ_{max} (log ε): 372 (3.70), 322 (3.80), 302 (3.20), 283 (3.10), 258 (3.60) nm

IR(KBr) ν_{max}: 3353 3103, 1610, 1521, 1475, 1276 cm^{-1}.

Cytotoxicity assay

Compounds were tested according to procedures described by Rubinstein *et al.* [14] against L-1210, P-388, A-549, and HCT-8 cancer cell lines. Samples were prescreened at 40, 4, 0.4, and 0.04 or at 50, 5, 0.5, and 0.05 μg/mL, against selected HTCL with duplicate dose treatments and three-day exposure. Results between replicates varied by no more than 5%.

Anti-HIV assay

This assay was performed using methods as described previously in the literature [15].

Acknowledgments

The authors are grateful for financial support from the National Science Council, Taiwan, Republic of China (NSC 96-2628-M-006-002) awarded to T. S. Wu. This study was also supported in part by NIH grant number AI33066 awarded to K. H. Lee and the Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004). Thanks are also due to Dr L.M. Cosentino of Biotech Research Laboratories, Rockville, MD, for the anti-HIV assay.

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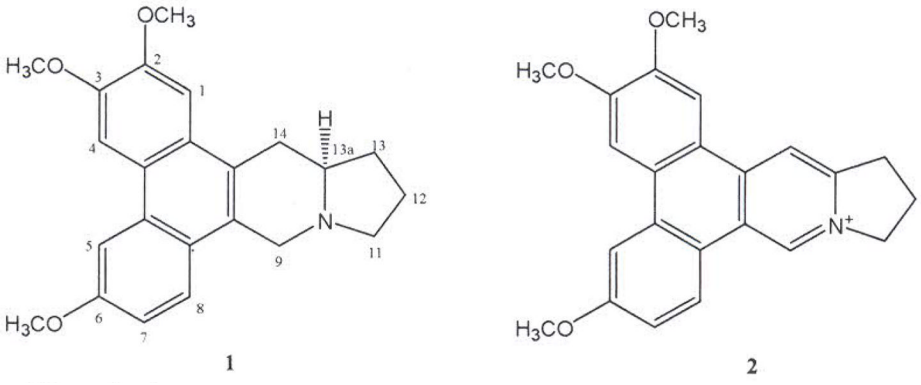

Figure 1.
Chemical structures of (−)-antofine (**1**) and dehydroantofine (**2**).

Table 1

^{1}H and ^{13}C NMR spectroscopic data of dehydroantofine (**2**) in CD_3OD.

position	δ_c	δ_{II}	HMBC	NOESY
1	106.7	7.50 (1H, s)		H-14, OCH_3-2
2	151.0		H-1, H-4, OCH_3-2	
3	159.3		H-1, H-4, OCH_3-3	
4	105.1	7.20 (1H, s)		H-5, OCH_3-3
4a	128.3		H-1, H-4	
4b	132.0		H-4, H-5, H-8	
5	117.1	7.22 (1H, d, *J*=2.4Hz)	H-7	H-4, OCH_3-6
6	161.6		H-5, H-7, H-8, OCH_3-6	
7	106.2	7.10(1H, dd, *J*=8.8, 2.4 Hz)	H-8	OCH_3-6, H-8
8	125.8	8.20 (1H, d, *J*=8.8Hz)	H-7	H-7, H-9
8a	119.4		H-5, H-7, H-9	
8b	125.3		H-14	
9	137.6	9.68 (1H, s)	H-11	H-8
11	59.5	4.87 (2H, t, *J*=7.2 Hz)	H-12, H-13	H-12
12	22.8	2.60(2H, p, *J*=7.2 Hz)	H-11, H-13	H-11, H-13
13	32.3	3.53 (2H, t, *J*=7.2 Hz)	H-11, H-12, H-14	H-12
13a	151.2		H-9, H-11, H-13	
14	117.3	8.51 (1H, s)	H-13	H-1
14a	139.3		H-1, H-9	
14b	120.4		H-4, H-14	
OCH_3-2	56.7	3.98 (3H, s)		H-1
OCH_3-3	56.2	3.99 (3H, s)		H-4
OCH_3-6	57.0	3.95 (3H, s)		H-5, H-7

[a] Abbreviations: s single, d doublet, dd double doublet, t triplet multiplet.

[b] *J* in Hz.

Table 2

Cytotoxicity and anti-HIV data of **1** and **2** isolated from *Cryptocarya chinensis.*

Cmpd	EC_{50}(μg/mL)				Anti-HIV
	L-1210	P-388	A-549	HCT-8	
1	0.1	0.1	0.002	0.001	-
2	3.29	2.14	1.73	2.82	1.88

-: no activity, L-1210: leukemia, P-388: murine lymphocytic leukemia, A-549: human pulmonary cell line, HCT-8: human colon cancer cell line, HIV: human immunodefic virus.