In Vitro Cytotoxic Activity of Phenanthroindolizidine Alkaloids from *Cynanchum vincetoxicum* and *Tylophora tanakae* against Drug-Sensitive and Multidrug-Resistant Cancer Cells

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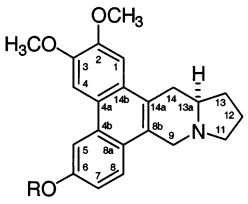
Two known phenanthroindolizidine alkaloids, (−)-(*R*)-13aα-antofine (**1**) and (−)-(*R*)-13aα-6-*O*-desmethylantofine (**2**), and two new natural products, (−)-(*R*)-13aα-secoantofine (**3**) and (−)-(*R*)-13aα-6-*O*-desmethylsecoantofine (**4**), were isolated from *Cynanchum vincetoxicum*. The structures of all compounds were established by means of NMR methods including COSY, NOESY, HSQC, and HMBC experiments, supported by HRMS and optical rotation data. Cytotoxic activity of the isolated alkaloids, and of three other alkaloids previously isolated from *Tylophora tanakae*, (−)-(*R*)-13aα-tylophorine (**5**), (−)-(*R*)-13aα-7-*O*-desmethyltylophorine (**6**), and (+)-(*S*)-13a*β*-isotylocrebrine (**7**), was assessed in vitro using a drug-sensitive KB-3-1 and a multidrug-resistant KB-V1 cancer cell line. Structure−activity relationships in this series of alkaloids are discussed. The IC_{50} values of some of the alkaloids are in the low nanomolar range, being thus comparable to the activity of clinically used cytotoxic drugs. Previously reported adverse side effects of these alkaloids could possibly be overcome by modern tissue-specific drug targeting techniques.

Phenanthroindolizidine alkaloids are found primarily in the genera *Cynanchum*, *Pergularia*, and *Tylophora* of the Asclepiadaceae,[1−4] but have also been reported from *Hypoëstes verticillaris* (Acanthaceae),[5] *Cryptocarya phyllostemmon* (Lauraceae),[6] and *Ficus hispida* and *F. septica* (Moraceae).[7,8] It is a small group of alkaloids known for their profound cytotoxic activity.[5,9−15] It has been shown that the toxicity of phenanthroindolizidine alkaloids is due to inhibition of protein and nucleic acid synthesis,[14,16−22] but the knowledge of structure−activity relationships is scarce. In 1965, tylocrebrine was submitted for Phase I clinical trials, but the study was halted due to central nervous side effects manifested as disorientation and ataxia.[23] However, these side effects could possibly be minimized by use of more polar analogues that are unable to pass the blood−brain barrier or by use of tissue-specific drug targeting techniques. Furthermore, a recent study showed that multidrug-resistant cancer cells are susceptible to phenanthroindolizidine *N*-oxide alkaloids.[15] Because development of resistance to chemotherapeutic agents is a major cause of mortality of cancer patients, this finding emphasizes the potential of phenanthroindolizidine alkaloids as therapeutic leads.

The present study describes in vitro cytotoxic activity of a series of phenanthroindolizidine alkaloids (tertiary amines) against wild type and multidrug-resistant cancer cell lines, and the structure−activity relationships are discussed. In addition to alkaloids **1**−**4** isolated from *Cynanchum vincetoxicum* (L.) Pers., alkaloids **5**−**7** previously isolated[24] from *Tylophora tanakae* Maxim. ex Franch. & Sav. are assessed for in vitro cytotoxic activity.



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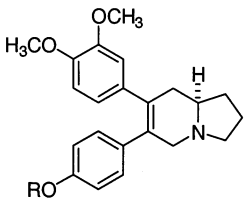

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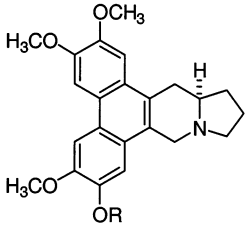

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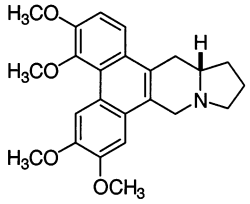

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Results and Discussion

In a continuation of the work on the cytotoxic activity of phenanthroindolizidine *N*-oxide alkaloids from *C. vincetoxicum* (L.) Pers. (syn. *Vincetoxicum officinale* Moench, *V. hirundinaria* Medic.),[15] tertiary amines were isolated from the aerial parts of *C. vincetoxicum*. The alkaloid fraction was analyzed by TLC, and visualization by Dragendorff's reagent showed the presence of several alkaloids. The alkaloids were separated using normal-phase preparative HPLC, which gave compounds **1**−**4** in an amount corresponding to a total of 0.003% of dry weight of the plant.

