Absolute Configuration of a Tetrahydrophenanthrene from *Heliotropium ovalifolium* by LC-NMR of Its Mosher Esters

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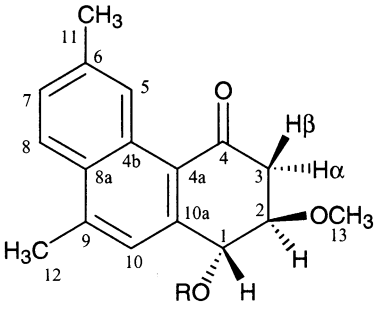
A new tetrahydrophenanthrene (**1**, (1*R*,2*R*)-1-hydroxy-2-methoxy-6,9-dimethyl-2,3-dihydrophenanthren-4(1*H*)-one (heliophenanthrone)) has been isolated from the aerial parts of *Heliotropium ovalifolium*. Its structure was elucidated on the basis of spectroscopic data, and the absolute configuration of the asymmetric centers was determined from LC-NMR data of the Mosher ester derivatives.

Heliotropium ovalifolium Forsk. (Boraginaceae) is a herbaceous plant responsible for several cases of cattle and sheep intoxications due to its content of pyrrolizidine alkaloids such as helifoline and retronecine.[1−3] This species is also suspected to cause the flaccid trunk paralysis occurring in free-ranging elephants of Lake Kariba in Zimbabwe.[4] Besides the known toxic pyrrolizidinic products, we recently reported the presence of two antifungal and antibacterial benzoquinones from the aerial parts of *H. ovalifolium*.[5] In our continuing search for new lead compounds from higher plants, our interest was focused on one of the major products of the CH_2Cl_2 extract from the aerial parts of the plant. As the data collected from a preliminary LC/UV/MS dereplication analysis did not allow the identification of this product directly from the crude extract, we undertook its isolation and structural determination, which led to the discovery of a new dihydrophenanthrene derivative named heliophenanthrone (**1**). This is the second example of the use of the Mosher esterification method in conjunction with LC-NMR analysis for the elucidation of absolute configurations of microquantities of available pure substance.[6]



1 R = H
1a R = (R)-MTPA
1b R = (S)-MTPA

Results and Discussion

Heliophenanthrone (**1**) was isolated as a white amorphous powder. The molecular formula was established as $C_{17}H_{18}O_3$ by high-resolution mass spectroscopic analysis, implicating nine centers of unsaturations and/or ring structures in compound **1**. The ^{13}C NMR spectrum of heliophenanthrone recorded in acetone-d_6 indicated 10 sp^2-hybridized carbon atoms (Table 1), of which four had protons attached and six were quaternary. In accord with the high number of unsaturations and/or rings, these 10 sp^2-hybridized carbons suggested the presence of a naphthalene moiety in compound **1**. The ^1H NMR spectrum of heliophenanthrone recorded in acetone-d_6 showed four protons located in the aromatic region at δ_H 7.44 (H-7), 7.64 (H-10), 7.99 (H-8), and 9.32 (H-5). Inspection of the gDQF-COSY spectrum indicated a vicinal relation between signals H-7 and H-8, confirmed by their coupling constants (J = 8.7 Hz). In addition, two singlet signals were seen in the ^1H NMR spectrum, each integrating for three protons, at δ_H 2.52 (H-11) and 2.74 (H-12), associated with two methyl groups. Long-range $^1H-^{13}C$ couplings deduced from the gHMBC spectrum of **1** (Table 1) revealed that the first methyl group at δ_H 2.52 correlated with three carbons positioned on the naphthalene unit at δ_C 138.8 (C-6), 128.9 (C-7), and 127.2 (C-5). The gHMBC spectrum also exhibited $^1H-^{13}C$ correlations between the second methyl group at δ_H 2.74 (H-12) and five other carbons of the naphthalene skeleton at δ_C 146.1 (C-10a), 142.4 (C-9), 131.9 (C-4b), 131.6 (C-8a), and 126.8 (C-10). These different structural elements gave the complete substitution pattern of the naphthalene moiety, except for the 4a and 10a positions. Examination of the contour maps of the gradient gDQF-COSY and TOCSY experiments allowed distinction of an additional spin system characterized by chemical shifts at δ_H 2.70 (1H, dd, J = 16.1, 8.3 Hz, H-3β), 3.21 (1H, dd, J = 16.1, 3.9 Hz, H-3α), 3.81 (1H, ddd, J = 8.3, 6.4, 3.9 Hz, H-2), 4.91 (1H, dd, J = 6.4, 4.9 Hz, H-1), and 4.97 (1H, d, J = 4.9 Hz, OH-1). The resonances at δ_H 2.70 and 3.21 could be assigned to the carbon signal at δ_C 43.5 (C-3) according to the gHSQC spectrum and were identified as CH_2 protons (J = 16.1 Hz). In addition, the gHMBC spectrum of heliophenanthrone suggested that this methylene moiety was linked to a carbonyl function [δ_C 198.0 (C-4)]. The signal at δ_H 4.97 did not correlate with any carbon atom in the gHSQC spectrum of compound **1** and was characterized as a hydroxyl signal. This fact was corroborated by observing its correlation in the gHMBC spectrum with the carbon C-1 at the downfield resonance [δ_C 72.1 and δ_H 4.91 (H-1)]. In the ^1H NMR spectrum of heliophenanthrone recorded in $CDCl_3$ (see Table 1), this signal appeared as a broad singlet shifted to δ_H 3.00,

