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Aouicha Elkhamlichi, Ali El Antri, Hanane El Hajaji, Brahim El Bali, Hassan Oulyadi, et al.. Phytochemical constituents from the seeds of *Calycotome villosa* subsp. *intermedia*. *Arabian Journal of Chemistry*, 2017, 10 (S2), pp.S3580-S3583. 10.1016/j.arabjc.2014.03.005 . hal-02046274

HAL Id: hal-02046274

<https://normandie-univ.hal.science/hal-02046274>

Submitted on 22 May 2024

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ORIGINAL ARTICLE

Phytochemical constituents from the seeds of *Calycotome villosa* subsp. *intermedia*



Aouicha Elkhamlichi ^a, Ali El Antri ^a, Hanane El Hajaji ^a, Brahim El Bali ^b, Hassan Oulyadi ^c, Mohammed Lachkar ^{a,*}

^a Engineering Laboratory of Organometallic and Molecular Materials “LIMOM”, CNRST (URAC 19), Faculty of Sciences Dhar El Mahraz, University Sidi Mohamed Ben Abdellah, P.O. Box 1796 (Atlas), 30000 Fez, Morocco

^b Laboratory of Mineral Solid and Analytical Chemistry ‘LMSAC’, Department of Chemistry, Faculty of Sciences, University Mohamed I, P.O. Box 717, 60000 Oujda, Morocco

^c UMR 6014 COBRA & FR3030 INC3M, Equipe Analyse et Modélisation, Université de Rouen – IRCOF, Rue Tesnière, 76821 Mont Saint-Aignan, France

Received 26 July 2013; accepted 5 March 2014

Available online 13 March 2014

KEYWORDS

Calycotome villosa subsp. *intermedia*;
Seeds;
Dihydroisoquinoline-N-oxide;
Methyl 4-hydroxybenzoate;
X-ray crystal structure determination

Abstract A new dihydroisoquinoline-N-oxide alkaloid **1** together with the previously known methyl 4-hydroxybenzoate **2** were isolated for the first time from the seeds of *Calycotome villosa* subsp. *intermedia*. Their structures were elucidated by extensive spectroscopic analysis and in the case of the new alkaloid 1-hydroxymethyl-6,7-dimethoxy-3,4-dihydroisoquinoline-N-oxide **1** by X-ray diffraction analysis.

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1. Introduction

Calycotome villosa (Poiret) Link (Leguminosae), a shrub that grows mostly in cool places, is very common in the Mediterranean area (Gibbs, 1968; Tutin, 1972). However, *C. villosa* (Poiret) Link subsp. *intermedia* (C. Presl) Quezel and Santa, a 50–150 cm spiny shrub with yellow flowers during the spring season, grow

especially in the north of Africa and Spain (Greuter et al., 1989). *Calycotome villosa* subsp. *intermedia* has intricate, angular, pubescent branches, which are green when young and become greyish when mature. It shows sharp terminations and alternate leaves; the lower leaves are elongate, oval and trifoliate, and covered by sericeous down. The flowers are yellow and grouped, and have a bell-shaped calyx, thickly downy legume with a protuberant upper rib, and round dark olivaceous seeds. This shrub grows mostly in cool places, on asperities from 500 to 900 m above sea level (Camarda and Valsecchi, 1983). *Calycotome villosa* subsp. *intermedia* is not used in the Moroccan folk medicine. Medicinal uses have been reported only for *C. villosa* (Poiret) Link as antitumoral agent (Hartwell, 1982) and for the treatment of furuncle, cutaneous abscess and chilblain in the Sicilian folk medicine (Lentini et al., 1993).

* Corresponding author. Tel.: +212 671 556 742; fax: +212 535 733 171.

E-mail address: lachkar.mohammed@gmail.com (M. Lachkar).

Peer review under responsibility of King Saud University.



