

Amino Phenolics from the Fruit of the Argan Tree *Argania spinosa* (Skeels L.)

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Z. Naturforsch. **69c**, 363 – 367 (2014) / DOI: 10.5560/ZNC.2014-0123

Received July 7 / October 23, 2014 / published online November 12, 2014

A new phenolic-type compound containing a nitrogenous, heterocyclic-fused ring from the fruit of the argan tree, *Argania spinosa* (Skeels L.), is described. This and another already known compound also isolated in the course of the work belong to an obscure and rare class of natural products, the amino phenolics.

Key words: *Argania spinosa*, Amino Phenolics, Structural Elucidation, Natural Products

Introduction

As part of our continuing studies in the search for bioactive compounds and new anticancer drugs for the treatment and prevention of cancer, we have previously examined the fruit of the oleaginous tree *Argania spinosa* (Skeels L.) (Khallouki *et al.*, 2003, 2005, 2008). For the argan tree, belonging to the Sapotaceae family that contains eight genera of which the genus

Argania consists of a single species endemic to Morocco and second only to oak in forest acreage (Ayad, 1989), there is much evidence for the beneficial effects of its products for human health.

Of the numerous compounds identified in this plant, a peculiar one is 4,4'-dihydroxy-3,3'-imino-di-benzoic acid (**1**) (Fig. 1). This compound has been reported previously as a natural product from the rhizome of the fern *Drynaria fortunei* (Liang *et al.*, 2010) but

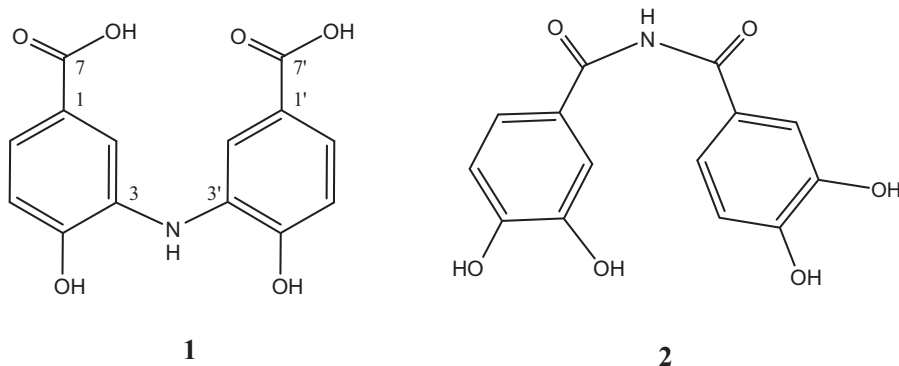


Fig. 1. 4,4'-Dihydroxy-3,3'-imino-di-benzoic acid (**1**) isolated from argan fruit and the compound *N*-(3,4-dihydroxybenzoyl)-3,4-dihydroxybenzamide (**2**) purportedly isolated from Pu-erh tea (Zhang *et al.*, 2011).

the data on its characterization are difficult to access. Interestingly, a related structure, ostensibly *N*-(3,4-dihydroxybenzoyl)-3,4-dihydroxybenzamide (**2**) (Fig. 1) isolated from Pu-erh tea (*Camellia assamica*), but with ^1H NMR data identical to those of **1** (*vide infra*), has also been reported (Zhang *et al.*, 2011). This appears quite impossible given the differing nature of the ring substituents with regard to the chemical shifts, even though an AMX pattern with very similar ^1H , ^1H coupling constant ($J_{\text{H,H}}$) values could be expected for the two compounds given that they are both 1,3,4-trisubstituted benzene ring systems. The same authors had also reported earlier (Zhang *et al.*, 2010) the synthesis of **2**, but did not report the NMR data of their synthesized compound nor did they compare the NMR data of the isolated natural product with those of the synthetic compound. Although compounds **1** and **2** are isomeric, it is highly unlikely that any synthesis could have led to compound **1** rather than compound **2** given the disparities in their structures.

