ACTA CHEMICA SCANDINAVICA 16 (1962) 142–148

3-(3-Carboxy-4-hydroxy-phenyl)-L-alanine (*m*-Carboxy-L-tyrosine), a New Amino Acid Isolated from Seeds of *Reseda odorata* L.*

P. OLESEN LARSEN and ANDERS KJÆR

Department of Organic Chemistry, The Royal Veterinary and Agricultural College, Copenhagen, Denmark

A new amino acid, isolated from the free amino acid fraction of seeds of *Reseda odorata* L., is demonstrated to be 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine (*m*-carboxy-L-tyrosine) (I).

The isolation was accomplished by means of strongly acid and weakly basic ion exchange resins and the final purification by recrystallization from water. Elemental analysis, ultra-violet and infra-red absorption spectra, protolytic properties and other data suggested 3-(3-carboxy-4-hydroxy-phenyl)-alanine as a likely structure for the isolate. Authentic material with L-configuration was synthesized by a series of steps departing from 3-nitro-L-tyrosine (II) and proceeding *via* 3-(3-cyano-4-hydroxy-phenyl)-L-alanine (IV). On comparison, the synthetic specimen proved identical with the natural amino acid. The latter represents the third example of *m*-carboxy-substituted aromatic amino acids encountered in higher plants.

During investigations of the contents of free amino acids in certain plants containing isothiocyanate-producing glucosides, attention was directed to species of the family *Resedaceae*. When a seed extract of the popular flower garden subject mignonette (*Reseda odorata* L.) was subjected to paperchromatographic amino acid analysis, a compound, exhibiting a greyish-blue colour with ninhydrin, was noticed next to a strong spot of glutamic acid on two-dimensional chromatograms (Fig. 1). The same compound possessed the notable property of exhibiting a brilliant blue or violet-blue fluorescence on exposure of the paper chromatograms to light of the wave-lengths 254 mμ and 350 mμ, respectively. Paper electrophoresis indicated that the unknown substance was a strongly acid amino acid. Efforts were hence directed towards isolation and characterization of the new amino acid.

On a preparative scale, 1 kg of ground seeds of *Reseda odorata* L. was defatted with carbon tetrachloride and subsequently extracted with aqueous

* Presented in abstract before the *1st Scandinavian Symposium on Natural Product Chemistry*, June 1961, Bornholm, Denmark.

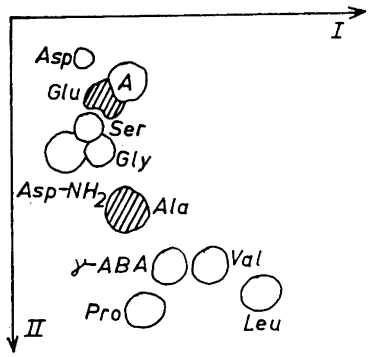


Fig. 1. Two-dimensional paper-chromatogram of the amino acid fraction of *Reseda odorata* L. (1) solvent: butanol:acetic acid:water (12:3:5); (2) solvent: phenol:water: conc. NH_3 (120:30:1). Developed with ninhydrin in acetone. A: 3-(3-Carboxy-4-hydroxy-phenyl)-L-alanine; other abbreviations are those commonly used.

methanol. The total amino acid fraction was separated from the extract by means of a strongly acid ion exchanger. When the amino acid fraction was applied to a weakly basic ion exchanger in the acetate form all acid amino acids including the unknown were retained. Aspartic and glutamic acid were eluted with acetic acid, whereas the new amino acid could be obtained virtually uncontaminated with other amino acids on subsequent elution with hydrochloric acid. The substance crystallized from the concentrated eluate at pH 2.5 in a total amount of 640 mg. It could readily be obtained in pure form as colourless needles on recrystallization from water.

