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Cytotoxic Activity of Some Phenanthroindolizidine *N*-Oxide Alkaloids from *Cynanchum vincetoxicum*

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Two previously known phenanthroindolizidine alkaloids, (−)-10β-antofine *N*-oxide (**1**) and (−)-10β,13aα-14β-hydroxyantofine *N*-oxide (**2**), and a novel alkaloid, (−)-10β,13aα-secoantofine *N*-oxide (**3**), were isolated from aerial parts of *Cynanchum vincetoxicum*. Their structures were established by means of NMR methods, including COSY, NOESY, HSQC, and HMBC experiments, as well as from their CD spectra. Cytotoxic activity of the alkaloids was assessed in vitro using both a drug-sensitive KB-3-1 and a multidrug-resistant KB-V1 cancer cell line. The antofine derivatives (**1** and **2**) showed pronounced cytotoxicity against the drug-sensitive cell line (IC$_{50}$ values about 100 nM), whereas the secoantofine derivative (**3**) was considerably less active. The KB-V1 cell line showed a marginal resistance against all alkaloids, demonstrating that these compounds are poor substrates for the P-glycoprotein (P-170) efflux pump.

The phenanthroindolizidine alkaloids are characteristic constituents of *Cynanchum*, *Pergularia*, *Tylophora*, and some other genera of the Asclepiadaceae.[1−4] The alkaloids exhibit pronounced cytotoxicity[5−9] and inhibit enzymes involved in the synthesis of DNA.[10,11] In this work, we describe the cytotoxic activity of three phenanthroindolizidine *N*-oxides, isolated from *Cynanchum vincetoxicum* (L.) Pers. (syn. *Vincetoxicum officinale* Moench, *Vincetoxicum hirundinaria* Medic.), against wild-type and multi-drug-resistant cancer cell lines. In addition to the previously known (−)-10β,13aα-antofine *N*-oxide (**1**) and (−)-10β,13aα-14β-hydroxyantofine *N*-oxide (**2**), a novel alkaloid, (−)-10β, 13aα-secoantofine *N*-oxide (**3**), is described.

1 R = H
2 R = OH
3
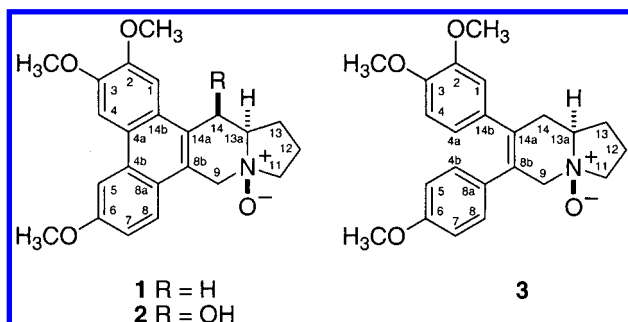
C. vincetoxicum is a small, polymorphic herb frequently encountered in Europe. The plant has been known for its poisonous and medicinal properties since medieval times. Previous studies have demonstrated the presence of several phenanthroindolizidine alkaloids of the antofine type,[12−15] as well as some unidentified alkaloids.[16]

Analysis of the alkaloid fraction of *C. vincetoxicum* by TLC showed the presence of an apolar cluster and a polar cluster of alkaloids. Because the bases of *C. vincetoxicum* have already been well investigated,[12−15] the present study was confined to the analysis of polar alkaloids. The polar fraction was resolved by normal-phase preparative HPLC,

to give three phenanthroindolizidine *N*-oxides (**1**−**3**) in a total amount corresponding to 0.00152% (w/w) of dry weight of the plant.

HRMS of **1** and **2** established their molecular formulas as C$_{23}$H$_{25}$NO$_4$ and C$_{23}$H$_{25}$NO$_5$, respectively, suggesting **2** to be a hydroxylated analogue of **1**. Comparison of ^{1}H and ^{13}C NMR spectra (see Supporting Information) and optical rotation data with those from the literature[14] suggested **1** to be (−)-10β-antofine *N*-oxide. Examination of coupling patterns in the ^{1}H NMR spectra and connectivities in the COSY and NOESY spectra of **1** and **2** allowed identification of the latter as a 14β-hydroxy derivative of **1**. Thus, the β-configuration of the 14-hydroxy group was shown by a small coupling and a strong NOE between H-13a and H-14. The α-configuration of H-13a in both compounds follows from NOEs between this proton and H-9α and H-11α.[17] The relative positions of the substituents in the two aromatic rings in **1** and **2** were confirmed by NOEs between H-1 and H-14 and between H-8 and H-9. The β-configuration of the *N*-oxide group was inferred from a strong deshielding of H-9β and H-11β by axial oxygen.[13] All ^{13}C NMR signals were assigned from HSQC and HMBC experiments.

