

Carotamine, a Unique Aromatic Amide from *Daucus Carota* L. Var *Biossieri* (Apiaceae)

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Abstract: The unique aromatic peptide 4-(*p*-aminobenzoylamino)-2-aminobenzoic acid, carotamine, together with 2,4-diaminobenzoic acid, isolated for the first time from a plant source, were identified from the aqueous alcoholic extract of the aerial parts of *Daucus carota* L. var. *boissieri* (Apiaceae). The structures were determined through conventional methods of analysis and confirmed by LC-ESI/MS and NMR spectral analysis.

Keywords: *Daucus carota* L. var *boissieri* (Apiaceae); aromatic peptide; 4-(*p*-aminobenzoylamino)-2-aminobenzoic acid; aminoacids; 2,4-diaminobenzoic acid

Introduction

Daucus L. (Apiaceae) [1] includes about 60 species distributed mostly in Europe, Africa, West Asia and few ones in North America and Australia [2]. In Egypt, the genus *Daucus* L. is represented by 6 wild species [1] among which the two varieties *Daucus carota* *boissieri* [3] and *Daucus carota* *sativus* [4] are widely cultivated for their fleshy edible roots (Bailey, 1960). *Daucus carota* has been reported to contain several constituents such as flavonoids [6,7], essential oils [8,9], polyacetylenes [10,11] and phenylpropanoids [12]. *Daucus carota* is well known in the Egyptian folk medicine as a stimulant, carminative and diuretic [13]. The decoction of carrot is used for infantile diarrhoea and as an antihelmentic [14]. The fruit essential oil has been proven to be hypotensive, cardiac and CNS

depressant [15], antibacterial [16], antibilharzial [17], and fungicidal [18]. Carrots also showed a significant protective activity in the alleviation of chloroform-induced hepatocellular injury in the mouse [19].

The present study reports on the isolation and identification of 2,4-diaminobenzoic acid (**1**) and the unique aromatic peptide, 4-(*p*-aminobenzoylamino)-2-aminobenzoic acid (**2**) or carotamine, which is the first aromatic peptide reported to occur in nature. Extensive EI and LC-ESI/MS techniques were applied together with ^1H - and ^{13}C -NMR spectral analysis to verify the full structure of both compounds.

Results and discussion

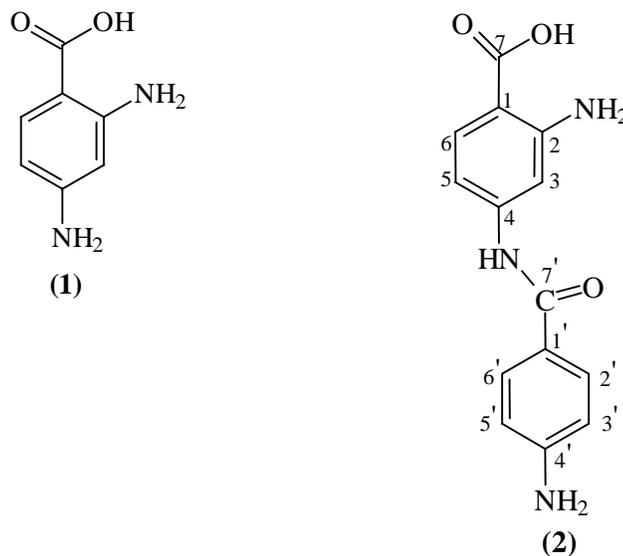
The aqueous alcoholic extract of the ground meal of the aerial *Daucus carota* parts, dried under vacuum, was defatted through exhaustive extraction with CHCl_3 . The residue left after CHCl_3 extraction was shown by two-dimensional chromatography to contain a mixture of polar compounds (high R_f values in aqueous solvents and low R_f values in organic solvent) mainly of phenolic nature (positive FeCl_3 test). The chromatograms also revealed the presence of two non-polar compounds that under UV light appeared as canary yellow (compound **1**) and dark purple (compound **2**) spots, respectively. A combination of column chromatography on Sephadex LH-20, using water saturated butanol as an eluent and preparative paper chromatography using 6% acetic acid as solvent afforded two pure samples of compounds **1** and **2**.