The elemental composition, $C_{10}H_{11}NO_5$, together with the infra-red spectrum (Fig. 2) clearly revealed the aromatic character of the new amino acid. The strong band at 1650 cm^{-1} was tentatively interpreted as the C=O-stretching

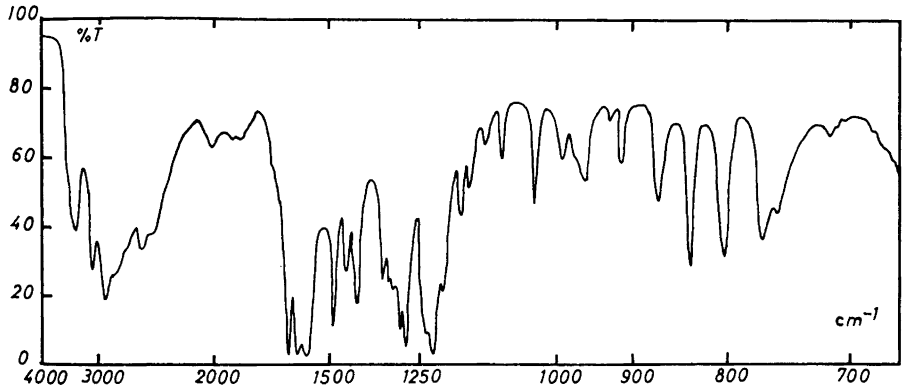


Fig. 2. Infra-red spectrum (in KBr) of 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine.

mode of a highly chelated carboxyl-group whereas the band at 1560 cm^{-1} was suggestive of a carboxylate ion. The two peaks at 870 cm^{-1} and 805 cm^{-1} provided evidence for a 1,2,4-aromatic substitution pattern. Application of the diagnostic assay developed in this laboratory [1] indicated that the unknown was an α-amino acid. With ferric chloride a strong violet colour was produced, indistinguishable from that given by salicylic acid, which also exhibits visible fluorescence in ultra-violet light. The characteristic ultra-violet spectra of the amino acid also provided useful information. The amino acid exhibited bands at 308 mμ, 236 mμ and 212 mμ (Fig. 3) in acid solution, a pattern reminiscent of that of salicylic acid [2]. According to the rule of Doub and Vandelbelt [3] for the absorption patterns of trisubstituted aromatic compounds, the observed UV-spectrum is consistent with a formulation of the new amino acid as 3-(3-carboxy-4-hydroxy-phenyl)-alanine (I); this structure is considered preferable to the isomeric 3-(3-hydroxy-4-carboxy-phenyl)-alanine because of the recent discovery in higher plants of the formally analogous *m*-carboxyphenylglycine [4] and *m*-carboxyphenylalanine [5].

The potentiometric titration curve (Fig. 4), revealing four protolytic functions, is in agreement with the proposed structure. The first dissociation step (pK 2) is due to the aliphatic carboxylic group. The second step (pK 3.4) can be assigned to the aromatic carboxylic group, the third step (pK 9.3) to the ammonium group whereas the last step (pK 12—13) indicates ionization of the phenol function, in agreement with the pK_2-value of 13 reported for sali-

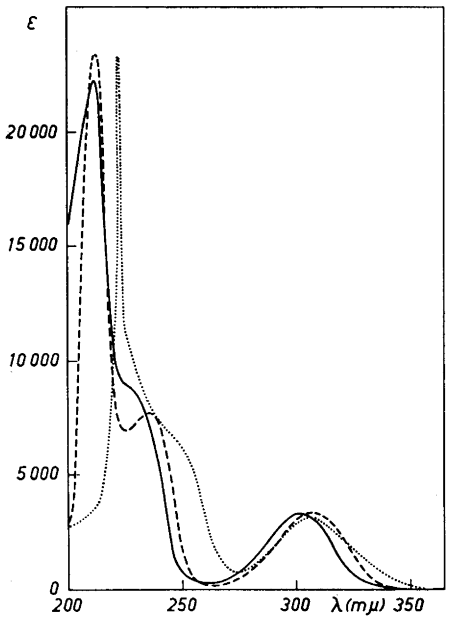


Fig. 3. Ultra-violet spectrum of 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine. — — — —: in 0.5 N HCl, ... : in phosphate buffer, pH 7, ——— :in 0.5 N NaOH.

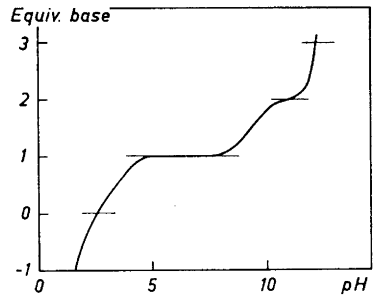
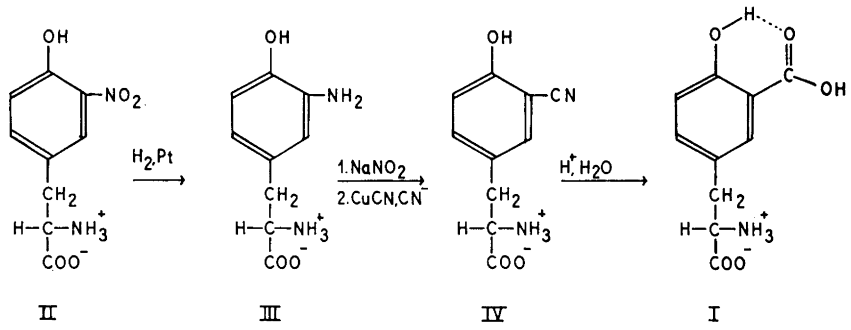