Compounds **1** and **2** were assigned the molecular formulas $C_{23}H_{25}NO_3$ and $C_{22}H_{23}NO_3$, respectively, based on FABMS. ^1H and ^{13}C NMR spectral data suggested **2** to be

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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectral Data for Compounds **3** and **4**[a]

	3		**4**	
position	^{13}C	^{1}H	^{13}C	^{1}H
1	113.44	6.51 (d, $J_{1,4a} = 2.0$)	112.91	6.33 (d, $J_{1,4a} = 1.7$)
2	147.55		148.04	
3	148.30		147.54	
4	111.00	6.47 (d, $J_{4,4a} = 8.3$)	110.62	6.38 (d, $J_{4,4a} = 8.3$)
4a	121.01	6.66 (dd, $J_{4,4a} = 8.3$, $J_{1,4a} = 2.0$)	120.70	6.46 (dd, $J_{4,4a} = 8.3$, $J_{1,4a} = 1.7$)
4b	130.39	6.99 (AA′XX′)	130.20	6.67 (AA′XX′)
5	113.65	6.61 (AA′XX′)	115.25	6.47 (AA′XX′)
6	158.23		155.21	
7	113.65	6.61 (AA′XX′)	115.25	6.47 (AA′XX′)
8	130.39	6.99 (AA′XX′)	130.20	6.67 (AA′XX′)
8a	133.32		131.64	
8b	132.64		132.64	
9	57.87	α: 3.03 (d, $J_{9\alpha,9\beta} = 15.9$)	56.17	α: 3.00 (d, $J_{9\alpha,9\beta} = 15.4$)
		β: 3.84 (d, $J_{9\alpha,9\beta} = 15.9$)		β: 3.67 (d, $J_{9\alpha,9\beta} = 15.4$)
11	54.26	α: 2.11 (m)	53.69	α: 1.81 (m)
		β: 3.21 (m)		β: 3.14 (m)
12	21.72	α: 1.66 (m)	21.35	α: 1.77 (m)
		β: 1.86 (m)		β: 1.56 (m)
13	30.84	α: 1.93 (m)	30.14	α: 1.84 (m)
		β: 1.53 (m)		β: 1.49 (m)
13a	60.62	2.32 (m)	60.79	2.19 (m)
14	38.48	α: 2.68 (d, $J_{14\alpha,14\beta} = 16.4$)	36.55	2.46 (m)
		β: 2.48 (dd, $J_{14\alpha,14\beta} = 16.4$, $J_{13a,\,14\beta} = 11.3$)		
14a	132.64		132.64	
14b	134.86		134.04	
MeO-2	55.44	3.39 (s)	55.58	3.30 (s)
MeO-3	55.49	3.49 (s)	55.73	3.45 (s)
MeO-6	54.80	3.38 (s)		

[a] In C_6D_6−$CDCl_3$, 7:9, δ values relative to internal TMS, coupling constants given as numerical values in Hz.

an *O*-desmethyl analogue of **1**, as confirmed by comparison with the NMR data reported in the literature.[25] A full assignment of all ^{1}H and ^{13}C resonances of **1** and **2** was obtained from COSY, NOESY, HSQC, and HMBC experiments (see Supporting Information). Optical rotation measured for **1** ($[\alpha]_D$ −124°) is close to that reported for natural antofine (*R*-form) by Faber and Wiegrebe ($[\alpha]_D$ −131°),[26,27] although values ranging from −32° to −165° have been reported.[6,7,25,28−30] Optical rotation measured for **2** ($[\alpha]_D$ −69°) is between the values reported ($[\alpha]_D$ −52° and $[\alpha]_D$ −125°) for natural 6-*O*-desmethylantofine (*R*-form).[25,30]

