Heliotropamide, a Novel Oxopyrrolidine-3-carboxamide from Heliotropium ovalifolium

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Heliotropamide, a Novel Oxopyrrolidine-3-carboxamide from *Heliotropium ovalifolium*

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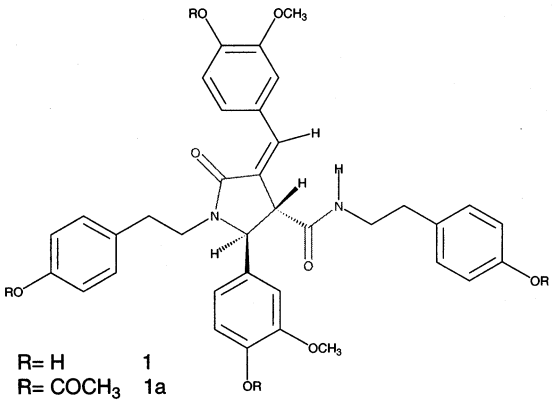
Heliotropamide (**1**), a new alkaloid with a novel oxopyrrolidine-3-carboxamide central moiety, has been isolated as the major product of the dicholoromethane extract of *Heliotropium ovalifolium* aerial parts. Its structure was elucidated by spectrometric methods including ESI-HR, EI, D/CI mass spectrometry, ^{1}H, ^{13}C, and 2D NMR experiments, and chemical derivatization. Neither heliotropamide nor its acetylated derivative (**1a**) showed any antifungal activity against *Cladosporium cucumerinum* and *Candida albicans*, antibacterial activity against *Bacillus subtilis*, radical-scavenging properties in the DPPH test, or inhibitory potential toward acetylcholinesterase.

Since the hypothesis of a plant intoxication was suggested to explain flaccid trunk paralysis in free-ranging elephants on the southern shore of Lake Kariba in Zimbabwe,[1,2] we have focused our investigations on the flora of Fothergill Island off the shore of Lake Kariba. After observation of the elephant feeding habits, *Heliotropium ovalifolium* Forsk. (Boraginaceae) was suspected to be responsible for the floppy trunk syndrome. *H. ovalifolium* is one of the 14 *Heliotropium* species that grow in East and Southern Africa. This erect or decumbent annual or perennial herb can grow up to 90 cm high and possesses white flowers in scorpiod cymes. The Boraginaceae family is well known for containing pyrrolizidine alkaloids (PAs) that are responsible for several cases of human and cattle intoxication resulting in chronic hepatotoxicity. The PAs also induce important risks of carcinogenicity, mutagenicity, and teratogenicity. *H. ovalifolium* has already caused several cases of cattle intoxication[3,4] due to its content in PAs, including helifoline and retronecine.[5]

Different extracts of the plant have been tested for their neurotoxic properties on rat neuronal cell lines, but none of them demonstrated in vitro toxic effects (personal communication, P. Jenner, Neurodegenerative Diseases Research Center, King's College London) nor did these extracts exhibit toxicity in multidimensional neuropharmacological tests concerning behavioral, neurological, and autonomic patterns (personal communication, P. Gómez-Serranillos, Departamento de Farmacologia, Universidad Complutense, Madrid). Besides these assays, the same extracts were also tested against different microorganisms. The dichloromethane extract of *H. ovalifolium* aerial parts was reported to possess antifungal properties against *Cladosporium cucumerinum*[6] and *Candida albicans*[7] as well as antibacterial activities toward *Bacillus subtilis*.[8] A consecutive bio-guided procedure resulted in the isolation of two benzoquinones and one tetrahydrophenanthrene from this extract.[9,10] We describe in this work the isolation and the structure elucidation of the UV quantitatively major compound of the same extract which displayed interesting UV and MS features in a HPLC/DAD-UV/APCI-MS preliminary analysis.