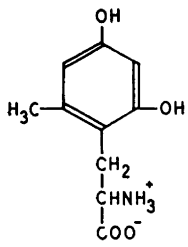
Fig. 4. Potentiometric titration curve of 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine in water. Determined by titration with 1 N NaOH of 19.33 mg of the amino acid in 100 μl 1 N HCl + 1 ml of water. The curve is strongly deformed in the pH-region above 11.5.

cylic acid [6]. The isoelectric point for the amino acid, deducible from the titration curve, is 2.5.

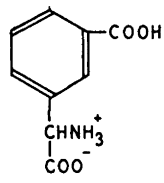
As a first synthetic approach, the O,N-diacetate of *m*-nitro-L-tyrosine was prepared by the method employed by Sealock [7] for preparing O,N-diacetyl-L-tyrosine. Reduction of the diacetate, however, was accompanied by undesired acyl migrations, even under acid conditions. Hence, this approach was abandoned.



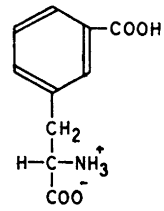
Final corroboration of the deduced structure, including information on the stereochemistry, was, however, obtained by the following unambiguous synthesis of the heretofore unknown 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine (I), by a route analogous to that previously developed for 3,4-dihydroxyphenylalanine [8]. 3-Nitro-L-tyrosine [9] (II) was reduced catalytically to 3-amino-L-tyrosine (III) which was, in turn, diazotized and treated with cuprous cyanide in cyanide to yield 3-(3-cyano-4-hydroxy-phenyl)-L-alanine (IV). Acid hydrolysis converted this compound into the desired 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine which was compared with the amino acid isolated from *Reseda* seeds. The two preparations were identical as judged from paper chromatography, infra-red spectra and optical rotation data.



V



VI



VII

Within the last few years several new aromatic amino acids have been encountered in higher plants. Thus, Schneider [10] identified 2,4-dihydroxy-6-methylphenylalanine (V) as a constituent of seeds of *Agrostemma githago* L., whereas Morris *et al.*[4] made the remarkable discovery of *m*-carboxyphenylglycine (VI) in bulbs of *Iris tingitana* Wedgw. Very recently, the latter group has also reported the isolation of the higher homologue, *m*-carboxy-L-phenylalanine [5] (VII), from the same source. The finding of three *m*-carboxylated aromatic acids, of course, raises the question as to a possible common or related biogenetic pathway of these. In this connexion it is noteworthy that the family *Resedaceae* systematically is very remote from the monocotyledonous family *Iridaceae*, a fact which suggests a much broader distribution of *m*-carboxy substituted aromatic compounds in higher plants than previously assumed.

During the present investigation, the occurrence of 3-(3-carboxy-4-hydroxyphenyl)-L-alanine in seeds of several other species of various genera within the family *Resedaceae* has been noticed. Among various species tested of the phylogenetically allied family *Cruciferae*, the new amino acid seems to be present in seeds of *Lunaria annua* L. and *L. rediviva* L.

EXPERIMENTAL

Microanalyses were performed by Mr. G. Cornali.

Rotations were measured in a 1 dm tube. Infra-red spectra were determined in potassium bromide pellets on a Perkin-Elmer 'Infracord'-instrument. Melting points were determined in capillary tubes in an Anschütz-Hershberg apparatus equipped with fully immersed thermometers. The standard rate of heating was 2° per min.

