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Pyrrolizidine Alkaloids from *Onosma kaheirei* Teppner (Boraginaceae)

Ioanna Maria Orfanou[1], Harilaos Damianakos[1], Ioannis Bazos[2], Konstantia Graikou[1] and Ioanna Chinou[1*]

[1] *Department of Pharmacy, Division of Pharmacognosy and Natural Product Chemistry, University of Athens, 15771 Greece*

[2] *Department of Biology, Division of Ecology and Systematics, University of Athens, 15771 Greece*

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Abstract: The new pyrrolizidine alkaloid (PA) 3′-*O*-acetylechinatine *N*-oxide (**7**), along with two more known PAs (**5**, **6**), two known flavonoids (**3**, **4**), one known alkannin (**1**), two known triterpenoids, one known sterol, and allantoin (**2**) were isolated from the aerial parts of *Onosma kaheirei*. In addition, the retention indeces of the reduced PAs **6** and **7** were determined in a DB-5 WCOT column, to aid their detection by GC/MS in the future.

Keywords: Boraginaceae; *Onosma kaheirei*; 3′-*O*-acetylechinatine *N*-oxide; pyrrolizidine alkaloids; alkannins.

1. Introduction

The genus *Onosma* L. *(*Boraginaceae*)* includes about 86 species distributed mainly in the East and Central Asia and the Mediterranean area. The phytochemical reports for this genus reveal that it comprises mainly aliphatic ketones, lipids, alkaloids, flavones and naphthazarines [1]. Among them, the most important are naphthazarines and pyrrolizidine alkaloids (PAs).

Naphthazarines occur typically in the roots of Boraginaceae as derivatives of the enantiomeric compounds alkannine and shikonin. The latter are lipophilic red pigments with effective wound healing properties [2].

PAs are hydroxylated 1-methylpyrrolizidines (necines), esterified with one or two monocarboxylic necic acids (acyclic PAs) or with one dicarboxylic necic acid (macrocyclic PAs) [3]. PAs are plant toxins associated with disease in livestock and a serious health risk to humans. The contamination of staple foods of animal origin such as meat, milk and dairy products, eggs, as well as honey, pollen products, grain and herbal teas with PAs has been reported [4].

In particular, the transformation of 1,2-unsaturated PAs in liver, into reactive alkylating agents, has been demonstrated to be responsible for the toxic effects. PA consumption over long periods is mainly known to damage liver, lung or blood vessels [4]. Genotoxic effects (mutations,

[*] Corresponding author: E-Mail: ichinou@pharm.uoa.gr; Phone:+30-210-7274595 *Fax:* +30-210-7274115

sister chromatid exchanges, chromosomal aberrations) in plants and several cell culture systems after metabolic activation, have been reported as well [5]. PAs are also known to act as teratogens and abortifacients. In humans, PA poisoning is usually manifested as acute veno-occlusive disease (VOD) and childhood chirrhosis. PAs are also possible carcinogens for humans, since a number of them have been demonstrated to induce cancer in experimental animals [4].

Having considered the importance of these compound classes, we were prompted to investigate the chemical constituents of the rare endemic Greek species of *Onosma kaheirei* Teppner. *O. kaheirei* is a cushion-forming perennial herb with lignified base, large patent bristles, lanceolate leaves, and lemon yellow corolla, growing in rocks and rocky slopes, openings in evergreen woodland, at altitudes of 500-1200 m [6].

