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Phenanthrenes and a dihydrophenanthrene from *Tamus communis* and their cytotoxic activity

Adriána Kovács [a], Peter Forgo [b], István Zupkó [c], Borbála Réthy [c], György Falkay [c], Pál Szabó [d], Judit Hohmann [a,*]

[a] *Department of Pharmacognosy, University of Szeged, Eötvös u. 6, H-6720 Szeged, Hungary*
[b] *Department of Organic Chemistry, University of Szeged, Dóm tér 8, H-6720 Szeged, Hungary*
[c] *Department of Pharmacodynamics and Biopharmacy, University of Szeged, Eötvös u. 6, H-6720 Szeged, Hungary*
[d] *Institute of Chemistry, Chemical Research Centre, Hungarian Academy of Sciences, H-1525 Budapest, Hungary*

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Abstract

From the petroleum ether extract of the rhizomes of *Tamus communis*, the 7-hydroxy-2,3,4,8-tetramethoxyphenanthrene (**1**) was isolated, together with the known 2,3,4-trimethoxy-7,8-methylenedioxyphenanthrene (**2**), 3-hydroxy-2,4,-dimethoxy-7,8-methylenedioxyphenanthrene (**3**), 2-hydroxy-3,5,7-trimethoxyphenanthrene (**4**) and 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene (**5**), through cytotoxic assay guidance. The structures were determined by means of HREIMS, ^{1}H NMR, JMOD and NOESY experiments. The cytotoxic effects of the isolated compounds were tested on cervix adenocarcinoma (HeLa) cells, with the MTT assay. The results demonstrated that, with the exception of **2**, all these compounds displayed pronounced cytotoxic activity; especially **1** and **3** exhibited significant cell growth inhibitory effects, with $IC_{50} = 8.52 \pm 0.70$ and 3.64 ± 0.12 μM, respectively.

Keywords: *Tamus communis*; Dioscoreaceae; Structure elucidation; Cytotoxicity; Phenanthrenes; Dihydrophenanthrene

1. Introduction

Only four species of the family Dioscoreaceae are native in Europe: *Dioscorea balcanica* Košanin, *Borderea pyrenaica* Miégeville, *Borderea chouardii* (Gausen) Heslot and *Tamus communis* L. *Dioscorea* and *Borderea* species are endemic in the Balkan Peninsula and the Pyrenees, whereas *T. communis* displays a broader distribution in South, South-Central and West Europe (Tutin et al., 1972).

T. communis (black bryony) is a climbing plant with large tubers which causes irritation when rubbed on the skin (Schmidt and Moult, 1983). Both the rhizomes and the berries have a reputation in folk medicine as effective rubefacients, and they have therefore traditionally been used in several countries for the treatment of rheumatism, arthrosis, lumbago and dermatosis (Duke, 2002). Moreover, different parts of the plant have been applied in traditional medicine for the treatment of polyps and tumours (Hartwell, 1969). Previous phytochemical investigations revealed the presence of spirostane and furostane glycosides (Aquino et al., 1991), sterols (Capasso et al., 1983), histamine (Schmidt and Moult, 1983) and hydroxy/alkoxy-substituted phenanthrenes and dihydrophenanthrenes (Reisch et al., 1969, 1972, 1973; Aquino et al., 1985a,b). The aim of the present work was an investigation of the cytotoxic activity of the petroleum ether extract from the fresh rhizomes of *T. communis*, and identification of the compounds responsible for the cytotoxicity. The present paper reports the isolation, structure elucidation and tumour cell growth-inhibitory activities of one new **1** and three known phenanthrenes **2**–**4**, together with a dihydrophenanthrene **5**, all obtained from the petroleum ether extract through cytotoxic assay guidance.

* Corresponding author. Tel.: +36 62 546453; fax: +36 62 545704.
E-mail address: hohmann@pharma.szote.u-szeged.hu (J. Hohmann).

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2. Results and discussion

The MeOH extract of the fresh rhizomes of *T. communis* was subjected to solvent–solvent partitioning, to yield petroleum ether-, $CHCl_3$- and H_2O-soluble phases. The petroleum ether extract exhibited high cytotoxic activity on cervix adenocarcinoma (HeLa) cells, with IC_{50} 8.02 ± 0.20 (mean ± SEM) μg/ml. Fractionation of the petroleum ether extract by vacuum liquid chromatography resulted in nine main fractions, among which fractions VI and VII exhibited cell growth-inhibitory activities of $90.8 \pm 1.01\%$ and $74.6 \pm 1.3\%$ (mean ± SEM), respectively, at a final concentration of 10 μg/ml. The active fractions (VI and VII) were subjected to multiple chromatographic purifications, which afforded compounds **1**, **3** and **4**. From the less active fractions III and V, chemically similar constituents **2** and **5** were isolated with a view to obtaining further compounds for structure–activity relationship studies.