Compound **1** was isolated as an amorphous white powder with LC/UV absorption maxima at 227, 274 and 312 nm. The IR spectral analysis revealed two intense absorption bands at ν_{max} 3449.9 and 1661.7 cm^{-1} , consistent with amino and hydroxyl groups and a carbonyl group, respectively. The EI/MS gave a molecular ion at m/z 152. In LC-ESI-ve/MS (see Experimental) compound **1** exhibited a R_t of 3.48 min. and a molecular ion at m/z 151, corresponding to a molecular weight of 152. Under Collision Induced Dissociation (CID) conditions fragment ions at m/z 135, 108 and 91 have been observed and are attributed to the $[\text{M}-\text{NH}_3]^+$, $[\text{M}-\text{COO}]^+$ and $[\text{M}-(\text{NH}_2+\text{COO})]^+$ ions, respectively. The above given data suggest a diaminobenzoic acid structure for compound **1**. To resolve any ambiguity about the structure of **1**, ^1H and ^{13}C -NMR spectral analysis were then undertaken. The ^1H -NMR spectrum ($\text{DMSO}-d_6$, room temperature) revealed, in the aromatic region, the presence of a resonance pattern at δ 6.3 (*d*, $J=2$ Hz), 6.4 (*dd*, $J=2$ Hz & $J=7.5$ Hz) and 7.8 (*d*, $J=7.5$ Hz) ppm, typical of a 1,2,4-trisubstituted benzene [20], and assigned to H-3, H-5 and H-6 in the proposed 2,4-diaminobenzoic acid structure of (**1**). The spectrum also revealed a downfield resonance appearing as a sharp singlet at δ 12.7 ppm attributable to a hydrogen bonded proton (between the carbonyl carboxyl group at position 1 and the *o*-amino group at position 2, thus confirming the structure of (**1**) as 2,4-diaminobenzoic acid. Further confirmation of the structure was obtained through ^{13}C -NMR analysis. The recorded spectrum showed seven distinct aromatic carbon resonances among which the most downfield resonance at δ 168.0 ppm was assigned to the carboxyl carbon resonance while the most upfield resonance at δ 100.1 ppm was assigned to the quaternary C-1 carbon. Assignment of the remaining carbon resonances was

aided by calculating the expected chemical shifts deduced by applying the additive substituent rules to the reported chemical shifts of anthranilic acid [21]. Consequently, the carbons that bear the amino groups, C-2 and C-4, were found resonating at δ 148.9 and 152.6 ppm, respectively. The protonated carbons C-3, C-5 and C-6 gave three signals at δ 103.2, 108.5 and 134.1 ppm, respectively, which all agree well with the 2,4-diaminobenzoic acid structure proposed for **1**. It should be mentioned that this is the second reported natural occurrence of this compound, which has been characterised once before as a metabolite of *Streptomyces flocculus* [22].