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Table 1. NMR Data of Heliophenanthrone (**1**)

position	δ_C[a]	δ_H, mult (J in Hz)[a]	δ_H, mult (J in Hz)[b]	gHMBC (H to C)[a]
1	72.1	4.91 dd (6.4, 4.9)	4.90 d (8.3)	146.1, 125.1, 126.8, 81.4, 43.5
2	81.4	3.81 ddd (8.3, 6.4, 3.9)	3.70 ddd (10.7, 8.3, 4.6)	198.0, 72.1, 57.2
3α	43.5	3.21 dd (16.1, 3.9)	3.30 dd (16.1, 4.6)	198.0, 125.1, 81.4, 72.1
3β		2.70 dd (16.1, 8.3)	2.65 dd (16.1, 10.7)	198.0, 125.1, 81.4, 72.1
4	198.0			
4a	125.1			
4b	131.9			
5	127.2	9.32 s	9.28 s	131.6, 128.9, 125.1, 22.2
6	138.8			
7	128.9	7.44 dd (8.7, 1.7)	7.40 dd (8.8, 1.5)	131.6, 127.2, 22.2
8	124.9	7.99 d (8.7)	7.92 d (8.8)	142.4, 138.8, 131.9
8a	131.6			
9	142.4			
10	126.8	7.64 s	7.70 s	131.6, 125.1, 72.1, 20.3
10a	146.1			
11	22.2	2.52 s	2.56 s	138.8, 128.9, 127.2
12	20.3	2.74 s	2.75 s	142.4, 131.6, 126.8
13	57.2	3.46 s	3.51 s	81.4
OH		4.97 d (4.9)	3.00 br s	

[a] Measured in acetone-d_6. [b] Measured in $CDCl_3$.

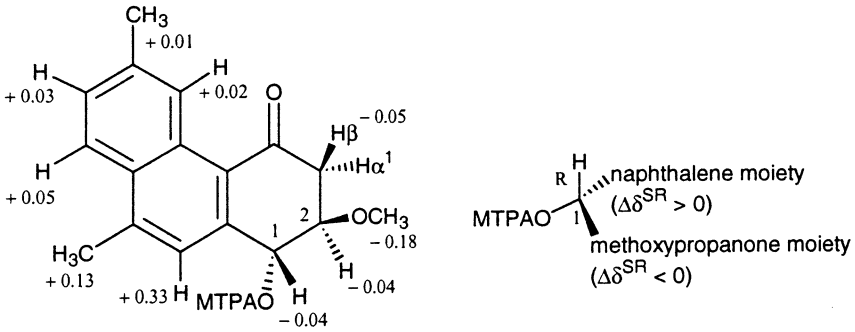

Figure 1. $\Delta\delta^{SR}$ values for MTPA derivatives **1a** and **1b** and spatial consequences.