Results and Discussion

The HPLC/DAD-UV/APCI-MS analysis of the CH_2Cl_2 extract of the *Heliotropium* aerial parts revealed that compound **1** was characterized by a UV spectrum showing interesting patterns not attributable to any class of compounds and a molecular ion at *m*/*z* 625. These data motivated the selective isolation of **1**.



Compound **1** was obtained after fractionation of the raw CH_2Cl_2 extract by open silica gel column chromatography (fractions I−V) followed by semipreparative chromatography of fraction V using a μBondapak C_{18} radial compression column (see Experimental Section). Due to the complexity of the structure elucidation, larger amounts of the compound of interest were needed. Peracetylation of fraction V was found to facilitate the reisolation of **1**. The acetylated compound showed a molecular ion at *m*/z 793 $[M + H]^+$, indicating the presence of four acetyl groups. The peracetylated compound measured in $CDCl_3$ gave a better resolution of the ^{1}H NMR signals when compared with the data of the nonacetylated compound recorded in CD_3OD (Figure 1). The HRESIMS analysis of compound **1** confirmed the findings of the HPLC/DAD-UV/APCI-MS analysis that suggested a molecular weight of 624, but was not precise enough to establish a molecular formula. This was probably due to the presence of a minor peak accounting for 10% of

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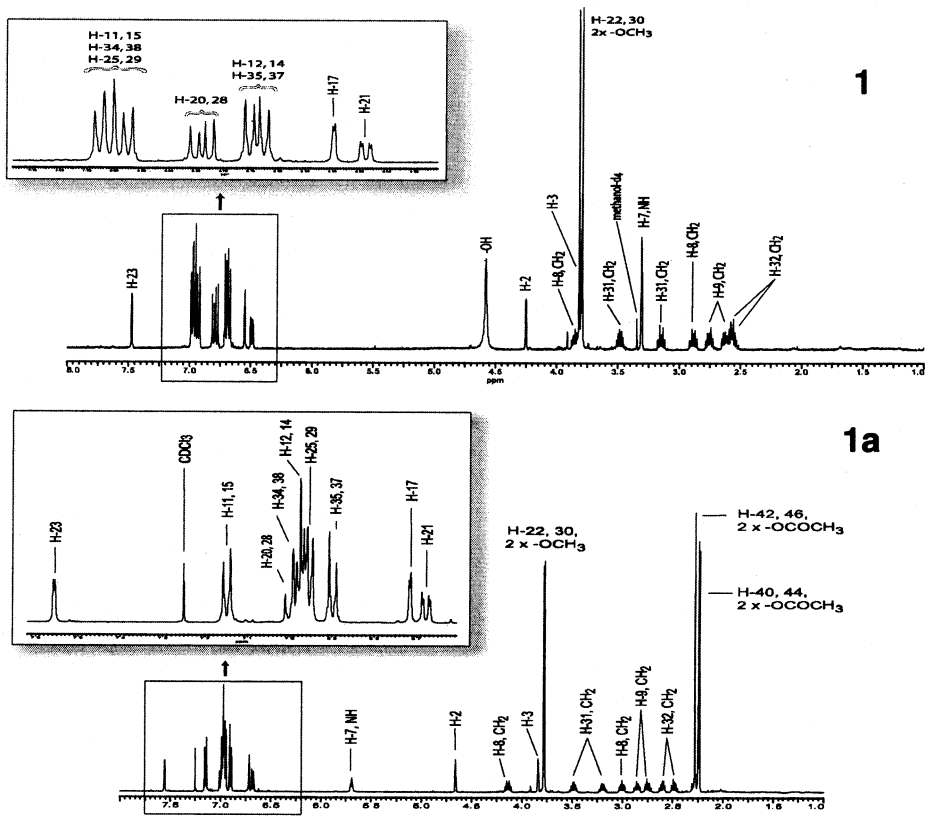

Figure 1. ^{1}H NMR spectrum of compounds **1** and **1a** (500 MHz, CD_3OD and $CDCl_3$, respectively).