Thus, there is some confusion regarding the structure and the NMR data of compounds **1** and **2**. Herein we confirm the structure and NMR data of compound **1** and additionally report the structure of a new and closely related compound, **3**, which was also isolated from argan fruit. These two compounds belong to an unusual class of natural products, the amino phenolics, which have been known for some time but are rarely reported. Amino phenolics have been isolated both from bacterial sources such as *Streptomyces* species (Gould *et al.*, 1996; Hu *et al.*, 1997) as well as from higher plants such as oats (Collins, 2011).

Materials and Methods

NMR analysis

NMR spectra were acquired using Bruker (Karlsruhe, Germany) Avance II (III) NMR spectrometers equipped with either 5-mm, normal- or inverse-configuration probes with triple-(z)-axis gradient capability at a field strength of 14.1 (9.4) T operating at 600.1 (400.1), 150.9 (100.6), and 60.8 MHz for the ^1H , ^{13}C , and ^{15}N nuclei, respectively, at 303 K. Pulse widths were calibrated following the described protocol (Klika, 2014). The chemical shifts of ^1H and ^{13}C nuclei are reported relative to tetramethyl silane (TMS) ($\delta = 0$ ppm for both ^1H and ^{13}C) using the solvent signals as secondary internal references ($\delta_{\text{CHD}_2\text{OD}} = 3.31$ ppm and $\delta_{\text{CHD}_2\text{S(O)CD}_3} = 2.50$ ppm for ^1H and $\delta_{\text{CD}_3\text{OD}} = 49.05$ ppm and

$\delta_{(\text{CD}_3)_2\text{SO}} = 39.52$ ppm for ^{13}C) while ^{15}N spectra were referenced externally to 90% nitromethane in CD_3NO_2 ($\delta_{\text{N}} = 0$ ppm). General NMR experimental and acquisition details for 1D ^1H , $^{13}\text{C}\{^1\text{H}\}$, DEPT, and selective NOESY ($\tau_{\text{m}} = 0.5$ s) and standard, gradient-selected 2D COSY, $^1\text{H}\{^{13}\text{C}\}$ -HSQC, $^1\text{H}\{^{15}\text{N}\}$ -HSQC, $^1\text{H}\{^{13}\text{C}\}$ -HMBC, and $^1\text{H}\{^{15}\text{N}\}$ -HMBC spectra have been previously described (Mäki *et al.*, 2005; Virta *et al.*, 2005) for routine spectral assignment and structural analysis.

4,4'-Dihydroxy-3,3'-imino-di-benzoic acid (1): ^1H NMR (CD_3OD): $\delta = 7.873$ (d, $J_{\text{H6}} = 2.04$ Hz, H2), 7.499 (dd, $J_{\text{H5}} = 8.28$ Hz, $J_{\text{H2}} = 2.04$ Hz, H6), 6.876 (d, $J_{\text{H6}} = 8.28$ Hz, H5), 2.806 (br s, H7), other labile H atoms were not observed. – ^{13}C NMR (CD_3OD): $\delta = 170.81$ (C7), 152.84 (C4), 132.70 (C3), 124.58 (C6), 123.76 (C1), 118.71 (C2), 115.08 (C5). – ^1H NMR (d_6 -DMSO): $\delta = 7.708$ (d, $J_{\text{H6}} = 2.04$ Hz, H2), 7.402 (dd, $J_{\text{H5}} = 8.26$ Hz, $J_{\text{H2}} = 2.04$ Hz, H6), 6.925 (d, $J_{\text{H6}} = 8.26$ Hz, H5), 6.687 (s, NH), other labile H atoms were not observed. – ^{13}C NMR (d_6 -DMSO): $\delta = 167.36$ (C7), 151.00 (C4), 130.81 (C3), 122.83 (C6), 121.69 (C1), 116.37 (C2), 114.25 (C5). – ^{15}N NMR (d_6 -DMSO): $\delta = -309.2$.