Compound **3** has the molecular formula $C_{23}H_{27}NO_3$ as determined by HRMALDIMS, suggesting **3** to be a *seco* analogue of **1**. As previously reported for other *seco* phenanthroindolizidine alkaloids,[15] use of aromatic solvent-induced shifts (C_6D_6−$CDCl_3$, 7:9) prevented overlap of ^{1}H NMR resonances of **3** and confirmed the presence of a 1,4-disubstituted and a 1,2,4-trisubstituted benzene ring. Combination of COSY, NOESY, HSQC, and HMBC experiments (see Supporting Information) allowed unambiguous assignment of all ^{1}H and ^{13}C resonances of **3** (Table 1). The relative position of the two benzene rings was determined by observation of NOEs from H-1 and H-4a to H-14α and from H-4b and H-8 to H-9β. NOEs from H-13a to H-14α and H-9α showed that H-13a is α-axial, whereas NOEs from H-1 and H-4 to 2-OCH_3 and 3-OCH_3, respectively, and from H-5 and H-7 to 6-OCH_3 proved the methoxylation pattern of the benzene rings. Selected NOE correlations are shown in Figure 1, and selected connectivities from HMBC experiments optimized for a maximum intensity of correlations based on three-bond couplings in aromatic rings ($^3J_{C,H} = 7$ Hz) are shown in Figure 2. *Seco* phenanthroindolizidine alkaloids with *R* configuration at C-13a exhibit negative rotations measured at the sodium D line,[15,26,31,32] and thus this configuration is assigned to the levorotatory **3**. Compound **3** is a novel natural product, which has only been reported as a product of *O*-methylation

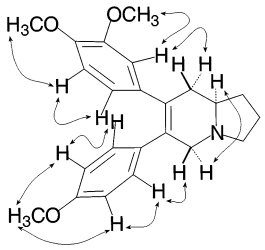

Figure 1. Selected NOE correlations observed for **3** (mixing time 700 ms).

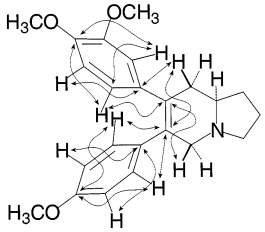

Figure 2. Selected HMBC connectivities observed for **3**. The experiment was optimized for $^nJ_{C,H} = 7$ Hz.

of phyllostemine[6] or as a synthetic racemate.[33] A complete assignment of ^{1}H and ^{13}C resonances is reported for the first time.

Compound **4** has the molecular formula $C_{22}H_{25}NO_3$, as determined by HRMALDIMS, suggesting **4** to be a *seco* analogue of **2**. An assignment of all ^{1}H and ^{13}C resonances of **4** (Table 1) is similarly based on COSY, NOESY, HSQC, and HMBC experiments (see Supporting Information). The observed connectivities were the same as for **3**, except for those involving the 6-OCH_3 group. Thus, the levorotatory **4** is (*R*)-13aα-6-*O*-desmethylsecoantofine, which is a novel alkaloid.

Compounds **1**−**4** together with **5**−**7** previously isolated from *Tylophora tanakae*[24] were tested for in vitro cytotoxicity using two human carcinoma cell lines, a drug-sensitive

Table 2. Cytotoxic Activity of Phenanthroindolizidine Alkaloids **1**−**7**

compound	IC_{50} (nM)	
	KB-3-1 cells	KB-V1 cells
antofine (**1**)	16 ± 5	14 ± 2
6-*O*-desmethylantofine (**2**)	7 ± 3	10 ± 4
secoantofine (**3**)	2630 ± 490	2440 ± 330
6-*O*-desmethylsecoantofine (**4**)	403 ± 83	425 ± 82
tylophorine (**5**)	214 ± 60	173 ± 45
7-desmethyltylophorine (**6**)	15 ± 3	17 ± 2
isotylocrebrine (**7**)	43 ± 3	54 ± 16
rhodamine 123	2200 ± 420	>500000

KB-3-1 cell line and a multidrug-resistant KB-V1 cell line. The KB-V1 cells display a complete multidrug-resistance phenotype including expression of the P-170 glycoprotein efflux pump.[34−36] However, the multidrug-resistant KB-V1 cells showed no significant resistance when tested with the alkaloids **1**−**7** (Table 2). Resistance index of the KB-V1 cells relative to the KB-3-1 cells was higher than 225 when tested with rhodamine 123 (Table 2). For vinblastine, the reported resistance index is 210, being even higher for other cytotoxic drugs.[34,37] Thus, the alkaloids **1**−**7** are poor substrates for the glycoprotein efflux pump, as previously shown for several phenanthroindolizidine *N*-oxide alkaloids.[15]

The alkaloids **1**, **2**, and **6** exhibited pronounced cytotoxicity, with IC_{50} values of 7−17 nM. The *seco* analogues **3** and **4** showed a remarkably decreased cytotoxicity. This demonstrates that a rigid phenanthrene structure is a prerequisite for a high cytotoxicity of the free bases, as previously demonstrated for the *N*-oxide alkaloids.[15] A 7-fold increase in toxicity was observed for **4** relative to **3**, indicating that a hydroxy group in the 6-position is of importance. This was also observed for **2**, which is by a factor 2 more toxic than its 6-methyl analogue **1**. Similarly, a 14-fold increase in toxicity was observed on going from **5** to the 7-desmethyl analogue **6**. It appears, therefore, that the compounds belonging to the 13a*R* series and having a free phenolic function at C-6 or C-7 show especially high cytotoxicity toward the KB cancer cell lines. Isotylocrebrine (**7**), which has the *S* configuration at C-13a, gave IC_{50} values around 50 nM for both cell lines (Table 2). Although **7** is less cytotoxic than **1**, **2**, and **6**, the high cytotoxicity against both cancer cell lines is important, because earlier studies showed equal or even higher cytotoxicity for the 13a*S* series than for the 13a*R* series.[13]

