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HYDROXYLATED GLUTAMIC ACIDS IN *PHLOX*, *LEPIDIUM* AND *RHEUM* SPECIES

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Key Word Index—*Phlox decussata*; *P. paniculata*; *P. arendsii*; Polemoniaceae; *Ledenbergia roseo-aenea*; Phytolaccaceae; *Pandanus veitchii*; Pandanaceae; *Lepidium sativum*; Cruciferae; *Rheum rhaponticum*; Polygonaceae; non-protein amino acids; (2*S*,4*R*)-4-hydroxyglutamic acid; (2*S*,4*S*)-4-hydroxyglutamic acid; diastereoisomers of (2*S*)-4-hydroxy-4-methylglutamic acid; 3,4-dihydroxyglutamic acids; glutathione.

Abstract—The two diastereoisomers, (2*S*,4*R*)-4-hydroxyglutamic acid and (2*S*,4*S*)-4-hydroxyglutamic acid, occur in characteristic concentration ratios in *Phlox* species. The second of these compounds is the principal free amino acid in the green parts of the plants. The presence of (2*S*,4*R*)-4-hydroxyglutamic acid in plants is reported for the first time. No other 4-substituted acidic amino acids were detected in the *Phlox* species analysed, although special attention was paid to the possible presence of 4-hydroxy-4-methylglutamic acids which have previously been reported in plants. It was found, however, that both diastereoisomers of (2*S*)-4-hydroxy-4-methylglutamic acid co-exist in *Ledenbergia roseo-aenea* and also in *Pandanus veitchii*. Although the presence of 3,4-dihydroxyglutamic acids in green parts of *Lepidium sativum* and *Rheum rhaponticum* has been previously reported, we were not able to detect or isolate any of the possible diastereoisomers from the green parts or seeds of these plants. We did isolate glutathione which was found to have some properties in common with those reported for the dihydroxy compounds.

INTRODUCTION

A relatively large group of 3- and/or 4-substituted acidic amino acids has been isolated from species in unrelated plant families [1–5] (see below). Some of these acidic amino acids co-exist within the same plant as do some of their diastereoisomeric forms [2–5]. The general co-occurrence of amino acids in this group might suggest that they are biosynthetically related. The hydroxylated compounds in this group are normally found in highest concentration in young actively growing green parts of the plants, such as the inflorescences of Resedaceae [4, 5] and the leaf primordia of Aspidiaceae and Aspleniaceae [3]. This suggests that they are produced from precursors closely related to primary photosynthetic products. However, little definitive biosynthetic information is available [5–7], and a re-examination of previously investigated plants [3, 4] using different methods [8] has revealed the presence of hitherto unidentified 3- and/or 4-substituted acidic amino acids.

4-Hydroxyglutamic acid has previously been found in species such as *Phlox decussata* (Polemoniaceae) [9], *Hemerocallis fulva* (Lilliaceae) [10, 11] and *Linaria vulgaris* (Scrophulariaceae) [12]. The chemical synthesis of the four stereoisomers, their separation from one another and their properties have been described [8, 12–15]. The plant product previously identified has been assigned the configuration (2*S*,4*S*)-4-hydroxyglutamic acid (**1**) [16]. The diastereisomer (2*S*,4*R*)-4-hydroxyglutamic acid (**2**) has not hitherto been reported as a plant constituent, but **2** is a well-known intermediate in the degradation of *trans*-4-hydroxy-(2*S*)-proline from collagen [17].

4-Hydroxy-4-methylglutamic acids are well-known plant constituents [1, 3–5] and they have been claimed as constituents of *P. decussata* [6]. The chemical synthesis, separation and properties of these compounds including their stereochemical characteristics have been reported ([8] and refs. cited therein). (2*S*,4*R*)-4-Hydroxy-4-methylglutamic acid (**3**) has been isolated from *Ledenbergia roseo-aenea*. (Phytolaccaceae) [18] and (2*S*,4*S*)-4-hydroxy-4-methylglutamic acid (**4**) has been isolated from *Pandanus veitchii* (Pandanaceae) [18], but **4** has not been reported in *L. roseo-aenea* and **3** has not been reported in *P. veitchii* and some doubt still exists concerning the configurations assigned to these compounds [8].