confirming the hydroxyl nature of this signal. The remaining signal in the ^{1}H NMR spectrum of compound **1** at δ_H 3.46 (3H, s, H-13) was associated with a methoxy group. Long-range ^{1}H−^{13}C couplings revealed that this methoxy unit was located on the 2-position [δ_H 3.81 (H-2) and δ_C 81.4 (C-2)]. On the basis of the gHMBC analysis, the 1-hydroxy-2-methoxy-4-oxobutyl partial structure was found to be linked to the naphthalene group at position 4a for the ketonic part and at position 10a for the alcoholic methine part. Therefore, the compound was identified as structure **1** and to our knowledge is a new natural product.

Present in heliophenanthrone were two asymmetric centers at positions C-1 and C-2. The relative stereochemistry of H-1 and H-2 (J = 6.4 Hz in acetone-d_6 and 8.3 Hz in $CDCl_3$) was determined to be *trans* by comparison with the literature.[7] This relation was also corroborated by the lack of correlation observed in the NOESY experiment between H-1 and H-2 signals.

Considering the small amount of purified **1** available, it was considered judicious to determine the absolute configuration of the secondary alcohol at C-1 by the Mosher ester method,[8,9] in conjunction with LC-NMR analysis of derivatives **1a** and **1b**. This methodology permitted the determination of the absolute configuration at the microscale level by the direct analysis of the crude reaction mixture.[6] The ^{1}H NMR data were then recorded using the LC-NMR stop-flow mode. Since similar retention times for **1a** and **1b** in the LC separation of both crude reaction mixtures were observed, the flows were stopped at the same solvent compositions. The spectra of the two esters revealed a slight difference of the δ_H due to the diamagnetic effect of the MTPA benzene ring. Calculation of the $\Delta\delta^{SR} = \delta_H(S) - \delta_H(R)$ by Mosher's method allowed assignment of a *R*-configuration to the secondary alcohol C-1 (Figure 1).[9] The *trans* relation between the two asymmetric centers C-1 and C-2 indicated then an *R*-configuration for the carbon in position 2. Thus, heliophenanthrone (**1**) was established as (1*R*,2*R*)-1-hydroxy-2-methoxy-6,9-dimethyl-2,3-dihydrophenanthren-4(1*H*)-one.

A final comment should be made about the biosynthesis of compound **1**. Two biogenetic pathways can be considered for the formation of the phenanthrenes. The first includes compounds isolated mostly from the Orchidaceae,[10,11] the Dioscoreaceae,[12] the Combretaceae,[13] and, less frequently, the Betulaceae;[14] these have characteristic unsaturated and polyoxygenated structures, associated usually with stilbene derivatives.[10] The second pathway is rare but is found in some Euphorbiaceae[15] and Juncaceae species,[16] in which phenanthrenes present base structures close to mevalonate derivatives.

Heliophenanthrone, though, exhibited different patterns, which suggested another biosynthetic pathway. In the family of *Heliotropium ovalifolium*, Boraginaceae, numerous quinonic derivatives have been reported such as alkannin and shikonin.[17] According to the biogenetic pathway of these compounds, the major biosynthetic intermediate occurs from the alkylation of the 4-hydroxybenzoic acid by geranyl pyrophosphate (GPP).[17] In the case of heliophenanthrone, a lavandulyl moiety could be considered in place of the geranyl part, followed by two cyclization steps to give the correct positions of the methyl groups. It should be noted that 6-formyl-2-methoxy-9-methyl-1,4-phenanthrendione, isolated from *Auxemma oncocalys* (Boraginaceae),[18] presented the same skeleton as heliophenanthrone.