the integration of the peak of compound **1**. Elemental analysis of compound **1** indicated the presence of nitrogen, but did not allow us to establish a molecular formula. The HRESIMS analysis of the acetylated compound (**1a**) finally suggested a molecular formula of $C_{44}H_{44}N_2O_{12}$, indicating a molecular formula of $C_{36}H_{36}N_2O_8$ for the nonacetylated product. Integration of the ^{1}H NMR spectrum confirmed the presence of 44 protons. Four distinct aromatic rings were determined from the ^{1}H NMR spectrum. Unless otherwise stated, all chemical shifts mentioned subsequently will be of the acetylated compound (**1a**). Signals at δ 3.77 and 3.78 (6H, OCH_3-22, 30) indicated the presence of two methoxyl groups. Eight multiplets located at lower field between δ 4.15 and 2.51 could be identified as CH_2 protons through correlations with their respective carbons in the gHSQC experiment. Protons at δ 3.21 and 3.50 could be attributed to the carbon at δ 40.6 (C-31), protons at δ 2.51 and 2.61 to the carbon at δ 34.6 (C-32), protons at δ 4.15 and 3.02 to the carbon at δ 42.2 (C-8), and the protons at δ 2.77 and 2.87 to the carbon at δ 32.7 (C-9). DQF-COSY correlations between these protons permitted us to assign their respective vicinal connections (see Figure 2).

The study of the gHMBC correlations gave evidence for two 4-acetoxyphenylethyl moieties, one 4-acetyloxy-3-methoxybenzylidene unit, and one 4-acetoxy-3-methoxyphenyl unit. Elucidation of the central pattern of the molecule was deduced from the observation of a signal at δ 5.73 (H-7), integrating for 1H in the ^{1}H NMR spectrum, but showing no correlation with any carbon in the gHSQC experiment. DQF-COSY correlations of this signal with the α and β protons geminally connected to the ^{13}C signal at δ 40.6 (C-31) and gHMBC correlations with ^{13}C resonances at δ 40.6 (C-31) and 169.0 (C-6) led to the hypothesis of a carboxamide central moiety that was proved by specific ^{1}H$-^{15}$N gHMBC experiments (Figure 3) and other HMBC correlations. The ^{1}H$-^{15}$N gHMBC measurements revealed

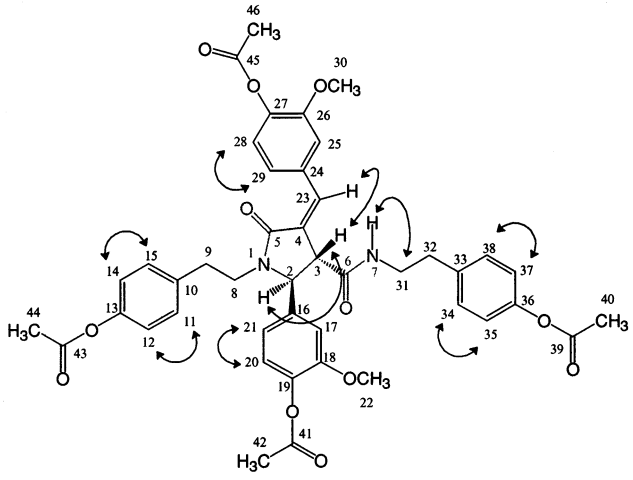

Figure 2. DQF-COSY correlations of **1a**.

cross-peaks for the protons at δ 2.51, 2.61 (H-32, 2H) and δ 3.85 (H-3) coupling in J_3 with the monoprotonated amide nitrogen (N-7) that was found to be correlated in J_1 with its proton located at δ 5.73 (H-7). The protons at δ 2.77, 2.87 (H-9, 2H) and 3.85 (H-3) are coupling in J_3 and the protons at δ 4.15 and 3.02 (H-8, 2H) in J_2 with the nitrogen (N-1) belonging to the second amide function. The presence of amide functions in the molecules was supported by the data of the IR spectrum of **1** with an absorption band at 1658 cm^{-1}. The IR spectrum also shows bands for hydroxyl groups (3346 cm^{-1}) and aromatic rings (1514 and 1449 cm^{-1}).