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As a part of a systematic research study on the constituents of Moroccan natural plants, we have reported, in previous phytochemical studies of *Calycotome villosa* subsp. *intermedia*, the isolation and characterization of flavone glucosides from the leaves and flowers (El Antri et al., 2004a) and tetrahydroisoquinoline alkaloids and isoquinoline-N-oxide alkaloid from the seeds (El Antri et al., 2004b,4c). Flavonoid compounds exhibit varied biological effects and have been identified as anti-tumor agents, antioxidants, and free radical scavengers (Manthy and Buslig, 1998; Bors et al., 1990). Substituted-tetrahydroisoquinoline alkaloids, in which C-1 is a quaternary stereogenic center, have been reported to display very interesting biological and pharmacological properties (He et al., 2000; Herbert, 1985). In a continuing search on the seeds of this species, the methanolic extract was found to contain a complex mixture of compounds. The present study describes the isolation and the structural elucidation of a new dihydroisoquinoline-N-oxide alkaloid together with a paraben derivative.

2. Materials and methods

2.1. Plant material

Seeds of *Calycotome villosa* subsp. *intermedia* were collected from the aerial part of the plant in June 2011 and again in June 2012 from area of Sefrou, Morocco. The plant was identified by Dr. Abdeslam Ennabili (Sidi Mohamed Ben Abdellah University, Fez, Morocco). A voucher specimen (n° EN008) has been deposited at the herbarium of the "Institut National des Plantes Médicinales et Aromatiques", Sidi Mohamed Ben Abdellah University, Fez, Morocco.

2.2. General procedures

Melting points were measured in open capillary tubes in a Büchi 530 apparatus and are uncorrected. The microanalyses have been performed at the "UATRS (Unités d'Appui Technique à la Recherche Scientifique), CNRST, Rabat, Maroc". UV-Visible spectra were obtained on a double-beam UV-Visible Cam-spec M550 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded in CDCl_3 on a Bruker (Wiessembourg, France) AM 300 spectrometer (300 and 75 MHz, for ^1H and ^{13}C NMR respectively). Chemical shifts are given as δ value with TMS as an internal standard and coupling constants are given in Hz. ESI-MS data were obtained on a Quattro II tandem quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with an electrospray ionization. EI-MS data were obtained on Micromass Autospec mass spectrometer. Silica gel GF₂₅₄ was used for TLC. Spots on chromatograms were detected under UV light (254 nm) and by Dragendorff's reagent. Column chromatography (CC) was carried out on silica gel 60 (70–230 mesh).

2.3. Extraction and isolation

The air dried plant material (150 g) was first extracted with hexane for 24 h and then with methanol for 48 h using a Soxhlet apparatus. The methanolic solution was evaporated to dryness and the resulting crude extract was dissolved in a 5% hydrochloric acid solution, and extracted with hexane and then with CH_2Cl_2 . The aqueous solution was basified to pH 10 with concentrated ammonia and extracted three times each with

200 ml of CH_2Cl_2 . The collected organic phases were dried over anhydrous sodium sulfate and evaporated under reduced pressure to give 2.6 g of the crude alkaloid extract. This crude alkaloid extract was subjected to column chromatography over silica gel (3 × 50 cm) using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient to give 15 mg of compound **2** (100% CH_2Cl_2) and 45 mg of compound **1** ($\text{CH}_2\text{Cl}_2/\text{MeOH}$: 97/3). Compound **1** was re-crystallized from methanol to afford crystals suitable for X-ray analysis.