2. Materials and Methods

2.1. General

Specific rotation $[\alpha]_D$ values were measured using a Perkin Elmer 341 polarimeter. 1D and 2D-NMR spectra were recorded on 400 and 200 MHz FT-NMR Bruker spectrometers (400 MHz for ^{1}H NMR, 50 MHz for ^{13}C NMR), using $CDCl_3$, DMSO-d_6, D_2O or MeOH-d_4 as solvents, and TMS as an internal standard. High resolution mass spectra (ESI+) were recorded on a Thermo Scientific LTQ Orbitrap Discovery mass spectrometer, using the infusion method. TLC plates Kieselgel 60 F_{254}, 0.2 mm layer thickness, were purchased from Merck Chemical Co. Zones on TLC plates were detected under UV light (254 and 366 nm) and/or after spraying with a methanolic solution of 2.5 % H_2SO_4 and 2.5 % vanillin, followed by heating at 105°C for 5 min. For the special spot detection of PAs (free bases or *N*-oxides) on TLC plates, the Mattocks-Molyneux visualizing reagents were applied [16]. Methanol or dichloromethane were used as extraction solvents for the compound zones obtained from preparative TLC, using 20 × 20 cm plates. The solvent or solvent combinations used in column chromatography with cellulose as stationary phase, had been formerly saturated in water, after shaking them with water in a separatory funnel (except for water miscible solvents). HPLC grade solvents were used. The stationary phases used for column chromatography were silica gel 60H and 230-400 mesh, as well as microcrystalline cellulose and silica gel 60 RP-18 (40-63 μm), all purchased from Merck Chemical Co. The GC-MS analyses were performed with a Hewlett Packard Gas Chromatograph 5890 Series II Plus (linked to a Hewlett Packard 5972 mass spectrometer), equipped with a 30 m long, 0.25 mm i.d. and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 100 to 300ºC at a rate of 4ºC/min. Helium was used as a carrier gas, flow rate was 0.7 mL/min, split ratio 1:20, injector temperature 220ºC, ionization voltage 70 eV. For the GC-MS PA identification, the same analytical method described by Witte and coworkers was applied [27], using a capillary fused-silica WCOT 30 m × 0.32 mm i.d., 0.25 μm film thickness HP-1-MS column, J & W Scientific, CA.

2.2. Plant material

The aerial parts of *O. kaheirei* were collected by Dr. Ioannis Bazos (Biology Dept. of the University of Athens), from a rocky site of Ymittos Mt., at an altitude of 850 m, in Attica, Greece, at 05/18/2010. The plant was botanically identified and deposited at the Herbarium of the Division of Pharmacognosy of the Pharmacy Dept. of the University of Athens, by Dr. I. Bazos (voucher specimen no. KL3825).

2.3. Extraction and isolation

An amount of 177 g of the dried aerial part of *O. kaheirei* were ground to powder. The powdered plant material was successively extracted with cyclohexane, CH_2Cl_2 and MeOH, by immersion in 2.5 L of the solvent for 24 h, twice, at room temperature. Filtration and in vacuo evaporation of the solvents, resulted in the crude extracts of cyclohexane (2.0 g), CH_2Cl_2 (1.7 g) and MeOH (6.1 g). A portion of the cyclohexane extract (1.2 g) was subjected to silica gel column chromatography, eluting with cyclohexane/EtOAc/HOAc 100:0:0, 99:1:0, 98:2:0 and 98:1:1, to afford 11 fractions (C1-C11), based on TLC analysis. Fraction C6 (62 mg) was chromatographed with cyclohexane/EtOAc/HOAc 88:11:1 using prep. TLC, to give pure lupeol (4.7 mg) and some more zones. One of them (11.6 mg) was further purified by prep. TLC (development with cyclohexane/EtOAc/HOAc 88:11:1), affording the pure compound **1** (2.0 mg). An amount of 1.3 g of the CH_2Cl_2 extract was subjected to silica gel column chromatography (elution with cyclohexane/CH_2Cl_2 100:0-50:50), to obtain 23 fractions (D1-D23) after TLC examination. Among them, D7 (7.8 mg) contained lupenone. Fraction D20 (13 mg) was further subjected to prep. TLC (development with cyclohexane/EtOAc/HOAc 88:11:1), yielding pure *β*-sitosterol (7.1 mg). The total amount (6.1 g) of the MeOH extract was subjected to exhaustive maceration with Et_2O three times, in order to remove non-polar components. An amount of 4.67 g of the obtained extract was submitted to column chromatography (ø 5 x 18.5 cm, cellulose bed), eluting (see *3.1 General*) with cyclohexane/EtOAc100:0-0:100, EtOAc/MeOH 99:1-80:20 and MeOH/H_2O 50:50, and resulted in the fractions M1-M38. Fractions M9-M11 were pooled together (130.7 mg) and subjected to column chromatography (Sephadex LH-20, MeOH) to afford the pure compound **2** (7.4 mg). Fractions M17-M21 were combined (134.5 mg) and further chromatographed by vacuum liquid chromatography on a short column (bed ø 4 x 4 cm, silica gel RP-18, eluting with H_2O/MeOH 0:100-80:20, 50:50), yielding the pure compound **3** (19.1 mg). Fraction M24 (40.8 mg) was further purified with silica gel prep. TLC (development with CH_2Cl_2/MeOH/HOAc 58:41:1) and the compounds **4** (34.1 mg), **5** (8.5 mg) and **6** (6.3 mg) were isolated, after extraction of the scraped-off zones with MeOH (also containing dissolved silica gel). Finally, part (82 mg) of the fraction M34 (231.9 mg) was subjected to reversed-phase prep. TLC (development with H_2O/MeOH 80:20), resulting in the pure compound **7** (12.6 mg), after extraction of the collected zone with MeOH.