The alkaloid **2** was previously isolated from *C. komarovii* Al. Iljinski,[18] identified on the basis of its ^{1}H NMR spectrum alone,[18] and assigned the absolute configuration 13a*R* from its CD spectrum.[19] 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,[20] the opposite is observed[19] for alkaloids with the 13a*R* configuration, such as (−)-antofine.[21,22] The CD spectra of **1** and **2** were in agreement with this rule (see Experimental Section). Thus, alkaloids **1** and **2** possess the 10*S*,13a*R* and the 10*S*,13a*R*,14*R* configurations, respectively.

Compound **3** has the molecular formula C$_{23}$H$_{27}$NO$_4$ as determined by HRMS. The aromatic region of a 400 MHz ^{1}H NMR spectrum of **3** in CDCl$_3$ showed a severe signal overlap. However, use of aromatic solvent-induced shift (C$_6$D$_6$−CDCl$_3$, 7:9) in conjunction with a high field (800 MHz) gave readily interpretable patterns of a 1,4-disubstituted and a 1,2,4-trisubstituted benzene ring, and COSY, NOESY, HSQC, and HMBC experiments allowed an unambiguous assignment of all ^{1}H and ^{13}C NMR resonances

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Table 1. 800 MHz ^{1}H and 100 MHz ^{13}C NMR Spectral Data for **3**

position	^{13}C^a	^{1}H^a	NOESY^b	COSY^b	HMBC^b
1	113.14	6.29 (d, $J_{1,4a}$ = 1.9)	MeO-3, H-4b/H-8, H-14α, H-14β	H-4a	C-3, C-4, C-4a, C-14a
2	148.38				
3	148.11				
4	110.70	6.39 (d, $J_{4,4a}$ = 8.2)	MeO-3, H-4b/H-8, H-4a		C-14b, C-1, C-2, C-4a
4a	120.76	6.43 (dd, $J_{4,4a}$ = 8.2, $J_{1,4a}$ = 1.9)	H-4, H-14α, H-14β	H-1	C-1, C-3, C-4a
4b	130.58	6.97 (d, $J_{4b,5}$ = 8.5)	H-1, H-4, H-5/H-7, H-9β	H-5/H-7	C-8a, C-5/C-7, C-6, C-8b
5	113.80	6.50 (d, $J_{4b,5}$ = 8.5)	MeO-6, H-4b/H-8	H-4b/H-8	C-8a, C-6
6	158.85				
7	113.80	6.50 (d, $J_{7,8}$ = 8.5)	MeO-6, H-4b/H-8	H-4b/H-8	C-8a, C-6
8	130.51	6.97 (d, $J_{7,8}$ = 8.5)	H-1, H-4, H-5/H-7, H-9β	H-5/H-7	C-8a, C-5/C-7, C-6, C-8b
8a	130.44				
8b	126.20				
9	66.58	α: 4.69 (s, br)	H-4b, H-8, H-9β, H-13a	H-9β	
		β: 5.28 (s, br)	H-4b, H-8, H-9α	H-9β	
11	67.08	α: 2.92 (m, br)	H-11β	H-11β, H-12A, H-12B	
		β: 4.46 (s, br)	H-11α, H-12B	H-11α	
12	19.56	A: 1.54 (m)	H-12B	H-12B, H-13α, H-13β	
		B: 1.98 (m)	H-11β, H-12A, H-13β	H-11α, H-12A, H-13α, H-13β	
13	26.44	α: 1.66 (m, overlap with H-13β)	H-13a	H-12A, H-12B, H-13a	
		β: 1.62 (m, overlap with H-13α)	H-14β	H-12A, H-12B, H-13a	
13a	70.23	2.82 (m)	H-9α, H-13α, H-14α	H-13α, H-13β, H-14α, H-14β	
14	31.89	α: 2.35 (dd, $J_{14α,14β}$ = 17.6, $J_{14α,13α}$ = 3.9)	H-13a, H-14β	H-13a, H-14β	
		β: 2.51 (dd, $J_{14α,14β}$ = 17.6, $J_{14α,1}$ J_{13a} = 11.8)	H-13β, H-14α	H-13a, H-14α	
14a	131.68				
14b	132.62				
MeO-2	55.44	3.27 (s)	H-1		C-1, C-2
MeO-3	55.44	3.41 (s)	H-4		C-3, C-4
MeO-6	54.82	3.31 (s)	H-5/H-7		C-6

^{a} In C_6D_6–$CDCl_3$ 7:9, δ values relative to internal TMS, coupling constants in Hz. ^{b} Signal correlating with ^{1}H resonance.