Compound 3: ^1H NMR (CD_3OD): $\delta = 7.826$ (ho m, large and small J , H6), 7.821 (ho m, small J , H2), 6.913 (ho m, large J , H5), 3.849 (2H, $\sim t$, $J = 7.1$ Hz, H9), 2.570 (2H, $\sim t$, $J = 8.1$ Hz, H11), 2.240 (2H, $\sim \text{qn}$, $J = 7.6$ Hz, H10), labile H atoms were not observed. – ^{13}C NMR (CD_3OD): $\delta = 178.19$ (C12), 172.41 (C7), 156.93 (C4), 131.63 (C6), 130.36 (C2), 128.21 (C3), 126.45 (C1), 117.44 (C5), 51.74 (C9), 32.33 (C11), 19.85 (C10). – ^{15}N NMR (CD_3OD): $\delta = -250.3$. – ^1H NMR (d_6 -DMSO): $\delta = 7.661$ (ho m, small J , H2), 7.655 (ho m, large and small J , H6), 6.866 (ho m, large J , H5), 3.691 (2H, $\sim t$, $J = 7.0$ Hz, H9), 2.390 (2H, $\sim t$, $J = 8.0$ Hz, H11), 2.087 (2H, $\sim \text{qn}$, $J = 7.5$ Hz, H10), labile H atoms were not observed. – ^{13}C NMR (d_6 -DMSO): $\delta = 174.15$ (C12), 168.7 (C7), 154.9 (C4), 129.05 (C6), 128.84 (C2), 124.94 (C3), 124.94 (C1), 115.57 (C5), 49.22 (C9), 30.82 (C11), 18.41 (C10). – ^{15}N NMR (d_6 -DMSO): $\delta = -252.9$.

MS analysis

High-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) was conducted on an Agilent 1100 HPLC system coupled to an Agilent single-quadrupole mass-selective detector (HP 1101; Agilent Technologies, Waldbronn,

Germany). Chromatography was performed using an RP-C18 column (250 mm \times 4 mm i. d. \times 5 μ m; Latek, Eppelheim, Germany) eluted with a gradient of 2% AcOH in double distilled water (A) and AcCN (B): initially 95% (v/v) A for 10 min, to 90% A within 1 min, to 60% A within 9 min, to 80% A within 10 min, to 60% A within 10 min, to 0% A within 5 min, and finally 0% A for 15 min. UV absorbance was monitored at 278 and 340 nm. Negative (positive)-ion mass spectra were acquired [fragmenter voltage, 100 V; capillary voltage, $-(+)$ 2500 V; nebulizer pressure, 30 psi; drying gas temperature, 350 $^{\circ}$ C; m/z scan range, 100–1500 Da].

Extraction and isolation

Fruits of *Argania spinosa* were harvested from Essaouira, Morocco, and the flesh was cut into small pieces, freeze-dried using a Christ lyophilizer (Osterode, Germany), pulverized into a fine powder (20 g), and then extracted with a Soxhlet apparatus using *n*-hexane (0.5 L) for 3 h to remove lipids followed by extraction with MeOH (3 \times 0.2 L, 3 h). The combined MeOH extracts were taken to dryness, suspended in absolute EtOH and then immobilized by Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) (5.0 g) before being deposited on a column of Sephadex LH-20 in EtOH. Fractionation was conducted by elution with increasing amounts of MeOH. Further fractionation after dissolution in 2% AcOH (2.0 mL) was performed using solid-phase extraction on C18 columns (Supelco, Bellefonte, PA, USA) eluting with increasing concentrations of MeOH in 2% AcOH. Final purification of phenolics was conducted by semi-preparative HPLC using conditions as described above.

Results and Discussion

The structural determinations of compounds **1** and **3** followed readily from the standard application of 1D and 2D NMR techniques. For compound **1**, the nominal mass was consistent with the ascribed formula and confirmed by high-resolution MS (HR-MS) (found, 289.0596; calcd. for $C_{14}H_{11}NO_6$, 289.0586). The positioning of the nitrogen atom on the ring was unequivocal, *e. g.* the chemical shift of C3, the HMBC correlations H2 to N and NH to C2 and C4, and the NOE between H2 and NH, as was the positioning of the OH and COOH groups at C4 and C1, respectively.

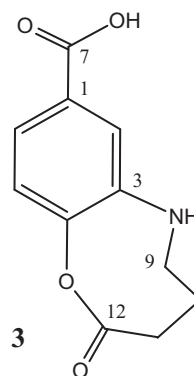


Fig. 2. Compound **3** isolated from argan fruit.

Thus we can categorically state that the NMR data are representative for structure **1** but not structure **2**.