Isolation of 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine (I) from seeds of Reseda odorata *L.* The seed, *i.e.* garden hybride material designated 'grandiflora', was purchased from I. E. Ohlsen's Enke, Copenhagen. One kilogram was portionwise suspended in carbon tetrachloride and disintegrated in a Waring blendor. After filtration, the residues were refluxed with the same solvent for 30 min, cooled and filtered. The procedure was repeated, whereupon the air-dried powder (680 g) was refluxed for 4 h with methanol:water (4 l, 7:3 (v/v)) and filtered again. The extraction was repeated twice with fresh portions of solvent. The combined filtrates were then evaporated *in vacuo* to a dark-brown syrup (81 g), which was partly dissolved in water (1 l) and centrifuged. The supernatant was divided into two equal parts, and the total amino acid fraction in each half was bound on a strongly acid ion exchange resin (Zeokarb 225, 4 × 50 cm) in the acid form. After washing the column with water (1 l), the amino acids were eluted with NH_3 (1.5 l, 1 N). The combined eluates from the two halves were evaporated to a dark-brown syrup (14 g), which was dissolved in water (200 ml) and fractionated on a weakly basic ion exchange resin (Dowex

3, 3.2 × 40 cm) in the acetate form. After washing with water (900 ml), glutamic and aspartic acid were eluted with acetic acid (3.6 l, 1 N). The unknown amino acid was retained even on washing with formic acid (3.6 l, 1 N) whereas it could be eluted with HCl (2 l, 1 N). The eluate was evaporated to give a tan, crystalline residue (960 mg). Excess HCl was removed on evaporation and the residue was dissolved in water (10 ml), treated with charcoal, and adjusted to pH 2.5 with ammonia. 3-(3-Carboxy-4-hydroxy-phenyl)-L-alanine separated as brownish crystals (540 mg). On concentration of the mother liquor a further crop (100 mg) could be obtained. Two recrystallizations from water afforded a pure specimen, m.p. 263—264° (decomp.), $[\alpha]_D^{25}$ + 1° (*c* 1.2, 1 N HCl), $[\alpha]_D^{25}$ −7.7° (*c* 0.9, 1 N NaOH) (Found: C 53.16; H 5.08; N 6.18. Calc. for $C_{10}H_{11}NO_5$: C 53.33; H 4.92; N 6.22). The infra-red spectrum is shown in Fig. 2. The ultra-violet spectra (Fig. 3) are measured at a concentration of 0.09 μequiv/ml. Extinctions below 240 mμ are highly dependent on the concentration, due to association. λ_{max} in mμ: (*a*) 0.5 N HCl: 308, 236, 212; (*b*) phosphate buffer, pH 7: 302, 211; (*c*) 0.5 N NaOH: 307, 222.

L-*2-Acetamido-3-(3-nitro-4-acetoxy-phenyl)-propionic acid.* *m*-Nitro-L-tyrosine (10 mmoles) was dissolved in NaOH (6 ml, 2 N) and water (3 ml), and the solution was placed in a pH-stat. Acetylation was accomplished in the course of 80 min at 0° with acetic anhydride (30 mmoles). pH was kept at 7.5 by automatic addition of NaOH (2 N). Conc. HCl was then added to pH 1.9, resulting in the separation of an oil which crystallized overnight to give L-2-acetamido-3-(3-nitro-4-acetoxy-phenyl)-propionic acid (2.65 g, 86 %). Three recrystallizations from ethyl acetate gave a pure specimen, m.p. 158—159°, $[\alpha]_D^{28}$ +37.1° (*c* 2.6, 96 % C_2H_5OH) (Found: C 50.26; H 4.56; N 8.87. Calc. for $C_{13}H_{14}N_2O_7$: C 50.32; H 4.55; N 9.03).