(Table 1). NOEs from H-1 and H-4a to H-14α and H-14β and from H-4b and H-8 to H-9β, established the relative position of the two benzene rings. A NOESY spectrum and chemical shifts of H-9β and H-11β were used to establish the relative stereochemistry of H-13a as α-axial and of the *N*-oxide group as β-axial, in a manner similar to that described for **1** and **2**. The CD spectrum of **3** showed positive Cotton effects at 250 and 304 nm and a negative Cotton effect at 281 nm. The dissimilarity between the CD spectra of **1** and **3** is expected because of the different aromatic chromophores. However, because *Cynanchum* species generally produce levorotatory alkaloids with the *R* configuration at C-13a and because *seco* compounds such as **3** are believed to be biosynthetic precursors of the alkaloids with the phenanthrene ring system,[23] the stereochemistry of **3** is tentatively assigned as 13a*R*.

Compounds **1**–**3** were tested in vitro for cytotoxicity using two carcinoma cell lines: the drug-sensitive KB-3-1 cell line and the multi-drug-resistant KB-V1 cell line. The KB-V1 cells display a complete multi-drug resistance phenotype, including expression of the P-170 glycoprotein efflux pump.[24–26] The resistance index of the KB–V1 cells relative to the KB-3-1 cells is 210 for vinblastine and even higher for other cytotoxic drugs.[24,27] The results of the cytotoxicity assays are shown in Table 2. Alkaloids **1** and **2** exhibited identical toxicity against the drug-sensitive cells, whereas the *seco* analogue (**3**) was shown to be considerably less active. Only slight (less than 2-fold) resistance was observed with the KB-V1 cells, whereas the resistance index for the test compound, rhodamine 123, was about 500. This finding indicates that phenanthroindolizidine alkaloids are poor substrates for the glycoprotein efflux pump, emphasizing an interest in their potential as therapeutic leads.[10,11]

Table 2. Cytotoxic Activity of Phenanthroindolizidine *N*-Oxide Alkaloids **1**–**3** Isolated from *C. vincetoxicum*

	IC$_{50}$ (μM)	
compound	KB-3-1 cells	KB-V1 cells
1	0.11 ± 0.04	0.16 ± 0.01
2	0.10 ± 0.04	0.16 ± 0.02
3	2.55 ± 0.35	3.30 ± 0.37
rhodamine 123	1.40 ± 0.17	>500

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. Circular dichroism (CD) and UV spectra were recorded at room temperature on a JASCO J-720 spectropolarimeter and on a Perkin-Elmer Lambda 2 spectrophotometer, respectively. NMR spectra were recorded at 25 °C on a Bruker AMX 400 or a Varian Unity Inova 800 spectrometer (proton frequency 400.13 and 799.809 MHz, respectively). NOESY spectra were obtained with mixing times of 800 ms. HMBC spectra were optimized for $^nJ_{C,H}$ = 7 Hz. Mass spectra were obtained on a JEOL JMS-AX505W double-focusing spectrometer with EI or FAB ionization (positive-ion mode). Column chromatography was performed on silica gel 60 (Merck, 0.063–0.2 mm). Fractions were monitored by TLC (Merck precoated Si gel 60 F$_{254}$ plates), using UV light and Dragendorff's reagent to visualize the spots. Preparative HPLC was carried out on a 250 × 16 mm Knauer column packed with Lichrosorb Si60, 5 μm, with spectrophotometric detection at 254 nm.

Plant Material. Aerial parts of *C. vincetoxicum* were collected along the northeastern coast of Zealand, Denmark, and the identity confirmed by Dr. Per Mølgaard, Department of Medicinal Chemistry, Royal Danish School of Pharmacy. A voucher specimen (DFHJJ3) has been deposited at Herbarium C (Botanical Museum, University of Copenhagen, Copenhagen).