Compound **1** was obtained as colorless crystals. Its HREIMS exhibited a molecular ion $[M]^+$ at *m/z* 314.1161 (calcd. for $C_{18}H_{18}O_5$, Δ 2 ppm), suggesting the molecular formula $C_{18}H_{18}O_5$. The UV absorption bands were indicative of a phenanthrene derivative (Reisch et al., 1969). In the ^{1}H NMR spectrum of **1**, two *ortho*-coupled doublet 1H pairs, an aromatic singlet signal and four methoxy signals were observed (Table 1). The JMOD spectrum revealed the presence of five *O*-substituted, four *C*-substituted aromatic quaternary carbons and five aromatic methines, in addition to four methoxy groups, indicating a pentasubstituted phenanthrene derivative. The positions of the substituents (one hydroxy and four methoxy groups) were determined by a NOESY experiment and the coupling constants. In the NOESY spectrum a series of correlation signals were observed between 2-OCH_3/H-1, H-1/H-10, H-10/H-9, H-9/8-OCH_3, 8-OCH_3/7-OH, H-6/H-5 and H-5/4-OCH_3 (Fig. 1), which provided evidence for the 2,3,4,8-tetramethoxy-7-hydroxyphenanthrene structure of compound **1**.

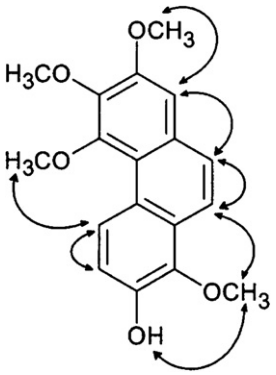

Fig. 1. Diagnostic Overhauser effects (↔) detected in the NOESY spectrum of **1**.

Compounds **2**–**4** proved to be 2,3,4-trimethoxy-7,8-methylenedioxyphenanthrene (**2**), 3-hydroxy-2,4-dimethoxy-7,8-methylenedioxyphenanthrene (**3**), and 2-hydroxy-3,5,7-trimethoxyphenanthrene (**4**), isolated earlier as compounds "TaI", "TaIV" and "TaVI", respectively, from the rhizomes of this plant (Reisch et al., 1969). After the first description of these compounds, the structures were revised in the 1970s (Letcher and Nhamo, 1972a; Letcher and Wong, 1978, 1979). In our experiments, the ^{13}C NMR signals of **2** and **3** were assigned for the first time, and in the cases of **2**–**4** the position of the substituents on the molecules were checked with the aid of the NOESY spectra.

Compound **5** was obtained as a white amorphous solid with the molecular formula $C_{17}H_{18}O_4$, as determined via the molecular ion peak at *m/z* 286.1217 in the HREIMS. Its UV spectrum exhibited absorption bands characteristic of a dihydrophenanthrene derivative (Letcher and Nhamo, 1972b). The ^{1}H NMR spectrum of **5** (Table 1) disclosed the presence of an isolated methylene, one *para*- and one *meta*-coupled proton pair, three methoxy groups and one hydroxy group (Table 1). The

Table 1
^{1}H NMR spectral data of the isolated phenanthrenes **1**–**5** [500 MHz, $CDCl_3$, δ ppm (J = Hz)]