3-(3-Cyano-4-hydroxy-phenyl)-L-alanine (IV). 3-Nitro-L-tyrosine (II) (20 mmoles) was dissolved in HCl (20 ml, 1 N) and reduced catalytically with Adam's PtO_2 catalyst (220 mg) and hydrogen (65 mmoles). After filtration, addition of HCl (12.5 ml, 4 N) and cooling to 0°, a solution of $NaNO_2$ (20.3 mmoles) in water (5 ml) was added. Solid Na_2CO_3 (28 mmoles) was added to bring pH to 3—4 and the solution was added in small portions to a second aqueous solution (40 ml), kept at 75° and containing CuCN (40 mmoles), NaCN (120 mmoles) and Na_2CO_3 (53 mmoles). A brisk evolution of nitrogen was observed. After 2 h at 75° and 1.5 h at 95°, the reaction mixture was cooled and adjusted to pH 2—3 with conc. HCl (hood!). CuCN was filtered off and the filtrate was brought to pH 6 with ammonia, whereupon the solution was diluted to a volume of 400 ml and applied to a strongly acid ion exchange resin (Zeokarb 225, 4 × 50 cm) in the acid form. The column was washed with water (500 ml), and the amino acids eluted with ammonia (1.3 l, 1 N). The eluate was evaporated to give a brown semi-crystalline solid (4.75 g), which was dissolved in water (10 ml). pH was adjusted to 6 with HCl, causing the 3-(3-cyano-4-hydroxy-phenyl)-L-alanine (2.3 g, 48 %) to precipitate. Several recrystallizations from water afforded a slightly yellow product retaining 1 3/4 moles of water of crystallization when air-dried, and decomposing above 250°, $[\alpha]_D^{28}$ +5° (*c* 1.5, 1 N HCl). (Found: C 50.27; H 5.82; N 11.49: H_2O (determined as weight loss at 120° over P_2O_5 at 0.01 mm Hg) 13.24. Calc. for $C_{10}H_{11}NO_5$, 1 3/4 H_2O: C 50.52; H 5.73; N 11.79; H_2O 13.26). The anhydrous product was hygroscopic and took up water to the same final weight when exposed to the atmosphere.

The nitrile seems to undergo decomposition in hot water as apparent from the production of a yellow contamination. In consequence hereof, nitrogen analyses tend to be low.

3-(3-Carboxy-4-hydroxy-phenyl)-L-alanine. (I). The nitrile (IV) (0.95 mmoles) was dissolved in conc. HCl (25 ml) and kept at room temperature for 26 h. Water (25 ml) was then added and the solution was refluxed for 27 h. After evaporation to dryness and removal of excess HCl, the residue was dissolved in water (3 ml) and pH was adjusted to 2.5 with ammonia, whereupon the 3-(3-carboxy-4-hydroxy-phenyl)-L-alanine (102 mg) separated. Concentration of the mother liquor gave a second crop (53 mg, total yield 73 %). A sample for analysis was produced by recrystallization from water, m.p. 263—264° (alone or in admixture with the natural compound), $[\alpha]_D^{26}$ −7.5° (*c* 0.9, 1 N NaOH) (Found: 53.39; H 4.88; N 6.11). The infra-red spectrum was indistinguishable from that of the natural product.

Attempts to produce the amino acid in one step from *m*-nitro-L-tyrosine proceeded satisfactorily as estimated from paperchromatographic analysis. However, the isolation of the pure amino acid in this case proved very tedious.

Paper chromatography. In accord with the strongly acid properties of the 3-(3-carboxy-4-hydroxy-phenyl)-alanine, R_F-values proved to be highly dependent on the pH of the applied solution, when solvent systems of low buffering capacity towards acids (*e.g.* butanol:acetic acid:water) were employed. The following R_F-values were determined for the new amino acid by descending chromatography on Whatman paper No. 1 at 25°: butanol:acetic acid:water (12:3:5): 0.31, when applied in neutral solution; 0.48, when applied in acid solution; phenol:water: conc.ammonia (120:30:1 (w/v/v)): 0.24.

The present work is part of investigations supported by *Kai Hansen's Fond.* A research grant to one of us (P.O.L.) from *The Carlsberg Memorial Foundation for Brewer I.C. Jacobsen* is gratefully acknowledged.

REFERENCES

1. Olesen Larsen, P. and Kjær, A. *Biochim. et Biophys. Acta* **38** (1960)148.
2. Doub, L. and Vandenbelt, J. M. *J. Am. Chem. Soc.* **71** (1949) 2414.
3. Doub, L. and Vandenbelt, J. M. *J. Am. Chem. Soc.* **77** (1955) 4535.
4. Morris, C. J., Thompson, J. F., Asen, S. and Irreverre, F. *J. Am. Chem. Soc.* **81** (1959) 6069.
5. Thompson, J. F., Morris, C. J., Asen, S. and Irreverre, F. *J. Biol. Chem.* **236** (1961) 1183.
6. Ågren, A. *Acta Chem. Scand.* **9** (1955) 49.
7. Sealock, R. R. *J. Biol. Chem.* **166** (1946) 1.
8. Waser, E. and Lewandowski, M. *Helv. Chim. Acta* **4** (1921) 657.
9. Bauer, H. and Strauss, E. *Ber.* **68B** (1935) 1108.
10. Schneider, G. *Biochem. Z.* **330** (1958) 428.

Received July 8, 1961.