Extraction and Isolation. Dried and powdered plant material (1 kg) was macerated three times with 3 L of MeOH–CH$_2$Cl$_2$–aqueous NH$_3$ (50:50:1), which yielded a total of 119 g of crude extract. The crude extract was dissolved in hydrochloric acid (0.016 M) and extracted with light petroleum. The aqueous fraction was made alkaline with aqueous NH$_3$ to pH 9–10 and re-extracted with EtOAc; the EtOAc extract was then evaporated and the residue (1.44 g) subjected to open column chromatography (silica gel, stepwise gradient from 7 to 75% MeOH in CH$_2$Cl$_2$, containing 1% of concentrated aqueous NH$_3$. The alkaloid content of the fractions was monitored by TLC using Dragendorff's reagent to visualize spots. The fractions containing polar alkaloids were pooled and the alkaloids separated by preparative HPLC using 8 mL/min of MeOH–CH$_2$Cl$_2$–aqueous NH$_3$ (10:89:1), to give, after repeated purification, 7.7 mg (0.00077%) of **1**, 2.5 mg (0.00025%) of **2**, and 5.0 mg (0.0005%) of **3**.

(−)-10β,13aα-Antofine N-oxide (1): colorless gum; [α]$^{21}_D$ −37.3° (*c* 0.13, MeOH), lit.[14] −35° (CHCl$_3$); CD λ_{extr}^{MeOH} ($\Delta\epsilon$, m^2/mol) 229 (+0.314), 253 (−0.169), 269 (−0.216) nm; HRFABMS *m/z* 380.1870 ([M + H]$^+$), C$_{23}$H$_{26}$NO$_4$ requires 380.1862.

(−)-10β,13aα-14β-Hydroxyantofine N-oxide (2): colorless gum; CD λ_{extr}^{MeOH} ($\Delta\epsilon$, m^2/mol) 211 (+0.055), 233 (+0.049), 268 (−0.047) nm; HRFABMS *m/z* 396.1772 ([M + H]$^+$), C$_{23}$H$_{26}$NO$_5$ requires 396.1810.

(−)-10β,13aα-Secoantofine N-oxide (3): colorless gum; [α]$^{21}_D$ −83.4° (*c* 0.15, CHCl$_3$); UV λ_{max}^{MeOH} (ϵ, L/mol × cm) 260 (7100), 280 (300) nm; CD λ_{extr}^{MeOH} ($\Delta\epsilon$, m^2/mol) 250 (+0.037), 281 (−0.157), 304 (+0.107) nm; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m/z* 382.1979 ([M + H]$^+$), C$_{23}$H$_{28}$NO$_4$ requires 382.2018; EIMS *m/z* 365 (27), 364 (33), 363 (79), 362 (100), 335 (45), 296 (26), 265 (35), 226 (15).

Assay for Cytostatic Activity. Mycoplasma-free carcinoma cell lines KB-3-1 (a HeLa subclone) and KB-V1, selected for resistance to vinblastine from the KB-3-1 cells,[24] were obtained from the Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD. The cells were maintained in monolayers at 37 °C in an atmosphere containing 5% CO$_2$ (humidity 98%), using Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, glucose (4.5 g/l), L-glutamine (0.58 g/L), sodium pyruvate (1 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). The KB-V1 cells were grown in the absence of cytostatic pressure from vinblastine, but the cells from passage numbers 5–15 preserved an unchanged degree of resistance to rhodamine 123[27] and were used for the assay. The KB-3-1 and KB-V1 cells were harvested by trypsinization at 70–80% and 60–70% confluence, respectively, applied into 96-well plates (4 × 10^3 of KB-3-1 cells or 7 × 10^3 of KB-V1 cells per well) in 75 μL of the culture medium, and grown for 24 h. Test substances were applied in 75 μL of a solution prepared by mixing 10 μL of a DMSO stock with 1.990 mL of the medium and appropriately diluted to required concentrations with the medium. Thus, no well contained more than 0.25% of DMSO, which was also present in the control wells. Six repeats of each concentration of the test substances were used, and the reported IC$_{50}$ values are the result of three separate determinations with different passages of the cells. Time of incubation and the amounts of the cells used were such that the cells in the control wells reached 70% (KB-3-1) or 60-70% (KB-V1) confluence at the end of the 72-h incubation period. After incubation, the medium was removed and the number of cells determined using CellTiter 96 aqueous cell proliferation assay kit from Promega Corporation. The MTS/PMS reagent[28] was freshly prepared by mixing 2.0 mL MTS solution (2 mg/mL) with 99.4 μL PMS solution (0.92 mg/mL) and 10.4 mL of the growth medium (without phenol red and serum). After addition of 120 μL of the reagent mixture to each well, the plate was incubated for 50 min and the absorbance determined at 492 nm.

Supporting Information Available: Table of NMR spectral data for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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