H	1	2	3	4	5
1	7.09 *s*	7.06 *s*	7.06 *s*	7.31 *s*	6.78 *s*
4		–	–	9.09 *s*	7.88 *s*
5	9.24 *d* (9.3)	9.10 *d* (9.1)	9.03 *d* (9.0)	–	–
6	7.31 *d* (9.3)	7.22 *d* (9.1)	7.23 *d* (9.0)	6.75 *d* (2.0)	6.46 *d* (2.5)
8	–	–	–	6.88 *d* (2.0)	6.44 *d* (2.5)
9	7.88 *d* (9.0)	7.68 *d* (8.9)	7.63 *d* (8.9)	7.52 *d* (8.7)	2.74 *m* (2H)
10	7.64 *d* (9.0)	7.55 *d* (8.9)	7.55 *d* (8.9)	7.59 *d* (8.7)	2.67 *m* (2H)
2-OCH_3	4.01 *s*	4.00 *s*	4.03 *s*	–	–
3-OCH_3	4.03 *s*	4.03 *s*	–	4.09 *s*	3.92 *s*
4-OCH_3	4.00 *s*	4.00 *s*	3.94 *s*	–	–
5-OCH_3	–	–	–	4.10 *s*	3.88 *s*
7-OCH_3	–	–	–	3.95 *s*	3.84 *s*
8-OCH_3	3.98 *s*	–	–	–	–
OH	5.78 *s*	–	6.03 *s*	5.84 *s*	5.57 *s*
–OCH_2O–	–	6.16 *s* (2H)	6.16 *s* (2H)	–	–

Table 2
Cytotoxic activity of the isolated phenanthrenes **1**–**5** on HeLa cells

Compound	$IC_{50} \pm SEM$ (μM)
1	8.52 ± 0.70
2	>30
3	3.64 ± 0.12
4	11.49 ± 0.68
5	14.21 ± 1.64
Doxorubicin	0.15 ± 0.028
Cisplatin	12.43 ± 1.05

substitution pattern was determined with the aid of a NOESY spectrum. Overhauser effects were detected between H-1/H-10, H-10/H-9, H-9/H-8, H-8/7-OCH_3, 7-OCH_3/H-6, H-6/5-OCH_3, 5-OCH_3/H-4 and H-4/3-OCH_3. These NOESY correlations led to the formulation of compound **5** as 2-hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene. This is the second reported isolation of this compound from a natural source: it was first obtained from the heartwood of *Combretum psidioides* (Letcher and Nhamo, 1972b). Interestingly, a similar compound, 5-hydroxy-2,3,7-trimethoxy-9,10-dihydrophenanthrene, differing only in the positions of two substituents, was identified earlier from the $CHCl_3$ extract of the rhizomes of *T. communis* (Aquino et al., 1985b).

The isolated compounds were tested for their cytotoxic activity on a HeLa cell line using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, compared with cisplatin and doxorubicin as positive controls. The cell growth-inhibitory potencies of compounds **1**–**5**, expressed as IC_{50} values, are shown in Table 2. 3-Hydroxy-2,4-dimethoxy-7,8-methylenedioxyphenanthrene (**3**) exhibited the most potent cytotoxic activity ($IC_{50} = 3.64 \pm 0.12$ μM), while compounds **1**, **4** and **5** displayed moderate cytotoxicities ($IC_{50} = 8.52$–14.21 μM), whereas **2** was found to be inactive.

3. Conclusions

The distribution of phenanthrene and dihydrophenanthrene in the plant kingdom appears to be limited; their occurrence has been reported to date in only a few plant families: Orchidaceae, Dioscoreaceae, Combretaceae, etc. These compounds are known to be endogenous plant growth regulators and potent phytoalexins. As concerns their pharmacological profile, a number of phenanthrenes have been reported to exert antiviral activity against vesicular stomatitis virus and human rhinovirus serotype 1B (Aquino et al., 1991), and smooth muscle-relaxing activity (Estrada et al., 1999). Some natural phenanthrenes and dihydrophenanthrenes have also been reported to display an antitumour effect (Pettit et al., 1988; Lee et al., 1995; Long et al., 1997; Shagufta et al., 2006). Our results provide further evidence that hydroxy/alkoxy-substituted phenanthrenes are promising antitumour agents. However, the low number of compounds **1**–**5** are not sufficient for a thorough structure-activity relationships, the cytotoxic potencies clearly indicate that the C(9)–C(10) double bond is not essential for the cytostatic effect, as compounds **4** and **5** are almost equally effective. A similar observation was reported earlier on structurally related alkoxy-substituted phenanthrenes and dihydrophenanthrenes (Pettit et al., 1988). The number and positions of the methoxy groups on the phenanthrene skeleton seem to be crucial factors as regards the efficacy, similarly as for the conformationally less restricted analogues, the *cis*-stilbene combretastatins. Although the highly active combretastatins require a trimethoxy-substituted benzene ring in the molecule (Srivastava et al., 2005), our results reveal that this is not essential for the phenanthrenes, since compound **2**, containing three methoxy substituents, proved to be the least active, and compound **3**, with only two methoxy groups, was the most effective in the MTT assay. Comparison of the effects of compounds **1**–**3** suggests that the presence of a hydroxy group on either ring A (**3**) or ring B (**1**) is favourable relative to full alkoxysubstitution (**2**). All of these findings demonstrate that the structure–activity relationships established for combretastatins cannot be applied to the congeners, e.g. to phenanthrenes. The present results can contribute to the design of further synthetic phenanthrenes, in which limited methoxylation is expected to be a favourable feature.