2.4. Characterization data

2.4.1. Compound 1

1-Hydroxymethyl-6,7-dimethoxy-3,4-dihydroisoquinoline N-oxide **1**, colorless powder; Mp 188–190 °C; UV λ_{max} nm (MeOH): 203, 240, 331; IR bands (KBr) ν_{max} cm^{-1} 3459–3349, 2970, 1610, 1520, 1465, 1352, 1274, 1186, 1142, 1029, 952 and 850; ^1H NMR (300 MHz, CDCl_3): 3.01 (2H, t, $J = 7.5$ Hz, H-4); 3.82 (3H, s, 6-OCH₃); 3.83 (3H, s, 7-OCH₃); 4.01 (2H, t, $J = 7.5$ Hz, H-3); 4.73 (2H, s, 1-CH₂); 6.66 (1H, s, H-5); 6.83 (1H, s, H-8); ^{13}C NMR (75 MHz, CDCl_3): 150.1(C-1); 148.3 (C-7); 144.6 (C-6); 124.9 (C-10); 120.5 (C-9); 110.9 (C-5); 107.6 (C-8); 57.75 (C-3); 57.7 (CH₂); 56.2 (CH₃-O); 56.1 (CH₃-O); 27.5 (C-4); EA: Found: C, 60.72; H, 6.39; N, 5.90. $\text{C}_{12}\text{H}_{15}\text{NO}_4$ requires: C, 60.75; H, 6.37; N, 5.91; O, 26.97%. HRMS ESI (m/z found 238.2591, calc.: 238.1001 for $\text{C}_{12}\text{H}_{15}\text{NO}_4$); ESI-MS m/z : 238 [M + H]⁺ ($\text{C}_{12}\text{H}_{15}\text{NO}_4$), 220, 190, 175.

2.4.2. Compound 2

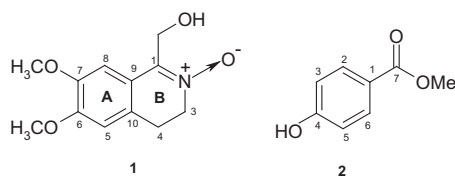
Methyl-4-hydroxybenzoate **2**, colorless needles; Mp 126–128 °C; UV λ_{max} nm (MeOH): 288, 325; ^1H NMR (300 MHz, CDCl_3): 7.95 (2H, d, $J = 8.7$ Hz, H-3, H-5); 6.84 (2H, d, $J = 8.7$ Hz, H-2, H-6); 5.57 (1H, s, OH); 3.86 (3H, s, OCH₃); ^{13}C NMR (75 MHz, CDCl_3): 167.0 (C-7); 159.8 (C-4); 131.9 (C-2 and C-6); 122.8 (C-1); 115.2 (C-3 and C-5); 52.0 (CH₃-O). HRMS ESI (m/z found 152.0445, calc.: 152.0473 for $\text{C}_8\text{H}_8\text{O}_3$); EI-MS m/z : 152 [M]⁺ $\text{C}_8\text{H}_8\text{O}_3$, 121, 93, 65.

2.5. X-ray crystal structure determination

Colorless single crystals of **1**, suitable for X-ray structure analysis, were obtained by slow evaporation of a methanol solution. The crystal has been collected on a STOE IPDS II two-circle-diffractometer using a MoK_α radiation ($\lambda = 0.71073$ Å). For further information on crystal structure and refinements data, CCDC 225920 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

3. Results and discussion

The seeds of *Calycotome villosa* subsp. *intermedia* were extracted with hexane and then with hot methanol. The methanolic extract was purified in the usual way for the isolation of alkaloids (Dalton, 1979). Fractionation and silica gel column chromatography yielded the new compound **1** and the



Scheme 1 A = phenyl ring and B = sofa six-membered ring.

known compound **2** (Scheme 1) besides the previously isolated alkaloids (El Antri et al., 2004b,4c). Compound **1** was the less polar of all alkaloids found in the methanolic extract of the seeds of *Calycotome villosa* subsp. *intermedia*.

Compound **1** was isolated in a crystalline form and completely characterized. The molecular formula of **1** was determined to be $C_{12}H_{15}NO_4$ on the basis of ESI-MS ($m/z = 238$ $[M + H]^+$) and elemental analysis. In the UV spectrum of **1**, the maximum bands are 203, 240 and 331 nm. Its IR spectrum showed absorption bands at 3459–3349, 2970, 1610, 1520, 1465, 1274, 1142, 1029 and 850 cm^{-1} . The ^1H NMR spectrum of **1** showed two aromatic protons at δ 6.83 (s, H-8) and 6.66 (s, H-5), two signals at δ 4.01 (t, 2H, $J = 7.5$ Hz) and 3.01 (t, 2H, $J = 7.5$ Hz) typical of H-4 and H-3 protons of a dihydroisoquinoline nucleus, and two singlets at δ 3.83 and 3.82 corresponding to the methoxyl groups at C-6 and C-7, which, in addition to the ^{13}C NMR spectrum and a prominent peak at $m/z = 238$ in the ESI mass spectrum, confirmed the dihydroisoquinoline skeleton in the molecule. The remaining ^1H and ^{13}C NMR data of **1** (singlet at δ 4.73 (2H), three CH_2 , two CH and five quaternary carbons) as well as the presence of an ion at $m/z = 190$ $[C_{12}H_{15}NO_4 + H - H_2O - NO]^+$ in the ESI-MS, evidenced a dihydroisoquinoline N-oxide in the molecule. Thus, the structure of **1** was determined as a 1-hydroxymethyl-6,7-dimethoxy-3,4-dihydroisoquinoline-N-oxide, which is a new natural product.