3,4-Dihydroxyglutamic acids have been reported as constituents of different plants, e.g. *Lepidium sativum* (Cruciferae) and *Rheum rhaponticum* (Polygonaceae). However, no information concerning the stereoisomers of these compounds is available. Although these amino acids have acquired general acceptance as plant products [1, 19], we have been unable to detect them in, or isolate them from *L. sativum* or *R. rhaponticum*. This paper also reports the presence of **1** and **2** and the absence of **3** and **4** from *Phlox* species and the co-occurrence of **3** and **4** in both *L. roseo-aenea*, and *P. veitchii*.

RESULTS AND DISCUSSION

Previously, we have employed 2D-PC and high voltage electrophoresis (HVE) to investigate the free amino acids in different plants [20] using synthetic reference compounds [8] for the identification of acidic amino acids [2–5]. These experiments showed that **1** is the

principal acidic amino acid in *P. paniculata*, and that small amounts of **2** are also present. We were unable to detect **3** and/or **4** in *P. paniculata* although they have previously been reported as constituents of *Phlox* species [6]. These investigations also showed that 4-substituted acidic amino acids are frequently accompanied by glutathione in the free amino acid pool [2–5]. This peptide has some properties in common with those reported for the 3,4-dihydroxyglutamic acids, e.g. R_f values and ionic mobilities on HVE. However, as we lacked reference samples of the 3,4-dihydroxyglutamic acids, it was decided to isolate all the acidic ninhydrin-reacting compounds from *L. sativum* and *R. rhaponticum* and characterize them unequivocally.

The acidic amino acids were isolated and identified by well-established methods [4, 5, 8]. To avoid transformation of the amino acids into lactones and lactams, the isolations were performed without refluxing at any state and all solutions were concentrated by lyophilization [21]. The configuration at C-2 was established using L-amino acid oxidase [3, 21].

The results obtained (Table 1) showed that all 3 *Phlox* species contained both **1** and **2**. Careful analyses of all fractions from the ion-exchange column in which 3- and/or 4-substituted acidic amino acids might be expected [8] revealed no trace of **3** and **4**. An unidentified acidic amino acid which was eluted from the anion-exchange column immediately before glutamic acid had R_f values on 2D-PC similar to those of **3** and **4**. Other properties, however, such as ionic mobility and R_t on the ion-exchange column [8] were different from those of **3** and **4**. If **3** and/or **4** are present in *P. decussata* at all, as reported previously [6], or in the other two *Phlox* species which we have studied, then they are present in concentrations of less than a 0.1–0.2 μg/g fr. wt. The previous report of **3** and/or **4** in *Phlox* species was based solely on PC data and radioactive tracer techniques, the compounds could not be detected by ninhydrin reaction and we suggest that these data may possibly have been misinterpreted.

The presence of **2** in plants has not previously been reported, but it is well known that the diastereoisomers of some 3- and/or 4-substituted acidic amino acids can co-occur in the same species [2–5]. As seen in Table 1 *P. veitchii* and *L. roseo-aenea* each contain both diastereoisomers of (2*S*)-4-hydroxy-4-methylglutamic acid. Previously, *L. roseo-aenea* has been reported to contain **3** and *P. veitchii* to contain **4** [18]. Using the methods of isolation and identification previously described [8] we have confirmed that **3** and **4** are the major acidic amino acids in *L. roseo-aenea* and *P. veitchii*, respectively. We have also shown, however, that lower concentrations of the second (2*S*) diastereoisomers are present in both species.

Previously, **2** has been reported as a constituent of micro-organisms and animals [1] being formed as an intermediate in the catabolism of (2*S*,4*R*)-4-hydroxyproline (**5**) from proteins [17, 22, 23]. The (2*S*,4*S*)-4-hydroxyproline (**6**) has also been reported as a free amino acid in plants [23, 24] but not as a constituent of proteins. It has been reported [17], that the 2-keto-4-hydroxyglutarates—the transamination products of **1** and **2**—are substrates for the 2-ketoglutarate dehydrogenase complex. Such enzyme activity has been detected in preparations from plants and animals [25], leading to the product L-malate when **1** is the substrate. It is also known that 2-keto-4-hydroxyglutarates are transformed in an aldolase-catalysed reaction into pyruvate + glyoxylate [17]. It is, unlikely, however, that the biosynthesis of **1** and **2** in plants is related to hydroxyproline catabolism.