4. Experimental

4.1. General experimental procedures

Melting points are uncorrected. UV spectra were recorded in MeOH on a Shimadzu UV-2101 PC UV–VIS spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DRX 500 spectrometer at 11.7 T (500 MHz for ^1H and 125 MHz for ^{13}C); the signals of the deuterated solvent were taken as the reference (7.26 ppm in ^1H NMR and 77.0 ppm in JMOD). Two-dimensional experiments (NOESY) were set up, performed and processed with the standard Bruker protocol. HREIMS spectra were obtained on a Finnigan MAT 95 S spectrometer. For vacuum liquid chromatography, silica gel (Kieselgel GF_{254} 15 μm, Merck) was used. Preparative TLC was carried out on silica gel (Kieselgel $60F_{254}$, Merck). The chromatograms were visualized under UV light at 254 and 365 nm, and by spraying with concentrated H_2SO_4, followed by heating at 110 °C for 10 min. Sephadex LH-20 (Pharmacia Fine Chemicals) was used for gel chromatography. Centrifugal chromatography was carried out on Chromatotron (Harrison Research) apparatus, using manually coated silica gel (60 GF_{254}, Merck) plates with 1.0 mm thickness.

4.2. Plant material

The rhizomes of *Tamus communis* L. were collected in the Mecsek Hills (Hungary) in June 2003. A voucher specimen

(no. 619) has been deposited at the Herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

4.3. Extraction and isolation of compounds

The fresh rhizomes of the plant (1.2 kg) were crushed and then percolated with MeOH (10 l) at room temperature. The MeOH extract was concentrated and extracted with petroleum ether 40–60 °C (5 × 400 ml) and $CHCl_3$ (5 × 400 ml). The petroleum ether fraction (1.32 g) was chromatographed by vacuum liquid chromatography, using a gradient system of cyclohexane–EtOAc (98:2, 95:5, 9:1, 85:15, 8:2 and 1:1, 15 × 20 ml of each). A total of 90 fractions were collected and combined on the basis of TLC monitoring, affording nine main fractions (I–IX). Fraction III was separated by preparative TLC on silica gel with the use of *n*-hexane–$CHCl_3$–Me_2CO (70:40:3) as mobile phase, yielding compound **2** (9.3 mg). Fraction V afforded a considerable amount of a crystalline material upon standing, which was identified as β-sitosterine. The mother liquor of this fraction was subjected to preparative TLC, using *n*-hexane–$CHCl_3$–Me_2CO (70:30:3) as developing system, and then further purified by gel chromatography on Sephadex LH-20, with elution with MeOH. Finally, the main compound from this separation was chromatographed by preparative TLC on silica gel with benzene–EtOAc (9:1), to furnish compound **5** (2.6 mg). Fractions VI and VII were processed in the same manner, first by centrifugal chromatography, using a gradient solvent system of cyclohexane–EtOAc (9:1, 85:15, 8:2, 7:3 and 1:1), and then by preparative TLC on silica gel in two steps. First, benzene–EtOAc (9:1) was used as solvent system, while in the second step *n*-hexane–$CHCl_3$–Me_2CO (50:49:1) was applied, which afforded compounds **1** (10.5 mg), **3** (16.3 mg) and **4** (2.6 mg).

4.3.1. 6-Hydroxy-2,3,4,8-tetramethoxyphenanthrene (*1*)

Colorless crystals; m.p. 183–186 °C; UV (MeOH) λ_{max} nm (log ε): 233 (4.26), 263 (4.80), 287 (4.11), 294 (3.99), 308 (3.97), 331 (3.26), 352 (3.31), 365 (3.38); ^{1}H NMR (500 MHz, $CDCl_3$): see Table 1. ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 105.3$ (C-1), 148.0 (C-2), 143.1 (C-3), 151.9 (C-4), 119.4 (C-4a), 124.8 (C-4b), 124.2 (C-5), 116.2 (C-6), 145.5 (C-7), 140.8 (C-8), 126.4 (C-8a), 119.3 (C-9), 127.3 (C-10), 128.4 (10a), 55.9, 60.2, 61.2, 61.9 (4×OCH_3), the signals were tentatively assigned; HREIMS *m*/*z* 314.1161 $[M]^+$ (calcd. for $C_{18}H_{18}O_5$, 314.1154).