Compound **2** was isolated as colorless needles, with molecular formula $C_8H_8O_3$ deduced from the molecular ion peak at m/z 152 in the EI-MS and supported by ^1H NMR and ^{13}C NMR data. The mass spectrum of compound **2** showed molecular ion peak at m/z 152 with ions corresponding to loss of OCH_3 ($M - 31$; $m/z = 121$) followed by loss of the carbonyl group ($M - 31 - 28$; $m/z = 93$). The ^1H NMR spectrum analysis re-

Table 2 Selected bond lengths and angles of **1**.

Bond lengths [\AA]			
C1 C2	1.383(2)	C5 C6	1.398(2)
C1 C6	1.411(2)	C5 C10	1.506(2)
C2 C3	1.412(2)	C7 N8	1.311(2)
C3 C4	1.386(2)	C9 C10	1.517(2)
Bond angles [$^\circ$]			
C1 C2 C3	119.8(1)	C7 N8 C9	121.9(1)
C3 C4 C5	120.8(1)	N8 C7 C6	119.6(1)
C5 C6 C1	119.2(1)	C5 C10 C9	111.5(1)
C5 C6 C7	120.3(1)	N8 C9 C10	111.1(1)

vealed the presence of a pair of doublets at δ 7.96 (d, $J = 8.2$ Hz, H-3 and H-5) and 6.88 (d, $J = 8.2$ Hz, H-2 and H-6), each integrating to two protons, suggesting the presence of two aromatic hydrogen in ortho position. Each singlet of hydroxyl and methoxyl signals was observed at δ 5.60 and 3.92, respectively. Its ^{13}C NMR spectrum showed six resonances (two quaternary carbons, two CH groups, one carboxyl and one methoxyl) and the signals of carboxyl $\text{C}=\text{O}$ (δ 167.0) and methoxyl (δ 159.8). Accordingly, compound **2** was assigned as a paraben derivative named methyl-4-hydroxybenzoate (Scheme 1). The physical and spectroscopic data found are in concordance with literature values (Cheng et al., 2001; Perveen et al., 2009). Although there are many types of paraben derivatives by organic synthesis (Crombie et al., 1979; Hirota et al., 1981), methyl-4-hydroxybenzoate **2** was isolated for the first time from *Calycotome villosa* subsp. *intermedia*.

The crystal structure of **1** has been determined on single crystal. Crystallographic data are given in Table 1. Selected geometrical parameters, bond lengths and angles are listed in Table 2 according to the numbering scheme displayed in Fig. 1. The unit cell contains two molecules $C_{12}H_{15}NO_4$. The main part of the molecule of alkaloid **1** is the dihydroisoquinoline molecular fragment, which consists of the phenyl ring (A) and the sofa six-membered ring (B) (see Scheme 1). The deformation of the molecule can be expressed in terms of the distortion angles between its different parts. According to the values of the corresponding distortion angles: C3 C4 C5 C10–175.5(2), C7 C6 C5 C4–176.7(2), C10 C5 C6 C1 176.3(2), C2 C1 C6 C7 177.1(2), C1 C2 C3 O31–178.4(2), we

Table 1 Crystal data and measurement conditions of compound **1**.