In conclusion, some of the phenanthroindolizidine alkaloids described in this work have IC_{50} values in the low nanomolar range, i.e., comparable to that of modern cytostatic drugs.[38,39] The alkaloids are equally cytotoxic to drug-sensitive and multidrug-resistant cells (Table 2). This contrasts many of the front-line antineoplastic drugs such as *Catharanthus* alkaloids, taxol, and tetracycline antibiotics, which are quite prone to the most common resistance mechanisms, including the multidrug-resistance based on the drug transporter glycoprotein (MDR1) expressed in the KB-V-1 cells. Although phenanthroindolizidine alkaloids have previously been found to exhibit serious central nervous side effects, a search for means to overcome these side effects, for instance using tissue-specific drug delivery systems,[40−44] appears attractive in the light of the very pronounced cytotoxicity of these alkaloids. This approach is now being pursued at this laboratory.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. NMR spectra were recorded at 25 °C on a Varian Gemini 2000 or a Bruker AMX 400 spectrometer (proton frequency 300.07 and 400.13 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 IonSpec Ultima 4.7 T Fourier transform mass spectrometer equipped with a matrix-assisted laser desorption ionization (MALDI) source with a 337 nm laser. Alkaloids **5**−**7** were isolated as described elsewhere.[24]

Plant Material. *C. vincetoxicum* was collected along the northeastern coast of Zealand, Denmark, and its 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. Air-dried and powdered leaves (750 g) were macerated twice with 3 L of CH_2Cl_2 and then twice with 3 L of MeOH−CH_2Cl_2 (1:1). This gave 19.2 g (extract A) and 31 g (extract B) of crude extract, respectively. Each of the crude extracts was dissolved in 600 mL of hydrochloric acid (0.016 M), and the solutions were extracted with light petroleum (5 × 200 mL). The aqueous solutions were made alkaline with aqueous NH_3 to pH 9−10 and re-extracted with ether (4 × 200 mL) to give alkaloid fractions A (37 mg) and B (148 mg). Fraction A was subjected to preparative reversed-phase HPLC [Lichrospher 100 RP18, 5 μm, 250 × 4.6 mm column, 20−90% of MeCN in 0.01 M ammonium formate (adjusted to pH 8.85) over 25 min, 1 mL/min, spectrophotometric detection at 254 nm], and alkaloid-containing fractions were identified by TLC using Dragendorff's reagent for visualization. These fractions were further purified by normal-phase preparative HPLC on Lichrosorb Si60 (5 μm, 250 × 16 mm column) with 8 mL/min of MeOH−CH_2Cl_2−aqueous NH_3 (5.5:93.5:1). The latter system was also used for isolation of alkaloids from fraction B. A second portion of leaves (700 g) was processed in a similar way, except that the alkaloid fraction A was subjected to purification by normal-phase preparative HPLC without initial separation by reversed-phase HPLC. Identical compounds were pooled to give, after repeated purification by normal-phase preparative HPLC, a total of 14.6 mg of **1** (0.001%), 13.5 mg of **2** (0.0009%), 13.7 mg of **3** (0.0009%), and 5.4 mg of **4** (0.00037%).