Experimental Section

General Experimental Procedures. Silica gel (63−200 μm, Merck) was used for open-column chromatography (CC). TLC employed precoated silica gel plates 60 F_{254} (Merck). Centrifugal partition chromatography (CPC) was achieved on a P.C. Inc. Ito multilayer coil separator-extractor model (capacity 226 mL; 2.6 mm) equipped with two constant-flow pumps (waters 6000A), a UV monitor (Knauer variable wavelength), a recorder (LKB Bromma 2210), and a sample inject valve with a 20 mL sample loop. HPLC/UV/DAD was performed with a Symmetry RP-18 column (4 μm, 3.9 × 150 mm; Waters) using a CH_3CN-H_2O gradient (20:80 → 100:0) in 25 min. The detection was performed at 210 and 254 nm. Semipreparative HPLC was performed with a Shimadzu LC-8 pump equipped with a Knauer UV detector using a μBondapak C_{18} prepacked radial-compression column (10 μm, 25 × 100 mm; Waters). Optical rotation was recorded on a Perkin-Elmer-241 polarimeter. The UV spectrum was measured on a Varian DMS 100S UV−vis spectrophotometer. NMR spectra were obtained on a Varian Unity Inova-500 spectrometer, at 500 (proton) and 125 MHz (carbon); δ in ppm relative to Me_4Si (internal standard), J in Hz. HRESIMS was recorded on a Bruker FTMS 4.7T instrument.

Plant Material. The aerial parts of *H. ovalifolium* were collected in March 1999 at Fothergill Island, Lake Kariba, Zimbabwe. A voucher is deposited at the Institut de Pharmacognosie et Phytochimie, Lausannne, Switzerland (No. 99034).

Extraction and Isolation. The air-dried powdered aerial parts (1.3 kg) of *H. ovalifolium* were extracted at room temperature with CH_2Cl_2 to afford 4.9 g of extract. This extract was first separated by column chromatography on silica gel with a petroleum ether−ethyl acetate gradient (from 9:1 to 1:4), giving five fractions. Fraction 3 (0.24 g) was further fractionated by CPC using a four-solvent heptane−ethyl acetate−MeOH−H_2O system (6:2:6:2); mobile phase, aqueous layer; flow rate, 2.0 mL/min; rotor speed, 800 rpm; detection at 254 nm, which gave 20 fractions. The final purification of compound **1** was achieved from fraction 8, by semipreparative chromatography using a SymmetryPrep C18 column (Waters) with isocratic conditions of H_2O−acetonitrile (70:30) and led to isolation of compound **1** (6.0 mg).

Heliophenanthrone, (1*R*,2*R*)-1-hydroxy-2-methoxy-6,9-dimethyl-2,3-dihydrophenanthren-4(1*H*)-one (1): amorphous powder; $[\alpha]^{25}_D$ −10.8° (*c* 0.60, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (3.06), 253 (2.87), 325 (2.48) nm; ^{1}H NMR (acetone-d_6 and $CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz), see Table 1; HRESI-MS (positive mode) *m*/*z* 293.1158 (calcd for $C_{17}H_{18}NaO_3$, $[M + Na]^+$, 293.1159).

Preparation of Mosher Ester Derivatives 1a and 1b. Compound **1** (0.5 mg, 1.9 mmol) in dry CH_2Cl_2 (0.8 mL) was transferred into a HPLC vial (2 mL, capacity) capped with a septum and then treated with dry pyridine (0.1 mL) and 50 mg (200 mmol) of (*S*)-(+)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (MTPA chloride). The mixture was stirred at RT under N_2 atmosphere for 4 h. The completion of reaction was monitored by TLC using Godin's reagent.[19] CH_2Cl_2 was removed with a N_2 flux. The total mixture containing **1a** was solubilized in 300 μL of MeCN (HPLC grade) and analyzed by LC-MS and by LC-NMR. Mosher ester derivative **1b** was prepared by using (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride reagent under the same conditions described above.

LC/APCI-MS Analysis of Mosher Ester Derivatives 1a and 1b. Separation of the crude reaction mixture containing **1a** was obtained with a Hewlett-Packard (Waldbronn, Germany) HPLC System Series 1100 photodiode array detector equipped with a Nova-Pak RP-18 column (150 × 3.9 mm i.d.; 4 μm; Waters). A gradient of MeCN in H_2O-CF_3COOH (0.05%) from 30 to 100% over 30 min was used. The crude reaction mixture containing **1a** (60 μg) was injected, and UV traces were measured at 210 and 254 nm. The flow rate was 1 mL/min. LC-MS was performed directly after UV-DAD measurements on a Finningan MAT TSQ-700 triple-stage quadrupole instrument (Finningan MAT, San Jose, CA). The APCI parameters were as follows: capillary temp 150 °C, vaporizer temp 70 °C, positive mode, sheath gas flow 60, discharge current 5 μA, spectra (150−900 mu). LC/APCI-MS analysis of the crude reaction mixture containing **1b** was performed under the same conditions.