Compound **2** was isolated as an amorphous yellow powder which exhibited in its LC/UV spectrum two fused absorption maxima at 363.8 and 336 nm as well as two shoulders at 237 and 302 nm. IR spectral analysis of **2** afforded a spectrum which revealed three absorption bands at ν_{\max} 3445.7, 1659.9 and 1640.5 cm^{-1} , consistent with amino and hydroxyl groups, a carboxyl carbonyl group and an amide carbonyl group, respectively. Standard alkaline hydrolysis (5% aqueous KOH, 100°C, ½ hour) of compound **2** yielded 2,4-diaminobenzoic acid (**1**) and *p*-aminobenzoic acid (CoPC). The EI/MS of **2** showed a molecular ion at m/z 271 and a base peak at 270, thus suggesting that the molecule of **2** is formed by two amino acids joined by an amide linkage (also detected by alkaline hydrolysis). In this spectrum the base peak at m/z 270 is therefore due to the loss of a carboxylic hydrogen or allylic proton from the amide bridge. The LC-ESI-ve/MS of **2** exhibited a R_t of 5.2 min. (see Experimental) and a molecular ion at m/z 270 corresponding to a molecular weight of 271. Under CID conditions the spectrum showed fragment ions at m/z 135, 120, 91 attributable to [aminobenzoic acid]⁻, [aminobenzoic acid-OH]⁻ and [M-(NH₂+COO)]⁻, respectively. The spectrum also showed a significant fragment ion at m/z 254 assignable to [M-NH₃]⁻ which also confirms that compound **2** is composed of 2,4-diaminobenzoic acid and monoaminobenzoic moieties linked through an amide bond. The results of ¹H-NMR spectral analysis of **2** lent further support to its suggested structure. The spectrum (DMSO-*d*₆, room temperature) showed distinct five proton resonances in the aromatic region at δ 6.4 (*d*, $J = 2.5$ Hz), 6.5 (*dd*, $J = 2.5$ and 7.5 Hz) and 7.8 (*d*, $J = 7.5$ Hz) ppm, respectively, corresponding to the 2,4-diaminobenzoic acid and at 7.1 (*d*, $J = 7.5$ Hz) and 8.2 (*d*, $J = 7.5$ Hz) ppm, assignable to H-3', H-5' and to H-2', H-6' in the symmetrical *p*-aminobenzoyl moiety. More interesting is the presence in this spectrum of a highly downfield sharp singlet resonance at 12.7 ppm, attributable to a hydrogen bonded proton. This reflected the presence of an unsubstituted COOH group at position 1 (see below) as well as the presence of a free vicinal amino group at position 2, responsible for the formation of the recognized hydrogen bond. Consequently, the structure of **2** is proven to be 4-(*p*-aminobenzoylamino)-2-aminobenzoic acid. Further support of this structure was then achieved through ¹³C-NMR spectral analysis (DMSO-*d*₆, room temperature) whereby the two most downfield resonances in the spectrum at δ 168.2 and 164.6 ppm are obviously due to the free carboxyl carbonyl carbon (C-7) and to the amide carbonyl carbon (C-7'). The most two intense resonances at δ 115.0 and 131.3 ppm are attributable to the C-3', C-5' and C-2', C-6' in the symmetrical *p*-aminobenzoyl moiety of **2**. Aromatic carbons bearing nitrogen functions (C-2, C-4 and C-4') appeared at δ 148.3, 154.4 and 153.1 ppm, respectively. The other carbon resonances in this spectrum exhibited chemical shift values which

agreed well with the proposed structure of **2** as 4-(*p*-aminobenzoylamino)-2-aminobenzoic acid, a new natural product.



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Experimental

General

LC/MS analyses were performed by reversed-phase HPLC on a Purosphere STAR RP-18 endcapped column (55x2 mm, 3 μ m, Merck, Darmstadt) using a Waters HPLC system, consisting of a Waters 2690 “Alliance” separation module coupled to a Waters 996 scanning UV detector. Flow injection analysis was performed by injecting 10 μ l of the extract into a solvent stream of methanol/water (1:1 by volume). Solvent A was 100% acetonitrile (HPLC grade, Merck); solvent B was water. Elution was performed at room temperature and at flow rate of 0.8 mL/min. The gradient program started at 5% A with an isocratic hold for 3 min, followed by a fast linear increase to 95% A at 4 min. The solvent composition was held for 1 min to flush the column, then changed back to initial conditions over 1 min and equilibrated for 4 min before the next sample injection; a shorter equilibration time lead to a shift in retention times. The total run time was 10 min. The eluent of the HPLC was split at a 1:4 ratio using an AcuRateTM flow splitter (LC Packings, via Omnilab, Mettmenstetten, CH) so that approximately 200 μ l/min entered the electrospray ion source of the mass spectrometer. The mass spectrometer used in this study was a Micromass Quattro-LC triple quadrupole mass spectrometer equipped with a “Z-Spray” electrospray ion source. The electrospray capillary