This belief is based on the observation that both diastereoisomers of (2*S*)-4-hydroxyglutamic acid co-occur, especially in actively growing plants, and **1** which is the major constituent of the mixture is structurally related to the non-protein amino acid **6**. A possible precursor of **1** and **2** is glutamic acid semialdehyde, but it is more tempting to propose that the condensation of a 3-carbon unit with glyoxylate in an aldolase type reaction may yield the 2-keto-4-hydroxyglutarates. These 2-ketoacids may then yield **1** and **2** in a transaminase-catalysed reaction as discussed elsewhere for other 3- and/or 4-substituted acidic amino acids [4, 5].

In the original report concerning the isolation of 3,4-dihydroxyglutamic acids from plants [26], it was mentioned that these amino acids moved more slowly than aspartic acid and 4-hydroxyglutamic acids on PC. In that report it was also mentioned that these amino acids were found together with the other acidic amino acids in mixed fractions after ion-exchange chromatography, but no information concerning differences in properties of the four possible diastereoisomeric 3,4-dihydroxyglutamic acids was provided. On the basis of this report [26] the 3,4-dihydroxyglutamic acids have been generally accepted as constituents of higher plants [1, 19], and we were interested to determine the nature of the natural diastereoisomer or diastereoisomers. The free amino acids were isolated by methods similar to the original procedure but without HCl-catalysed hydrolysis of the extracts [26]. The only acidic compound isolated from *L. sativum* and subsequently from *R. rhaponticum* with R_f values corresponding to those reported for the 3,4-dihydroxyglutamic acids was glutathione (GSSG). The identity of this peptide was confirmed by co-chromatography and coelectrophoresis and by the comparison of the ^{1}H NMR spectrum with that of an authentic sample. A comparison was also made between the oxidation products of the isolate and of commercial glutathione.

In conclusion, we have shown for the first time that (2*S*,4*R*)-4-hydroxyglutamic acid is a plant product which co-exists with (2*S*,4*S*)-4-hydroxyglutamic acid in *Phlox* species; that (2*S*,4*R*)-4-hydroxy-4-methylglutamic acid and (2*S*,4*S*)-4-hydroxy-4-methylglutamic acid co-exist in both *L. roseo-aenea* and in *P. veitchii*; that 3,4-dihydroyglutamic acids do not occur in appreciable amounts in those plants of *L. sativum* and *R. rhaponticum* which we have analysed, but that these plants do contain major concentrations of glutathione. Absolute proof concerning the absence of plant constituents is very difficult to obtain. Our findings suggest, however, that earlier reports of the occurrence of 3,4-dihydroxyglutamic acid(s) in plants and the presence of 4-hydroxy-4-methylglutamic acid(s) in *Phlox* species merit re-investigation, at least to establish which diastereoisomers possibly could be the plant products.

EXPERIMENTAL

Plant material. The *Phlox* species listed in Table 1 and *R. rhaponticum* were grown outdoors in the Botanical Garden of the Royal Veterinary and Agricultural University, Copenhagen. Seeds of *L. sativum*, which were purchased from J. E. Ohlsens

Table 1. Acidic amino acids in plants reported to contain hydroxylated glutamic acids*

Plants	Aspartic acid	Glutamic acid	4-Hydroxyglutamic acid		4-Hydroxy-4-methyl-glutamic acid		Glutathione
			1(2*S*,4*S*)	**2**(2*S*,4*R*)	**3**(2*S*,4*R*)†	**4**(2*S*,4*S*)†	
Phlox (Polemoniaceae)							
P. decussata L.	++	+++	++++	+	(−)	(−)	+
P. arendsii Arends	++	+++	++++	+	(−)	(−)	+
P. paniculata var. Spitfire L.	++	+++	++++	+	(−)	(−)	+
Ledenbergia (Phytolaccaceae)							
L. roseo-aenea Lem.	+++	+++	(−)	(−)	++++	+	+
Pandanus (Pandanaceae)							
P. veitchii (Hort.) ex Cogn. et March	+++	+++	(−)	(−)	+	++++	+
Lepidium (Cruciferae)							
L. sativum L.	+++	++++	(−)	(−)	(−)	(−)	+++
Rheum (Polygonaceae)							
R. rhaponticum L.	++	++++	(−)	(−)	(−)	(−)	+++

* The table shows relative amounts of the amino acids as observed from the intensity of the ninhydrin spots after PC and HVE or from the peaks by use of the amino acid analyser.