For compound **3** (Fig. 2), from the mass spectrum, a likely formula of $C_{11}H_{11}NO_4$ was inferred by the mass of 221 Da obtained in both positive- and negative-ion modes of low-resolution MS coupled with the carbon and hydrogen count from the NMR spectra. The formula was thence confirmed by HR-MS for the pseudomolecular $[M - H]^-$ ion (found, 220.0612; calcd. for $C_{11}H_{10}NO_4$, 220.0615). The presence of a COOH group was strongly indicated by the loss of 44 Da. From the NMR spectrum, the presence of an *n*-propyl chain with a nitrogen atom bound at one end, an 1,3,4-trisubstituted benzene ring with an oxygen atom bound to C4 as inferred by the δ_C and $J_{H,H}$ values, as well as two carboxylic-type carbonyl carbon atoms was concluded. These segments accounted for 6 of the 7 double bond equivalents (DBEs), thereby necessitating the construction of an additional ring in the struc-

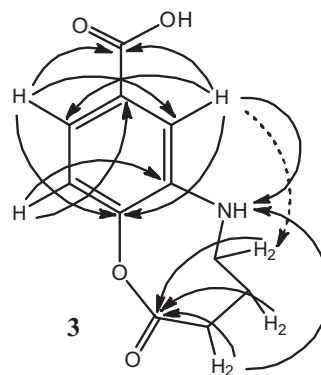


Fig. 3. The pivotal HMBC correlations (solid arrows) and NOE contact (dashed arrow) for compound **3**.

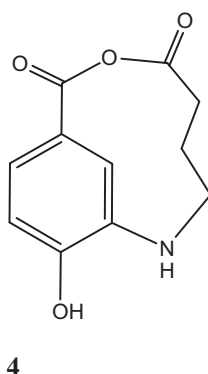


Fig. 4. A plausible alternative anhydride structure for the new compound **4** isolated from argan fruit.

ture. The required connectivities between the segments were established by HMBC and NOE contacts (Fig. 3) to unequivocally provide the final structure, particularly the H2 to N8 and H11 to N8 correlations as well as the NOE contact between the H9 and H2 nuclei. The chemical shifts of the assigned signals were also consistent with the proposed structure **3**.

It is worth noting that an alternative anhydride structure, **4** (Fig. 4), is conceivable linking the carboxy group at C1 and the carboxy group at the end of the *n*-propyl chain which is part of the ester functionality in the structure assigned to compound **3**. HMBC correlations could not be observed that would categorically distinguish between these two structures **3** and **4**, and this is not surprising, because 4- or 5-bond correlations would be required, *e. g.* H11 to C4 or H4 to C12 for **3**, or H11 to C7, H2 to C12, or H6 to C12 for **4**. While

an NOE contact between the H9 and H2 nuclei is consistent with both structures **3** and **4**, NOE contacts between the H2 and either the H10 or H11 nuclei were not observed, which could be anticipated given the observation of the NOE contact between the H9 and H2 nuclei. Furthermore, in *d*₆-DMSO, the phenolic proton that would exist for the alternate anhydride structure **4** was not observed, and which nevertheless might be expected, even though the NH and acidic protons were not observed in the NMR spectrum. Certainly, the potential for hydrogen bonding between the phenol group and the amine group would help facilitate either observation of the phenol proton, if the phenol group acted as a H donor, or observation of the amine proton, if the roles were reversed and the phenol group acted as a H acceptor. Whether an anhydride functionality would persist in the native state and survive the isolation process is questionable, however. In any case, the rapid motional averaging of the heterocyclic ring which leads to the chemical shift equivalence of the methylene pairs in the *n*-propyl chain would not be viable in the alternate anhydride structure **4**, because the ring inversion necessary for spin-site interchange would be strongly impeded by H2 due to steric interactions as indicated from physical models. Moreover, sizeable deshieldings would be expected in the alternate anhydride structure **4** for H2 due to steric compression, and similarly for H10 and H11 for the same reason. Additionally, H10 and H11 would be even further deshielded due to the anisotropy of the benzene ring to which they are constrained within close proximity as a result of the geometric limitations of the heterocyclic ring.

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