2.4. PA N-oxide reduction for GC-MS analysis

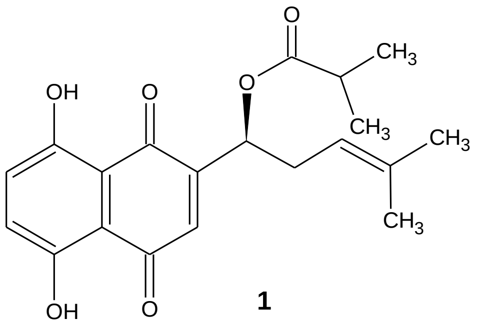
An amount of 6 mg of the PA *N*-oxide, was reduced by stirring with 32 mg (excess) $Na_2S_2O_4$ in 0.42 mL of 0.05 M HCl for 30 min, at room temperature. The resulting sulphur containing suspension was left to settle, and subsequently the supernatant was filtered in a syringe through a 0.45 μm HPLC sample filter, concentrated to a minimum volume of ~ 0.2 mL, and saturated with Na_2SO_4. The solution was alkalized to pH 10 with conc. $NH_3 \cdot H_2O$ solution, and repeatedly extracted with a Et_2O/n-BuOH 2:1 mixture. The extract was evaporated to dryness, redissolved in MeOH, and subjected to GC-MS analysis (see *General Experimental*). Prior to analysis, the reduced dry PAs were kept undissolved under a cyclohexane layer, in a refrigerator, to guard against oxidation.

2.5. 3' -O-acetylechinatine N-oxide (7)

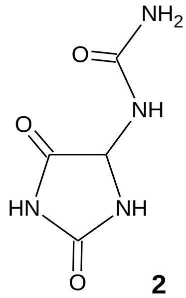
Pale yellow gummy residue; $[\alpha]^{20}_D$ - 4.1 (c 0.09, MeOH); ^{1}H-NMR (400 MHz, methanol-d_4), see Table 1; ^{13}C-NMR (50 MHz, methanol-d_4), see Table 1; Positive HR-ESI-MS *m/z* 380.1664 $[M+Na]^+$ (calcd. for $C_{17}H_{27}NO_7Na$, 380.1668)

2.6. 3'-O-acetylechinatine

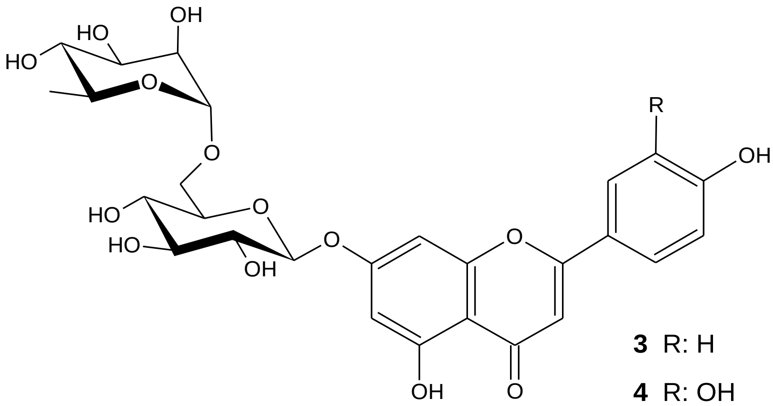
RI: 2242 (DB-5 column); positive EI-MS *m/z*: 173 (46), 159 (5), 138 (100), 129 (29), 120 (10), 94 (38), 93 (49), 80 (12), 75 (27), 67 (10), 59 (5), 41 (7).