† Concerning the configurations assigned to **3** and **4**, see Introduction.

(−) Not detectable; +, weak; ++, medium; +++, strong; ++++, very strong.

Enke, Copenhagen, were grown in a greenhouse. *P. veitchii* and *L. roseo-aenea* were obtained from Dr. G. A. Dardenne, Chemie Organique et Biologique, Faculté des Sciences Agronomiques de l'Etatt, Gembloux, Belgium. The plant material was stored at −20° until extractions were carried out.

^{1}H NMR spectroscopy, PC, prep. PC, HVE and amino acid analysis were performed as previously described [4, 8].

Isolation of acid amino acids. Leaves, stems and inflorescences of *P. decussata* (65 g) were homogenized in H_2O (400 ml) and filtered. The filtrates were taken to dryness by lyophilization and the isolation of the amino acids was performed by ion-exchange chromatography as desscribed elsewhere [8]. Plant material of *P. paniculata* (40 g) and *P. arendsii* (33 g) were treated in the same way. The fractions containing **1** and **2** were subjected to prep. HVE in buffer system (1) for 2 hr and/or in buffer system (2) for 3 hr [8] followed by ion-exchange chromatography on Dowex 50 W (×8, 200–400 mesh, H^+, 0.7 × 10 cm) [21] which afforded crystalline **1** and **2**. The compounds thus isolated were identical with the corresponding synthetic material [PC in solvent 1, 2, and 3; HVE in buffer system (1), (2), and (3) and by ^{1}H NMR] [8].

Leaves and stems of *P. veitchii* (20 g) and *L. roseo-aenea* (20 g) were treated in the same way as described above for *Phlox* species. Chromatographically pure **3** and **4** were isolated from both plant species (Table 1). The isolated compounds were identical with the synthetic diastereoisomers of 4-hydroxy-4-methylglutamic acid (PC, HVE and ^{1}H NMR) [8, 18].

Seeds and seedlings of *L. sativum* 30 and 50 g, respectively, were extracted and the amino acids isolated by use of cation-exchange chromatography as described for *Phlox* species. Amino acids with lower R_f values in solvent system 1 [8, 26] than aspartic acid were isolated by prep. PC in solvent system 1, followed by ion-exchange chromatography on Dowex 50 w (×8, 200–400 mesh, H^+, 0.7 × 10 cm) [21]. Evaporation of the pyridine eluate left a residue (40 mg) with a ^{1}H NMR spectrum identical with that of commercial glutathione [24]. PC and HVE data for glutathione, R_f values (in solvent): for GSH: 0.28 (1), 0.06 (2), 0.06 (3), for GSSG: 0.09 (1), 0.10 (2), 0.06 (3). HVE mobilities in cm (buffer): for GSH: 19.1 (pH 1.9), 9.1 (pH 3.6), 12.3 (pH 6.5) for GSSG: 21.2 (pH 1.9) 9.6 (pH 3.6), 16.2 (pH 6.5).

Leaves (100 g), stems (100 g), and inflorescences (100 g) of *R. rhaponticum* harvested in July were extracted and the amino acids isolated using cation-exchange chromatography as described for *Phlox* species. About 10% of each amino acid extract was purified by prep. PC in solvent system 1 as described for *L. sativum*. The acidic amino acids in the other 90% of the amino acid extract from the inflorescences were isolated by the Ecteola anion-exchange technique described elsewhere [5]. The amino acids were identified by 2D-PC in solvent systems 1–2 and 1–3, HVE in the buffer systems 1, 2 and 3, and by use of the amino acid analyser. The amino acids with lower R_f values than aspartic acid were isolated by prep. PC in solvent 1, and analysed by PC and HVE both before and after treatment with H_2O_2. It was not possible to detect 3,4-dihydroxyglutamic acids but glutathione in this fraction.