The presence of this carboxamide central unit was confirmed by the observation of ^{1}H$-^{13}$C NMR HMBC correlations between the signal at δ 7.57 (H-23) and the ^{13}C NMR signals at δ 53.4 (C-3), 126.5 (C-4), and 168.0 (C-5) as well as between δ 4.67 (H-2) and δ 53.4 (C-3), 127.4

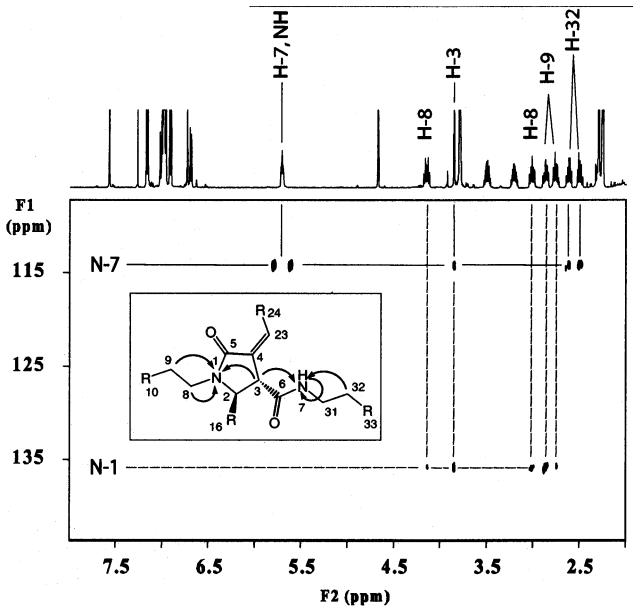

Figure 3. $^{1}H-^{15}N$ long-range gHMBC correlations of **1a** (^{15}N reference standard: $^{15}NH_4NO_3$).

(C-4), and 169.0 (C-6). Additional $^{1}H-^{13}C$ long-distance correlations between the ^{1}H NMR signal at δ 3.85 (H-3) and ^{13}C NMR resonances at δ 127.4 (C-4) and 168.0 (C-5) were in agreement with the proposed structure.

The NOE correlation observed between ^{1}H NMR signals at δ 4.67 and 3.85 suggested a *cis* configuration for protons H-2 and H-3. Due to the lack of NOE effects of ^{1}H NMR resonance of H-23 (δ 7.57), the relative configuration of the double bond between C-4 and C-23 could not be assigned. These results led us to conclude compound **1** to be 4-(4-hydroxy-3-methoxybenzylidene)-2-(4-hydroxy-3-methoxyphenyl)-*N*,1-bis[2-(4-hydroxyphenyl)ethyl]-5-oxopyrrolidine-3-carboxamide, named heliotropamide.

No natural product could be found in the literature presenting the same central oxopyrrolidine-3-carboxamide pattern, although substances with closely related structures exhibiting hydroxyl-phenylethyl and hydroxy-methoxy-bezylidene moieties were isolated from the fruits of *Cannabis sativa*.[11] Neither heliotropamide nor the acetylated derivative showed any activity in our in-house tests, including antifungal activity against *Cladosporium cucumerinum* and *Candida albicans*, antibacterial activity against *Bacillus subtilis*, radical-scavenging properties on DPPH, and inhibitory activity of acetylcholinesterase. Due to the novelty of the structure of **1**, other biological assays such as antiprotozoal tests are underway.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR: Varian Unity INOVA-500 NMR instrument. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and CD_3OD at 500.00 and 125 MHz, respectively. TMS: internal standard. $^{15}N-^{1}H$ NMR: the $^{15}N-^{1}H$ HMBC spectrum was measured with a Varian Unity INOVA-500 (50 MHz for ^{15}N) using an indirect detection probehead with a pulsed field *z*-gradient coil. UV: Varian DMS 100S UV−vis spectrophotometer. UV spectra were recorded in MeOH. $[\alpha]_D$: Perkin-Elmer-241 polarimeter. TLC: silica gel 60 F_{254} Al sheets (Merck) using petrol ether−EtOAc, 1:1. CC: silica gel (63−200 μm; 750 × 65 mm i.d. and 63−200 μm; 400 × 20 mm i.d., Merck). EIMS and D/CIMS: Finnigan MAT TSQ-700 triple-stage quadrupole instrument. HPLC/DAD-UV/APCIMS with a Nova-Pak RP-18 column (4 μm; 250 × 3.9 mm i.d.; Waters) using a CH_3CN-H_2O gradient (20:80 → 100:0) in 40 min. The detection was performed at 210 and 254 nm. The APCI used parameters: capillary temperature 150 °C, vaporizer temperature 450 °C, corona needle current 3.6 μA, and sheath gas flow 70% (=70 psi). Spectra (150−700 amu) were recorded in the positive ion mode. 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).