4.3.2. 2,3,4-Trimethoxy-7,8-methylenedioxyphenanthrene (*2*)

Yellowish-white crystals; 149–151 °C; UV (MeOH) λ_{max} nm (log ε): 233 (4.54), 268 (4.81), 277 (4.69), 293 (4.18), 305 (4.08), 315 (3.94), 360 (3.57), 375 (3.64); ^{1}H NMR (500 MHz, $CDCl_3$): see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$): δ 105.8 (C-1), 152.0, 151.9 (C-2, C-4), 143.1 (C-3), 117.6, 119.4 (C-4a, C-4b), 120.5 (C-5), 108.9 (C-6), 143.4 (C-7), 142.3 (C-8), 125.0 (C-8a), 118.6 (C-9), 127.1 (C-10), 129.1 (C-10a), 101.4 (–OCH_2O–), 55.8, 60.1, 61.2 (3 × OCH_3).

4.3.3. 3-Hydroxy-2,4-dimethoxy-7,8-methylenedioxyphenanthrene (*3*)

Yellowish-white crystals; m.p. 159–161 °C; UV (MeOH) λ_{max} nm (log ε): 232 (4.52), 268 (4.81), 276 (4.70), 294 (4.10), 307 (4.06), 320 (3.97), 340 (3.55), 355 (3.77), 373 (3.81); ^{1}H NMR (500 MHz, $CDCl_3$): see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$): δ 105.3 (C-1), 144.1, 146.7 (C-2, C-4), 139.4 (C-3), 117.8, 119.0 (C-4a, C-4b), 120.3 (C-5), 108.7 (C-6), 143.4 (C-7), 142.2 (C-8), 124.5 (C-8a), 117.1 (C-9), 127.3 (C-10), 125.9 (C-10a), 101.4 (–OCH_2O–), 56.1, 59.7 (2×OCH_3).

4.3.4. 2-Hydroxy-3,5,7-trimethoxyphenanthrene (*4*)

Yellowish-white crystals; m.p. 176–177 °C; UV (MeOH) λ_{max} nm (log ε): 234 (4.34), 251 (4.73), 261 (4.94), 284 (4.19), 320 (3.34), 330 (3.46), 345 (3.73), 362 (3.84); ^{1}H NMR (500 MHz, $CDCl_3$), see Table 1.

4.3.5. 2-Hydroxy-3,5,7-trimethoxy-9,10-dihydrophenanthrene (*5*)

White amorphous solid; UV (MeOH) λ_{max} nm (log ε): 218 (4.17), 268 (3.86), 277 (3.91), 303 (3.81), 313 (3.75); ^{1}H NMR (500 MHz, $CDCl_3$), see in Table 1; HREIMS: *m*/*z* 286.1217 $[M]^+$ (calcd. for $C_{17}H_{18}O_4$, 286.1205).

4.4. Bioassays

Cytotoxic effects were measured *in vitro* on a HeLa (cervix adenocarcinoma) cell line, using the MTT colorimetric assay. The cytotoxicity tests were carried out in 96-well microtitre plates, using 5000 cells per well in all cases, which were allowed to adhere overnight before the drugs were introduced. The original medium was then removed, 200 μl culture medium containing the compounds of interest was added and the cells were incubated for 72 h. The tested extracts and compounds were dissolved in DMSO. The final concentration of DMSO never exceeded 0.3%, and therefore had no essential effect on the cell growth. Next the living cells were assayed: aliquots (20 μl at 5 mg/ml) of the MTT stock solution were pipetted into each well and reduced by viable cells to an insoluble formazan product during a further 4 h. After this contact period, the medium was removed and the formazan crystals were dissolved in 100 μl DMSO by gentle shaking for 60 min. Finally, the absorbance was measured at 545 nm with a microplate reader (Mosmann, 1983). In this way the cell growth or drug toxicity was determined. The 50% inhibitory concentration (IC_{50}) was derived from the dose–response curves fitted to the measured points by GraphPad Prism 2.01. All *in vitro* experiments were carried out on two microplates with at least five parallel wells.

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