LC-NMR Analysis of Mosher Ester Derivatives 1a and 1b. A Varian Unity Inova 500 MHz NMR instrument equipped with a ^{1}H[^{13}C] pulse field gradient indirect detection microflow LC/NMR probe (flow cell 60 μL; 3 mm i.d.) was used. Reversed-phase HPLC of the two crude reaction mixtures was carried out on a Varian (Palo Alto, CA) modular HPLC system, comprising a Varian 9012 pump, a Valco injection valve, and a Varian ProStar 320 UV detector. The separation was achieved by using a μBondapak C_{18} prepacked column (100 × 8 mm i.d.; 10 μm; Waters) with a gradient step of CH_3CN in D_2O-CF_3COOH (0.05%), from 30 to 100% over 30 min. The flow rate was 1 mL/min. The crude reaction mixture (60 μL) of each compound (around 180 μg) was injected, and the UV traces were measured at 210 nm. The retention times for **1a** and **1b** were similar, corresponding to the same solvent composition (CH_3CN-D_2O, 96:4). The ^{1}H NMR data of **1a** and **1b** were recorded using the stop-flow mode of the peak, which afforded all the typical resonances with 256 scans. Solvent suppression was achieved with the "water suppression enhanced through T_1 effects" (WET) technique,[20] and acetonitrile was referenced at δ_H 2.00 ppm.

1-(*R*)-MTPA Ester (1a): LC-^{1}H NMR (CH_3CN-D_2O, 96:4, 500 MHz) stop-flow mode (around 180 μg of **1a**, NT256) δ 2.53 (3H, s, H-11), 2.59 (3H, s, H-12), 2.87 (1H, dd, J = 15.4, 6.6, H-3β), 3.43 (3H, s, OCH_3-MTPA), 3.64 (3H, s, H-13), 4.11 (1H, m, H-2), 6.49 (1H, d, J = 5.0 Hz, H-1), 7.01 (1H, s, H-10), 7.50 (4H, m, H-7 and phenyl MTPA), 7.62 (2H, m, phenyl MTPA), 7.99 (1H, d, J = 8.0 Hz, H-8), 9.13 (1H, s, H-5), H-3α is not discernible, perhaps under suppression peak at 3.29 for residual MeOH; LC-MS APCI-positive ion mode *m*/*z* (rel int) 487.3 $[M + H]^+$ (8), 293.7 (12), 254.1 (29), 252.9 (100).

1-(*S*)-MTPA Ester (1b): LC-^{1}H NMR (CH_3CN-D_2O, 96:4, 500 MHz) stop-flow mode (around 180 μg of **1b**, NT256) δ 2.54 (3H, s, H-11), 2.72 (3H, s, H-12), 2.82 (1H, dd, J = 16.5, 6.3, H-3β), 3.00 (1H, dd, J = 16.5, 2.3, H-3α), 3.44 (3H, s, OCH_3-MTPA), 3.46 (3H, s, H-13), 4.07 (1H, m, H-2), 6.45 (1H, d, J = 4.5 Hz, H-1), 7.34 (1H, s, H-10), 7.45 (3H, m, phenyl MTPA), 7.53 (3H, m, H-7 and phenyl MTPA), 8.04 (1H, d, J = 8.5 Hz, H-8), 9.15 (1H, s, H-5); LC-MS APCI-positive ion mode *m*/*z* (rel int) 487.1 $[M + H]^+$ (32), 293.7 (11), 254.1 (28), 252.9 (100).

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Supporting Information Available: Figure summarizing the alkannin biosynthetic pathway and the alternative postulated biosynthesis of heliophenanthrone. This material is available free of charge via the Internet at http://pubs.acs.org.

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