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

Omayma A. Eldahshan, Nahla A. Ayoub*, Abd-El Naser B. Singab and Mohamed M. El-Azizi.

Department of Pharmacognosy, Faculty of Pharmacy, Ain-Shams University, Cairo, Egypt.

*Author to whom correspondence should be addressed; e-mail: ayoub.n@link.com.eg

*Received: 11 May 2002; in revised form: 24 June 2002 / Accepted 26 June 2002 / Published: 30 June 2002

**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*-amino benzoylamino)-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 $^1$H- 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 $\nu$$_{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 [M-NH3]$^-$, [M-COO]$^-$ and [M-(NH$_2$+COO)]$^-$ ions, respectively. The above given data suggest a diaminobenzoic acid structure for compound 1. To resolve any ambiguity about the structure of 1, $^1$H and $^{13}$C-NMR spectral analysis were then undertaken. The $^1$H-NMR spectrum (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 $^{13}$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
Molecules 2002, 7

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 νmax 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 Rf 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-d₆, 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-aminobenzoylamo)n-2-aminobenzoic acid. Further support of this structure was then achieved through ¹³C-NMR spectral analysis (DMSO-d₆, 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.

![Structures](image_url)

**Acknowledgements**

The authors are deeply indebted to Dr. J. Hau, Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, P.O.Box 44, CH-1000 Lausanne 26, Switzerland, for the LC-ESI/MS measurements.

**Experimental**

**General**

LC/MS analyses were performed by reversed-phase HPLC on a Purosphere STAR RP-18 endcapped column (55x2 mm, 3µm, 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 10µ 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™ flow splitter (LC Packings, via Omnilab, Mettmenstetten, CH) so that approximately 200 µ/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’s software package MassLynx 3.4. $^1$H- and $^{13}$C-NMR spectra were obtained on a Bruker AMX 400 spectrometer. $^1$H 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$_2$O; (2) 6% HOAc or (3) BAW (n-BuOH-HOAc-H$_2$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-H$_2$O (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$_2$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 (H$_2$O), 0.60 (HOAc), 0.45 (BAW); LC/UV $\lambda_{\max}$ (nm): 227, 274 and 312; IR $\nu_{\max}$ cm$^{-1}$: 3449.9, 1661.7; $M_r$, 152, -ve ESI/MS [M-H]$^-$: 151; $^1$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); $^{13}$C-NMR: $\delta$ ppm 101.1 (C-1), 148.9 (C-2), 103.2 (C-3), 152.6 (C-4), 108.5 (C-5), 143.1 (C-6), 168.0 (C-7).

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

R$_f$-values: 0.20 (H$_2$O), 0.25 (HOAc), 0.85 (BAW); LC/UV $\lambda_{\max}$ (nm): 363.8, 336, 237, 302; IR $\nu_{\max}$ cm$^{-1}$: 3445.7, 1659.9, 1640.5; $M_r$, 271; -ve ESI/MS [M-H]$^-$: 270; $^1$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’); $^{13}$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’).
References

1. Täckholm, Vv. Student Flora of Egypt; Cairo University Press: Cairo, 1972, p193.
2. Jafri, S.M.H. Flora of Libya; 1. Ed.: Al Faateh University, Faculty of Science: Libya, 1985, p130.
3. Muschler, R. A Manual Flora of Egypt; R. Friedlander and Sons: Berlin, 1912; Vol. II, p 711.
4. Tutin, T.G.; Heywood, V.H.; Burges, N.A.; Moore, D.M.; Valentine, D.H.; Walters, S.M.; Webb, D.A. Flora Europaea; Cambridge University Press: Cambridge, 1981; Vol II, p 373.
5. Bailey, L.H. Manual of Cultivated Plants Growing in the United States and Canada; MacMillan: New York, 1960, p 747.
6. El Sayed, N.H.; El-Kubesy, T.M. Biochem. Syst. Ecol., 1994, 22, 762.
7. Singab, A.B.; Masuda, Y.; Okada, Y.; Mahran, G.; Khalifa, T.; Okuyama, T. Nat. Med., 1996, 49.
8. Kilibarda, V.; Nanusevic, N.; Dogovic, N.; Ivanic, R.; Savin, K. Pharmazie 1996, 51, 777.
9. Porchezhian, E.; Ansari, S.H.; Ali, M. Ind. J. Nat. Prod., 2000, 61, 24.
10. Lund, E.D. Phytochemistry 1992, 31, 3621.
11. Degen, T.; Buser, H.-R.; Stadler, E. J. Chem. Ecol., 1999, 25, 67.
12. Nagahashi, G.; Abney, C.D.; Doner, L.W. New Phytol., 1996, 133, 281.
13. El-Antaki, D. Tazkaret Oly-Al Albab; 3. Ed.; El-Azharia Press: Cairo, 1923, p122.
14. Watt, J.M.; Breyer, M.J. The Medicinal and Poisonous Plants of Southern and Eastern Africa; E. and S. Livingstone: London, 1962, p134.
15. Bodrug, M.V. Status and Prospects of Study and Use of Aromatic Plants in the Moldavian-SSR; USSR: Rastit, Resuuur, 1982, p 558.
16. Syed, M.; Sabir, A.W.; Chaudhary, F.M.; Bhatly, M.K. Pak. J. Sci. Ind. Res., 1986, 29, 189.
17. Halim, A.F.; Mashaly, M.M.; Sandra, P. 1st Anglo-Egyptian Conference of Pharm. Sci., Alexandria, Egypt, Nov, 1988, p15.
18. Dwivedi, S.K.; Pandey, V.N.; Dubey, N.K. Flavour Frag. J., 1991, 6, 295.
19. Bishayee, A.; Sarkar, A.; Chatterjee, M. J. Ethnopharmacol., 1995, 37, 69.
20. Hesse, M.; Meier, H.; Zeeh, B. Spectroscopic Methods in Organic Chemistry; George Thieme Verlag: Stuttgart, New York, 1997, p123.
21. Kalinowski, H.O.; Berger, S.; Braun, S. $^{13}$C-NMR Spektroskopie; Georg Thieme Verlag: Stuttgart, New York, 1984.
22. Gould, S.J. J. Antibiotics, 1988, 41, 688.

Sample availability: Available from the authors.

© 2002 by MDPI (http://www.mdpi.org). Reproduction is permitted for noncommercial purposes.