1

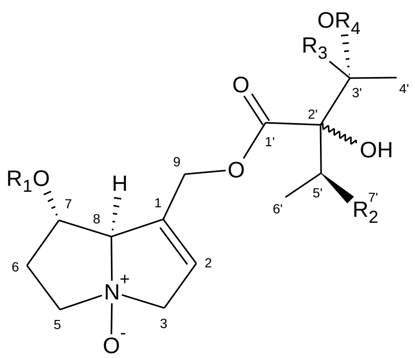


2



3 R: H

4 R: OH



	R_1	$C_{2'}$—OH	R_2	R_3	R_4
5	Ac	▬OH (wedge)	CH_3	H	H
6	H	·······OH (hashed)	OH	CH_3	H
7	H	▬OH (wedge)	CH_3	H	Ac

Figure 1. Structures of compounds 1-7

3. Results and Discussion

3.1. Structure elucidation

The cyclohexane extract of the aerial parts of *O. kaheirei* afforded after various chromatographic separations two known compounds, isobutyrylalkannin (**1**) [7] and lupeol [8]. Isohexenylnaphthazarins are well known constituents of certain plants of the Boraginaceae family [2]. The absolute configuration of the herein isolated **1** was established to be of the alkannin type, on the basis of its negative value of specific rotation [found $[\alpha]^{25}_{D}$: – 309 (c=0.001, CH_2Cl_2); lit. value $[\alpha]^{25}_{D}$: – 515 (c=0.00136, CH_2Cl_2)] [7]. The isolation of lupeol has been previously reported in the Boraginaceae family [9, 10], but is described here for the first time for the *Onosma* genus.

The dichloromethane extract was subjected to column chromatography and prep. TLC to yield two more known compounds. Their structures were established as lupenone [11,12] and β-sitosterol [13]. Lupenone is described for the first time in the Boraginaceae family, while β-sitosterol has been isolated twice in the *Onosma* genus *O. heterophylla* [14] and *O. limitaneum* [15].

Preliminary TLC examination of the methanol extract using the Mattocks-Molyneux spray reagent [16], confirmed the presence of PA *N*-oxides. The components of the methanol extract of *O. kaheirei* were sequentially separated and purified, to yield five previously known compounds (**2-6**), and a new natural compound (**7**). In specific, allantoin (**2**) was obtained as an amorphous white solid, and was recognized by TLC comparison with an authentic sample, as well as by means of its ^{1}H- and ^{13}C-NMR spectra [17, 18]. Allantoin has been also isolated from *Onosma erecta* [19]. In addition, the structures of **3** and **4** were elucidated by 1D and 2D NMR spectroscopy, and confirmed by comparison of their ^{1}H and ^{13}C NMR data with those reported in the literature for apigenin-7-*O*-rutinoside [20,21] and luteolin-7-*O*-rutinoside [20,22], respectively. These two flavonoids are reported for the first time within the Boraginaceae family. Furthermore, two known PA *N*-oxides, 7-*O*-acetylechinatine *N*-oxide (**5**) and 7-epiechimiplatine *N*-oxide (**6**) (both formerly isolated from *O. erecta*) were isolated and recognized, after comparison of their spectroscopic data with those reported in the literature [19].

