

## 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

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### 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  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectral analysis to verify the full structure of both compounds.

## Results and discussion

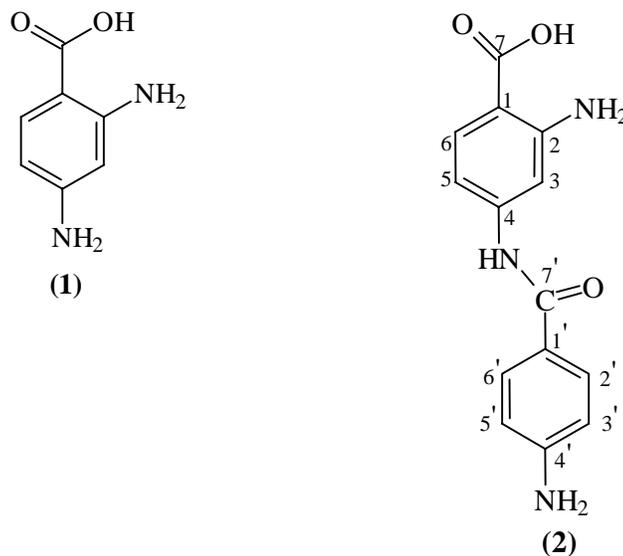
The aqueous alcoholic extract of the ground meal of the aerial *Daucus carota* parts, dried under vacuum, was defatted through exhaustive extraction with  $\text{CHCl}_3$ . The residue left after  $\text{CHCl}_3$  extraction was shown by two-dimensional chromatography to contain a mixture of polar compounds (high  $R_f$  values in aqueous solvents and low  $R_f$  values in organic solvent) mainly of phenolic nature (positive  $\text{FeCl}_3$  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  $\nu_{\text{max}}$  3449.9 and 1661.7  $\text{cm}^{-1}$ , 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**,  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral analysis were then undertaken. The  $^1\text{H}$ -NMR spectrum ( $\text{DMSO}-d_6$ , room temperature) revealed, in the aromatic region, the presence of a resonance pattern at  $\delta$  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  $\delta$  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  $^{13}\text{C}$ -NMR analysis. The recorded spectrum showed seven distinct aromatic carbon resonances among which the most downfield resonance at  $\delta$  168.0 ppm was assigned to the carboxyl carbon resonance while the most upfield resonance at  $\delta$  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  $\delta$  148.9 and 152.6 ppm, respectively. The protonated carbons C-3, C-5 and C-6 gave three signals at  $\delta$  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  $\nu_{\max}$  3445.7, 1659.9 and 1640.5  $\text{cm}^{-1}$ , 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]<sup>+</sup>, [aminobenzoic acid-OH]<sup>+</sup> and [M-(NH<sub>2</sub>+COO)]<sup>+</sup>, respectively. The spectrum also showed a significant fragment ion at  $m/z$  254 assignable to [M-NH<sub>3</sub>]<sup>+</sup> which also confirms that compound **2** is composed of 2,4-diaminobenzoic acid and monoaminobenzoic moieties linked through an amide bond. The results of <sup>1</sup>H-NMR spectral analysis of **2** lent further support to its suggested structure. The spectrum (DMSO-*d*<sub>6</sub>, room temperature) showed distinct five proton resonances in the aromatic region at  $\delta$  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 <sup>13</sup>C-NMR spectral analysis (DMSO-*d*<sub>6</sub>, room temperature) whereby the two most downfield resonances in the spectrum at  $\delta$  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  $\delta$  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  $\delta$  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 $\mu$ 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 $\mu$ 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 AcuRate<sup>TM</sup> flow splitter (LC Packings, via Omnilab, Mettmenstetten, CH) so that approximately 200  $\mu$ 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.  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra were obtained on a Bruker AMX 400 spectrometer.  $^1\text{H}$  spectra were measured relative to TMS and  $^{13}\text{C}$  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)  $\text{H}_2\text{O}$ ; (2) 6% HOAc or (3) BAW ( $n$ -BuOH-HOAc- $\text{H}_2\text{O}$ , 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- $\text{H}_2\text{O}$  (3:1). The aqueous alcoholic extract was dried in vacuum, and completely defatted with  $\text{CHCl}_3$ . The residue left, 10 g, was dissolved in methanol and subjected to column chromatography (CC) on Sephadex LH-20 using  $n$ -BuOH saturated with  $\text{H}_2\text{O}$  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 ( $\text{H}_2\text{O}$ ), 0.60 (HOAc), 0.45 (BAW); LC/UV  $\lambda_{\text{max}}$  (nm): 227, 274 and 312; IR  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3449.9, 1661.7;  $M_r$  152, -ve ESI/MS  $[\text{M-H}]^-$ : 151;  $^1\text{H}$ -NMR:  $\delta$  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);  $^{13}\text{C}$ -NMR:  $\delta$  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 ( $\text{H}_2\text{O}$ ), 0.25 (HOAc), 0.85 (BAW); LC/UV  $\lambda_{\text{max}}$  (nm): 363.8, 336, 237<sub>shoulder</sub>, 302<sub>shoulder</sub>; IR  $\nu_{\text{max}}$   $\text{cm}^{-1}$ : 3445.7, 1659.9, 1640.5;  $M_r$  271, -ve ESI/MS  $[\text{M-H}]^-$ : 270;  $^1\text{H}$ -NMR:  $\delta$  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');  $^{13}\text{C}$ -NMR:  $\delta$  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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