voltage was set to 3.0 kV, the source block temperature to 120°C. The cone gas was operated at 60 l/h, desolvation gas at 520 l/h and the desolvation temperature to 150°C. Spectra were acquired in profile mode alternating with 35 and 70 V cone voltage and scanning over the range m/z 50 to 1500 per second. Data acquisition was performed using Micromass' software package MassLynx 3.4. ^1H - and ^{13}C -NMR spectra were obtained on a Bruker AMX 400 spectrometer. ^1H spectra were measured relative to TMS and ^{13}C spectra were measured at 100 MHz, relative to DMSO- d_6 and converted to the TMS scale by adding 77 ppm. Paper chromatography (PC) was carried out on Whatman No. 1 paper, using either (1) H_2O ; (2) 6% HOAc or (3) BAW (n -BuOH-HOAc- H_2O , 4:1:5, top layer) as eluents; solvent 2 was used for preparative PC (PPC) on Whatman No. 3 mm paper.

Plant material, isolation and identification

Fresh aerial parts of *Daucus carota* L. var *boissieri*, were collected from Orman Botanical garden, Cairo, Egypt, during March 2000 and authenticated by Prof. Dr. Nabil El-Hadidi, Department of Botany, Faculty of Science, Cairo University, Egypt. A voucher specimen has been deposited in the Herbarium of the Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt. One kg of aerial parts of *Daucus carota*, dried in the shade in an air-draft, were comminuted to powder and exhaustively extracted with EtOH- H_2O (3:1). The aqueous alcoholic extract was dried in vacuum, and completely defatted with CHCl_3 . The residue left, 10 g, was dissolved in methanol and subjected to column chromatography (CC) on Sephadex LH-20 using n -BuOH saturated with H_2O for elution to yield 10 major fractions (**I-X**). Compound (**1**) (15 mg) was isolated from fraction **IV** by repeated PPC using 6% HOAc as a solvent. Compound (**2**) (20 mg) was obtained from fraction **X** by PPC using 6% HOAc as a solvent followed by Sephadex LH-20 CC using MeOH for elution.

2,4-Diaminobenzoic acid (1).

R_f -values: 0.55 (H_2O), 0.60 (HOAc), 0.45 (BAW); LC/UV λ_{max} (nm): 227, 274 and 312; IR ν_{max} cm^{-1} : 3449.9, 1661.7; M_r 152, -ve ESI/MS $[\text{M-H}]^-$: 151; ^1H -NMR: δ ppm 6.3 (d , $J = 2.5$ Hz, H-3), 6.4 (dd , $J = 7.5$ Hz and $J = 2.5$ Hz, H-5), 7.8 (d , $J = 7.5$ Hz, H-6); ^{13}C -NMR: δ ppm 100.1 (C-1), 148.9 (C-2), 103.2 (C-3), 152.6 (C-4), 108.5 (C-5), 134.1 (C-6), 168.0 (C-7).

4-(p-Aminobenzoylamino)-2-aminobenzoic acid (2).

R_f -values: 0.20 (H_2O), 0.25 (HOAc), 0.85 (BAW); LC/UV λ_{max} (nm): 363.8, 336, 237_{shoulder}, 302_{shoulder}; IR ν_{max} cm^{-1} : 3445.7, 1659.9, 1640.5; M_r 271, -ve ESI/MS $[\text{M-H}]^-$: 270; ^1H -NMR: δ ppm 6.4 (d , $J = 2.5$ Hz, H-3), 6.5 (dd , $J = 7.5$ and 2.5 Hz, H-5), 7.1 (d , $J = 7.5$, H-3' & H-5'), 7.8 (d , $J = 7.5$ Hz, H-6), 8.2 (d , $J = 7.5$, H-2' & H-6'); ^{13}C -NMR: δ ppm 100.5 (C-1), 148.3 (C-2), 103.5 (C-3), 154.4 (C-4), 108.5 (C-5), 133.1 (C-6), 168.2 (C-7), 118.8 (C-1'), 131.3 (C-2'), 115.0 (C-3'), 153.1 (C-4'), 115.0 (C-5'), 131.3 (C-6'), 164.6 (C-7').

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Sample availability: Available from the authors.