L-*Amino acid oxidase* (EC 1.4.3.2) from *Crotalus terr. terr.* venom was used to establish the configuration at the α-centre in **1** and **2** isolated from *P. decussata* [3, 21].

Oxidation of glutathione with H_2O_2. Glutathione (GSH and GSSG) (3 mg) dissolved in 0.1 ml 1 M NH_3 and 0.1 ml 30% H_2O_2 were mixed and left at room temp. After 6 and 24 hr the reaction mixture was analysed by PC and HVE. R_f values (in solvent): for the oxidized product GSO_2OH: 0.05 (1), 0.03 (2), 0.03 (3). HVE mobilities in cm (buffer): −3.5 (pH 1.9), 28.0 (pH 3.6), 25.5 (pH 6.5).

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REFERENCES

1. Fowden, L. (1970) *Progress in Phytochemistry* (Reinhold, L. and Liwschitz, Y., eds.) Vol. 2, p. 203. John Wiley, New York.
2. Dardenne, G., Casimir, J. and Sørensen, H. (1974) *Phytochemistry* **13**, 2195.
3. Meier, L. K. and Sørensen, H. (1979) *Phytochemistry* **18**, 1173.
4. Meier, L. K., Olsen, O. and Sørensen, H. (1979) *Phytochemistry* **18**, 1505.
5. Olsen, O. and Sørensen, H. (1980) *Phytochemistry* **19**, 1717.
6. Linko, P. and Virtanen, A. I. (1958) *Acta Chem. Scand.* **12**, 68.
7. Peterson, P. J. and Fowden, L. (1972) *Phytochemistry* **11**, 663.
8. Kristensen, E. P., Larsen, L. M., Olsen, O. and Sørensen, H. (1980) *Acta Chem. Scand. Sect. B* **34**, 497.
9. Virtanen, A. I. and Hietala, P. K. (1955) *Acta Chem. Scand.* **9**, 175.
10. Fowden, L. and Steward, F. C. (1957) *Ann. Botany N.S.* **21**, 53.
11. Fowden, L. (1959) *Ann. Rep. Prog.* **56**, 359.
12. Hatanaka, S. (1962) *Acta Chem. Scand.* **16**, 513.
13. Benoiton, L., Winitz, M., Birnbaum, S. M. and Greenstein, J. P. (1957) *J. Am. Chem. Soc.* **79**, 6192.
14. Fowden, L., Scopes, P. M. and Thomas, R. N. (1971) *J. Chem. Soc. C* 833.
15. Lee, Y. K. and Kaneko, T. (1973) *Bull. Chem. Soc. Jpn* **46**, 3494.
16. Fowden, L. (1964) *Annu. Rev. Biochem.* 173.
17. Gupta, S. C. and Dekker, E. E. (1979) *Arch. Biochem. Biophys.* **192**, 324.
18. Jadot, J., Casimir, J. and Loffet, A. (1967) *Biochim. Biophys. Acta* **136**, 79.
19. Bell, E. A. (1980) in *Encyclopedia of Plant Physiology*, New Series, Vol. 8 (Bell, E. A. and Charlwood, B. V. eds.) p. 403, Springer, Berlin.
20. Nawaz, R. and Sørensen, H. (1977) *Phytochemistry* **16**, 599.
21. Sørensen, H. (1970) *Phytochemistry* **15**, 1527.
22. Hansen, B. A., Lane, R. S. and Dekker, E. E. (1974) *J. Biol. Chem.* **249**, 4891.
23. Kuttan, R., Pattabhiraman, K. S. V. and Radhakrishnan, A. N. (1974) *Phytochemistry* **13**, 453.
24. Kristensen, I., Larsen, P. O. and Sørensen, H. (1974) *Phytochemistry* **13**, 2803.
25. Payes, B. and Laties, G. G. (1963) *Biochem. Biophys. Res. Commun.* **13**, 179.
26. Virtanen, A. I. and Ettala, T. (1957) *Acta Chem. Scand.* **11**, 182.