Table 1. ^{1}H NMR Data of **1** and **1a** (500 MHz)

H	**1** (in CD_3OD)	**1a** (in $CDCl_3$)
2	4.25 (1H, d, J = 2.44 Hz)	4.67 (1H, d, J = 1.6 Hz)
3	3.80 (1H, s)	3.85 (1H, t, J = 1.8 Hz)
7	3.30 (NH, t, J = 2.9 Hz)	5.73 (NH, t, J = 5.8 Hz)
8	2.88 (1H, m)	3.02 (1H, m)
	3.85 (1H, m)	4.15 (1H, m)
9	2.62 (1H, m)	2.77 (1H, m)
	2.76 (1H, m)	2.87 (1H, m)
11	6.97[a] (1H, m)	7.15[a] (1H, bd, 8.3)
12	6.69[b] (1H, m)	6.98[b] (1H, m)
14	6.69[b] (1H, m)	6.98[b] (1H, m)
15	6.97[a] (1H, m)	7.15[a] (1H, bd, 8.3)
17	6.54 (1H, d, J = 2.0 Hz)	6.73 (1H, d, J = 1.9 Hz)
20	6.77 (1H, d, J = 8.3 Hz)	6.99 (1H, m)
21	6.48 (1H, dd, J = 2.0, 7.8 Hz)	6.69 (1H, dd, J = 8.2, 1.9 Hz)
22	3.78 (3H, s)	3.77[c] (3H, s)
23	7.47 (1H, d, J = 2.0 Hz)	7.57 (1H, d, 1.9 Hz)
25	6.96[c] (1H, m)	6.96 (1H, m)
28	6.79 (1H, d, J = 8.3 Hz)	7.01 (1H, m)
29	6.93[c] (1H, m)	6.97 (1H, m)
30	3.81 (3H, s)	3.78[c] (3H, s)
31	3.14 (1H, m)	3.21 (1H, m)
	3.48 (1H, m)	3.50 (1H, m)
32	2.56 (1H, m)	2.51 (1H, m)
	2.58 (1H, m)	2.61 (1H, m)
34	6.92[c] (1H, m)	6.99 (1H, m)
35	6.93[c] (1H, m)	6.90[d] (1H, bd, 8.3)
37	6.93[c] (1H, m)	6.90[d] (1H, bd, 8.3)
38	6.92[c] (1H, m)	6.99 (1H, m)
40		2.23[e] (3H, s)
42		2.29[f] (3H, s)
44		2.24[e] (3H, s)
46		2.29[f] (3H, s)

[a−f] Values with the same symbol may be interchanged.

Plant Material. The aerial parts of *H. ovalifolium* were collected in March 1999 at Fothergill Island, Zimbabwe. A voucher (No. 99034) is deposited at the Insitute of Pharmacognosy and Phytochemistry, University of Lausanne, Lausanne, Switzerland.