Compound **7** was obtained as an optically active, pale yellow gummy residue. It was identified as a 1,2-unsaturated PA *N*-oxide of the retronecine/heliotridine type, by its purple spot obtained after visualization of TLC plates with the Mattocks-Molyneux reagent [16]. The molecular formula of **7** was established to be $C_{17}H_{27}NO_7$ by HR-ESI-MS (*m/z* 380.1664 $[M+Na]^+$, calcd. for $C_{17}H_{27}NO_7Na$ 380.1668). ^{13}C/^{1}H signal matching was based on HMQC correlations for all the non-quaternary carbons. ^{1}H-NMR chemical shifts of the ring protons were in close agreement with the values reported for other acyclic retronecine/heliotridine *N*-oxides [3]. In particular, the chemical shift values of the deshielded H-3α/H-3β (δ_H 4.63/4.33), H-5α/H-5β (δ_H 3.83/3.68) and H-8 (δ_H 4.67) protons, suggested the presence of the *N*-oxide group [3,23]. No NOESY interaction between H-7/H-8 (δ_H 4.73/4.67) was observed, indicating their trans orientation and that heliotridine is the necine moiety. The signals at δ_H 2.04 (3H, s) and at δ_C 18.5 and 172.0 were assigned to an acetyl group. The HMBC correrlation between H-3′ at δ_H 5.18 (1H, q, J = 6.5 Hz, H-3′) and the carbonyl carbon at δ_C 172.0, revealed that O-3′ was acetylated. The presence of a $\Delta^{1,2}$-double bond was confirmed by the signals of H-2 at δ_H 5.92 (1H, brs) and C-1/C-2 at δ_C 133.7/123.5 respectively, as well as from the COSY correlations between H-3α, H-3β/H-2 and H-8/H-2 (allylic coupling). The signal at δ_H 4.85 (2H, obscured by the OH peak) was assigned to the CH_2-9 group, according to the allylic COSY correlation between H_2-9 and H-2, and HMBC correlations between H-2 and C-9 (δ_C 62.4); H_2-9 and C-1; H_2-9 and C-8 (δ_C 97.2). The key HMBC correlation between H_2-9 and the carbonyl carbon atom at δ_C 170.6 (C-1′), indicated the presence of an esterified necic acid at O-9. One 1-acetoxyethyl group accounting for the signals at δ_H 5.18 (1H, q, J = 6.5 Hz, H-3′) and 1.29 (3H, d, J = 6.5 Hz, H-4′), was recognized from the ^{1}H-1D and COSY spectra, and was further supported by HMBC correlations (Table 1). Two methyl groups (H_3-6′/H_3-7′) resonating at δ_H 0.97 (3H, d, J = 6.9 Hz) and 0.91 (3H, d, J = 6.9 Hz) respectively, bonded to a methine group (CH-5′) at δ_H 2.17 (1H, sept, J = 6.9 Hz) were recognized likewise. In addition, the presence of an oxygenated quaternary carbon atom resonating at δ_C 82.0 was evident in the ^{13}C-NMR spectrum. The connection of the recognized fragments was accomplished on the basis of HMBC correlations. Only two heliotridine diastereomers corresponded to this structural formula, namely 3′-O-acetylrinderine *N*-oxide and 3′-O-acetylechinatine *N*-oxide. The former was ruled out, since its ^{1}H and ^{13}C NMR data [24] did not match with those obtained for **7**. Furthermore, all the diastereomeric PAs sharing this structural formula (echinatine, indicine, lycopsamine, intermedine and rinderine), had a common absolute configuration for C-2′ [3]. Thus, the spectral data of **7** could be attributed only to 3′-*O*-acetylechinatine *N*-oxide, the C-3′ epimer of 3′-O-acetylrinderine *N*-oxide. The PA 3′-*O*-acetylechinatine has been previously isolated from *Eupatorium portoricense* (Asteraceae) [25] and *Cynoglossum creticum* (Boraginaceae) [26], but not in the *N*-oxide form.

The retention index (RI) of reduced **7** (free PA), was determined (2242, DB-5 column) and its mass spectrum recorded, according to the sample preparation and GC-MS method described by Witte et al. [27]. The RI value of reduced **6** on a DB-5 column was also determined (2268), confirming the value reported previously [28].

Table 1. ^{1}H and ^{13}C NMR data of compound **7** (400 MHz for ^{1}H NMR, 50 MHz for ^{13}C NMR in MeOH-d$_4$, δ in ppm, J in Hz).

Position	δ_C	δ_H	COSY	HMBC[a]
1	133.7			2,3,8,9
2	123.5	5.92 (1H, *brs*)	3α,3β,8,9	3α,3β,8,9
3	79.1	α 4.63 (1H, *d*, J = 16.6)	2,3β,9	2,8,5α
		β 4.33 (1H, *d*, J = 16.6)	2,3α,9	
5	70.2	α 3.83 (1H, *m*)	5β,6α,6β	3α,3β,6α,7
		β 3.68 (1H, *m*)	5α,6α,6β	
6	35.7	α 2.60 (1H, *m*)	5α,5β,6β,7	5α,5β,7,8
		β 2.08 (1H, *m*)	5α,5β,6α,7	
7	70.8	4.73 (1H, *m*)	6α,6β,8	5α,5β,6β,8
8	97.2	4.67 (1H, *brs*)	7	2,9,7
9	62.4	4.85 (2H, *brs*) (obscured by the OH peak)	2	2,8
1′	170.6			9,3′
2′	82.0			3′,4′,6′,7′
3′	71.9	5.18 (1H, *q*, J = 6.5)	4′	4′
4′	16.6	1.29 (3H, *d*, J = 6.5)	3′	3′
5′	33.9	2.17 (1H, *sept*, J = 6.9)	6′, 7′	3′,6′,7′
6′	17.5	0.97 (3H, *d*, J = 6.9)	5′	5′,7′
7′	18.3	0.91 (3H, *d*, J = 6.9)	5′	5′,6′
*C*OCH$_3$	172.0			3′, COC*H$_3$*
CO*CH$_3$*	18.5	2.04 (3H, *s*)		

[a]HMBC correlations are from proton(s) stated to the indicated carbon.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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