(−)-13aα-Antofine [(*R*)-2,3,6-trimethoxy-9,11,12,13,13a,-14-hexahydrodibenzo[*f*,*h*]pyrrolo[1,2*b*]isoquinoline] (1): colorless gum; $[\alpha]^{25}_D$ −123.9° (*c* 0.15, $CHCl_3$), lit.[26] −131° ($CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.91 (1H, s, H-4), 7.90 (1H, d, *J* = 2.6 Hz, H-5), 7.83 (1H, d, *J* = 9.1 Hz, H-8), 7.31 (1H, s, H-1), 7.21 (1H, dd, *J* = 9.1, 2.6 Hz, H-7), 4.74 (1H, d, *J* = 15.1 Hz, H-9β), 4.11 (3H, s, 3-OMe), 4.07 (3H, s, 2-OMe), 4.02 (3H, s, 6-OMe), 3.72 (1H, d, *J* = 15.1 Hz, H-9α), 3.47 (1H, dt, *J* = 8.7, 2.0 Hz, H-11β), 3.35 (1H, ddd, *J* = 15.6, 3.8, 1.4 Hz, H-14α), 2.92 (1H, dd, *J* = 15.6, 10.5 Hz, H-14β), 2.53 (1H, m, H-13a), 2.51 (1H, q, *J* = 8.7 Hz, H-11α), 2.25 (1H, m, H-13α), 2.03 (1H, m, H-12β), 1.93 (1H, m, H-12α), 1.80 (1H, m, H-13β); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 157.75 (C-6), 149.63 (C-2), 148.60 (C-3), 130.39 (C-4b), 127.24 (C-14b), 126.68 (C-8b), 125.72 (C-14a), 124.48 (C-8), 124.28 (C-8a), 123.74 (C-4a), 115.08 (C-7), 104.84 (C-5), 104.11 (C-1), 103.94 (C-4), 60.35 (C-13a), 56.10 (3-OMe), 55.98 (2-OMe), 55.14 (C-11), 55.13 (6-OMe), 53.82 (C-9), 33.63 (C-14), 31.28 (C-13), 21.61 (C-12).

(−)-13aα-6-*O*-Desmethylantofine [(*R*)-6-hydroxy-2,3-dimethoxy-9,11,12,13,13a,14-hexahydrodibenzo[*f*,*h*]-pyrrolo[1,2*b*]isoquinoline] (2): colorless gum; $[\alpha]^{25}_D$ −68.6° (*c* 0.15, $CHCl_3$), lit.[25] −51.5° (*c* 0.47, pyridine); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.96 (1H, d, *J* = 2.3 Hz, H-5), 7.93 (1H, s, H-4), 7.75 (1H, d, *J* = 8.9 Hz, H-8), 7.32 (1H, s, H-1), 7.09 (1H, dd, *J* = 8.9, 2.3 Hz, H-7), 4.56 (1H, d, *J* = 15.7 Hz, H-9β), 4.00 (3H, s, 2-OMe), 3.95 (3H, s, 3-OMe), 3.57 (1H, d, *J* = 15.7 Hz, H-9α), 3.38 (1H, m, H-14α), 3.30 (1H, m, H-11β), 2.79 (1H, dd, *J* = 15.4, 10.3 Hz, H-14β), 2.34 (1H, m, H-13a), 2.33 (1H, q, *J* = 8.5 Hz, H-11α), 2.16 (1H, m, H-13α), 1.83 (2H, m, H-12), 1.65 (1H, m, H-13β); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 155.65 (C-6), 149.42 (C-2), 148.44 (C-3), 130.26 (C-4b), 126.58 (C-8b), 126.49 (C-14b), 124.71 (C-14a), 124.36 (C-

8), 123.01 (C-4a), 122.59 (C-8a), 116.59 (C-7), 106.71 (C-5), 104.44 (C-1), 104.29 (C-4), 60.16 (C-13a), 55.72 (3-OMe), 55.60 (2-OMe), 54.59 (C-11), 53.40 (C-9), 33.03 (C-14), 31.01 (C-13), 21.32 (C-12).

(−)-13aα-Secoantofine [(*R*)-6-(4-methoxyphenyl)-7-(3,4-dimethoxyphenyl)-1,2,3,5,8,8a-hexahydroindolizine] (3): colorless gum; $[\alpha]^{25}_D$ −81.9° (*c* 0.4, $CHCl_3$); HRMALDIMS *m*/*z* 362.1790 ($[(M - 2H_2) + H]^+$), $C_{23}H_{24}NO_3$ requires 362.1751.

(−)-13aα-6-*O*-Desmethylsecoantofine [(*R*)-6-(4-hydroxyphenyl)-7-(3,4-dimethoxyphenyl)-1,2,3,5,8,8a-hexahydroindolizine] (4): colorless gum; $[\alpha]^{25}_D$ −92.6° (*c* 0.2, $CHCl_3$); HRMALDIMS *m*/*z* 348.1560 ($[(M - 2H_2) + H]^+$), $C_{22}H_{22}NO_3$ requires 348.1594.

Assay for Cytotoxic Activity. Mycoplasma-free carcinoma cell lines KB-3-1 and KB-V1, selected for resistance with vinblastine from the KB-3-1 cells,[34] were obtained from the Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD. The assay was performed as previously described.[15,45]

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Supporting Information Available: Tables with COSY, NOESY, and HMBC connectivities for compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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