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 fractionated by column chromatography on silica gel with a petrol ether−EtOAc gradient (9:1 → 1:4) giving fractions I−V. Purification of fraction V (0.5 g) was performed by semipreparative chromatography using a μBondapak C_{18} (10 μm, 25 × 100 mm) radial compression column with CH_3CN-H_2O (24:78) as the mobile phase and led to the isolation of compound **1** (50 mg). The separation conditions were established with a Waters radial compression analytical module and scaled up to a semipreparative module.

Heliotropamide (1): amorphous powder; $[\alpha]_D^{21}$ +14° (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ϵ) 223 (4.16), 287 (3.86), 327 (3.94); IR (KBr) ν_{max} 3346, 1658, 1514, 1449, 1268, 1031, 923; ^{1}H NMR (500 MHz, CD_3OD), see Table 1; ^{13}C NMR (125 MHz, CD_3OD), see Table 2; EIMS *m/z* 624 $[M]^+$ (26), 488 (35), 157 (52), 137 (69), 107 (100); DCIMS *m/z* 625 $[M + H]^+$ (100), 489 (18); HRESIMS (positive mode) *m/z* 625.2537 (calcd for $C_{36}H_{36}N_2O_8$ $[M + H]^+$, 625.2544).

Preparation of the Acetyl Derivative (1a). Compound **1** (10 mg) was acetylated with Ac_2O−pyridine, 1:1. The reaction mixture was concentrated and dried, and the residue was dissolved in CH_2Cl_2, washed with a 1% $NaHCO_3$ solution, and evaporated under reduced pressure. The organic layer was purified by analytical HPLC using a Nova-Pak C_{18} (RCM 8 ×

Table 2. ^{13}C NMR Data (δ) of **1** and **1a** (125 MHz)

C	**1** (in CD_3OD)	**1a** (in $CDCl_3$)
2	66.9	64.1
3	54.6	53.4
4	126.5	127.4
5	170.9	168.0
6	172.8	169.0
8	44.4	42.2
9	34.0	32.7
10	130.7	135.6[a]
11	130.9[a]	129.5[b]
12	116.3[b]	121.6
13	156.9[c]	149.3
14	116.3[b]	121.6
15	130.9[a]	129.5[b]
16	132.5	138.6
17	111.0	110.0
18	149.5[d]	151.8[c]
19	148.1	139.9[d]
20	116.5[e]	123.5
21	120.5	118.0
22	56.5	56.0
23	136.1	134.5
24	127.6	132.4
25	113.9	113.5
26	149.6[d]	151.4[c]
27	149.2	141.0[d]
28	116.7[e]	123.3
29	125.6	122.8
30	56.5	56.0
31	42.1	40.6
32	35.3	34.6
33	130.7	135.5[a]
34	130.7[a]	129.4[b]
35	116.4[b]	121.6
36	157.0[c]	149.2
37	116.4[b]	121.6
38	130.7[a]	129.4[a]
39		169.3
40		21.0
41		168.5[e]
42		20.5
43		169.3
44		21.0
45		168.7[e]
46		20.5

[a−e] Values with the same symbol may be interchanged.

10 mm; Waters), with a MeCN−H_2O gradient of 20% to 100% of MeCN in 40 min, affording the acetyl derivative **1a** (t_R = 20.1 min; 10.5 mg, 83.3%).

Acetyl heliotropamide (1a): amorphous powder; $[\alpha]_D^{21}$ +25° (*c* 0.01, MeOH); UV (MeOH) λ_{max} (log ϵ) 252 (4.11), 284 (4.51); ^1H NMR (500 MHz, $CDCl_3$, TMS), see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$, TMS), see Table 2; EIMS *m*/*z* 792 $[M]^+$ (10), 750 (8), 643 (45), 601 (35), 587 (92), 544 (39), 502 (31), 450 (82), 425 (43), 164 (90); ESIMS *m*/*z* 793 $[M + H]^+$ (55), 352 (37); HRESIMS (positive mode) *m*/*z* 815.2771 (calcd for $C_{44}H_{44}N_2NaO_{12}$ $[M + Na]^+$, 815.2786).

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