Empirical formula	$C_{12}H_{15}NO_4$	
Formula weight (g/mol)	237.25	
Temperature (K)	100(2)	
Wavelength [λ , K α (\AA)]	0.71073	
Crystal system	Triclinique	
Space group	P-1	
Unit cell dimensions (\AA , $^\circ$)	a = 6.9573 (12)	$\alpha = 74.987(12)$
	b = 8.6906 (13)	$\beta = 87.066 (14)$
	c = 9.7297 (16)	$\gamma = 77.254 (13)$
Volume (\AA^3)	554.20 (16)	
Z	2	
$d_{\text{cal.}}$ (g/cm^3)	1.422	
Absorption coefficient (mm^{-1})	0.107	
$F(000)$	252	
Crystal size (mm^3)	$0.36 \times 0.32 \times 0.22$	
Reflections collected	6426	
Final R indices [$I > 2\sigma(I)$]	R1 = 0.0348, wR2 = 0.0802	

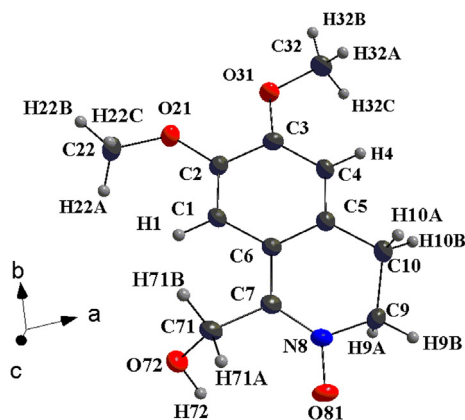


Figure 1 Perspective view of the molecule of **1**. Thermal ellipsoids drawn at 50% probability levels.

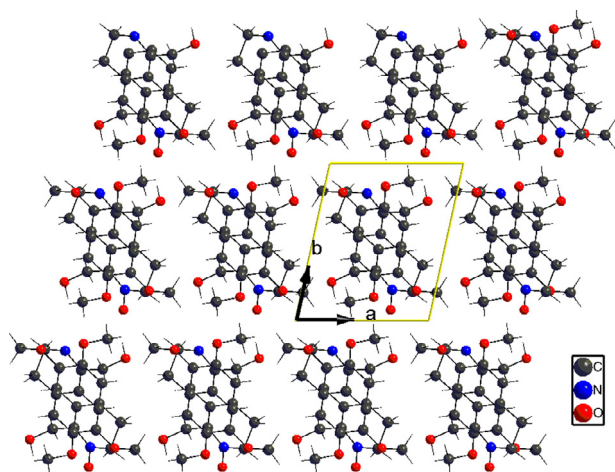


Figure 2 Projection of the crystal structure of alkaloid **1** onto [001].

state that the bonds C5–C10 and C6–C7 are in the plane of the ring. Same could be concluded about the orientation of the methoxy groups. The O31–C32 methoxy group lies in the plane of the ring (C4 C3 O31 C32 is -4.2°) while the methoxy group O21 C22 is out of the plane (C1 C2 O21 C22 is 16.7°). The hydroxyl group O72 has a gauche arrangement with respect to the H72–O72–C71–C7 torsion angle (65.0°).

A one restriction to the freedom of the molecule's motion is imposed by intramolecular H-bonds, between H72 and O81 ($2.711(2)$ Å). More such interactions are used to extend the molecular network in the three dimensions. Fig. 2 depicts a projection on the [001]. One can remark the formation of the “dimers” and their propagation in the ab-plane using H–O interactions.

4. Conclusion

The isolation and spectroscopic characterization of a new alkaloid together with a paraben derivative from the seeds of *Calycotome villosa* subsp. *intermedia* was described. The structure of the new alkaloid **1** was solved by X-ray analysis. Paraben derivative **2** was isolated for the first time from *Calycotome villosa* subsp. *intermedia*.

Acknowledgements

This work was funded by grants from the « Centre National de la Recherche Scientifique et Technique, Rabat, (URAC 19) » and the University Sidi Mohamed Ben Abdellah (Fez). The authors thank Dr. Abdeslam Ennabili (Sidi Mohamed Ben Abdellah University, Fez, Morocco) for identification of the plant.

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