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DIASTEREOISOMERIC 4-SUBSTITUTED ACIDIC AMINO ACIDS IN FERNS

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Key Word Index—Aspidiaceae; Aspleniaceae; non-protein amino acids; diastereoisomers of acidic amino acids; stereochemistry; chemotaxonomy.

Abstract—Diastereoisomeric 4-substituted acidic amino acids occur in characteristic associations in the green parts of some species of the Filicinae. Subspecies of *Phyllitis scolopendrium* accumulate 2(*S*),4(*R*)-4-methylglutamic acid, 2(*S*)-4-methyleneglutamic acid and the two diastereoisomers of 2(*S*)-4-hydroxy-4-methylglutamic acid, the last two occurring at relative concentrations of 3:1. All *Asplenium* species investigated were distinctive in accumulating 2(*S*),4(*R*)-4-methylglutamic acid, the two diastereoisomers of 2(*S*)-4-hydroxy-4-methylglutamic acid, and the two diastereoisomers of 2(*S*)-4-hydroxy-2-aminopimelic acid in a characteristic concentration ratio. Some *Polystichum* species do not accumulate 4-substituted acidic amino acids whereas others accumulate both diastereoisomers of 2(*S*)-4-hydroxy-4-methylglutamic acid and of 2(*S*)-4-hydroxy-2-aminopimelic acid, and thus resemble *Asplenium* species. The seasonal variation in the concentration of 4-substituted acidic amino acids in the green parts of *Phyllitis*, *Asplenium* and *Polystichum* species has also been determined.

INTRODUCTION

Investigations of free protein amino acids in ferns have previously been reported [1]. Different 4-substituted acidic amino acids are also known to occur in these plants: 4-methylglutamic acid, 4-methyleneglutamic acid, and 4-hydroxy-4-methylglutamic acid have been isolated from *Phyllitis scolopendrium* [2]; 4-hydroxy-4-methylglutamic acid has also been found in *Adiantum pedatum* [3, 4], *Polystichum acrostichoides* (Michaux) Schott [5], and in 9 out of 16 species of Filicinae investigated [6]. 4-Hydroxy-2-aminopimelic acid occurs in *Asplenium septentrionale*, *A. nidus*, *A. trichomanes*, and *A. viviparum* [7]. The configurations of these amino acids were not indicated in the above mentioned reports and no correlation was recognized between the distribution of the amino acids and the taxonomic position of the species [6]. In a few reports the stereochemistry of 4-substituted acidic amino acids in ferns has been considered, thus 2(*S*),4(*R*)-4-methylglutamic acid (**1**), 2(*S*)-4-methyleneglutamic acid (**2**) and 2(*S*),4(*S*)-4-hydroxy-4-methylglutamic acid (**3**) have been isolated from *P. scolopendrium* [8]. Also the presence or absence of **1** and **3** in some species of *Asplenium* and *Athyrium* has been used in a chemotaxonomic study of these plants [9]. However, results presented in a report from this laboratory [10] have brought into question the correctness of the 2(*S*),4(*S*) configuration assigned to **3**.

The present work is a continuation of our previous studies concerning the distribution of 3- and 4-substituted acidic amino acids in plants [11, 12]. Knowledge of the stereochemistry [10] and co-occurrence of these compounds, as well as the co-occurrence of diastereoisomeric forms, are considered to be of great importance in the biosynthetic study now in progress.

RESULTS AND DISCUSSION

Isolation of the amino acids was performed by established methods, including ion-exchange chromatography, preparative PC and preparative high voltage electrophoresis (prep-HVE). The isolated compounds were identified by co-chromatography in solvents 1, 2 and 3, co-electrophoresis at pH 1.9, 3.6 and 6.5, and by ^{1}H NMR. Corresponding spectra were obtained with synthetic material (see Experimental). By HVE in buffer systems 1 and 2, it was possible to separate **1** from the diastereoisomer **1'** (highest pKa_2 value), **3** from the diastereoisomer **3'** (lowest pKa_2 value). This system was also employed to separate the two diastereoisomeric forms of 2(*S*)-4-hydroxy-2-aminopimelic acid in the compound with lowest pKa_2 value (**4**) and the compound with highest pKa_2 value (**4'**).

The compounds **3**, **3'**, **4** and **4'** are easily transformed into lactones and lactams, especially in acidic solution, both **4** and **4'** are easily transformed into two structurally different lactones as described elsewhere [13]. Therefore, the isolation of the acidic amino acids was performed without the use of reflux at any step and with all concentrations performed by lyophilization. This method was used for a relative estimate of concentrations of the acidic amino acids in leaves of some fern species harvested in July. The results obtained are presented in Table 1 and demonstrate that all of the Aspleniaceae investigated contain more than one of the 4-substituted acidic amino acids. By comparison with results obtained with synthetic compounds, the configuration at C-2 in **1**, **2**, **3**, **3'**, **4** and **4'** was established as 2(*S*) by use of L-amino acid oxidase. The absolute configuration of **1** is then 2(*S*),4(*R*)-4-methylglutamic acid [14] since HVE in buffer system 2

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Table 1. Acidic amino acids in leaves of ferns harvested in July*

Plant	Aspartic acid	Glutamic acid	4-Methylglutamic acid 1	1′	4-Methylene-glutamic acid 2	4-Hydroxy-4-methylglutamic acid 3	3′	4-Hydroxy-2-amino-pimelic acid 4	4′	Yellow compound†
Aspleniaceae										
Asplenium nidus L.	+	+	+ +	(—)	(—)	+ +	+	+ + + +	+	(—)
A. septentrionale (L.) Hoffm.	+	+ + +	(—)	(—)	(—)	(—)	+ +	+	(—)	(—)
A. trichomanes L.	+	+ +	+ +	(—)	(—)	+ +	+	+	(—)	(—)
A. bulbiferum Forst.	+	+ +	+ + + +	(—)	(—)	+	+	+	+ + +	(—)
Phyllitis scolopendrium crispa Newm.	+	+ +	+ + + +	(—)	(—)	+	+	(—)	(—)	(—)
P. scolopendrium [*A. scolopendrium*] Newm.	+	+	+ + + +	(—)	+	+ + + +	+ +	(—)	(—)	+ +
P. scolopendrium undulatum Newm.	+	+ +	+ + + +	(—)	+	+ + +	+	(—)	(—)	+ +
P. scolopendrium fissilis Monk-Manii Newm.	+	+	+ + + +	(—)	+ +	+ + + +	+ +	(—)	(—)	(—)
P. scolopendrium vulgare Newm.	+	+ +	+ + + +	(—)	+ +	+ + + +	+ +	(—)	(—)	+ +
Aspidiaceae										
Polystichum setosum grandidens Schott.	+ +	+ + + +	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
P. munitum Pr.	+ +	+ + +	(—)	(—)	(—)	(—)	(—)	(—)	+	(—)
P. acrostichoides Schott.	+ +	+ + +	(—)	(—)	(—)	(—)	+ +	(—)	+	(—)
P. setiferum proliferum R. Br.	+ +	+ + +	(—)	(—)	(—)	(—)	+ +	(—)	+	(—)

* The table shows relative amounts of amino acids as observed from the intensity of the ninhydrin spots after PC and HVE: (—) = not detectable; + = weak; + + = medium; + + + = strong; + + + + = very strong.

† An unidentified acidic amino acid producing a yellow colour with ninhydrin (see Results and Discussion).

reveals that **1** is the diastereoisomer with the lowest pKa_2 value [11]. The absolute configuration assigned to **3** and **3′** is not firmly established [10 and refs. cited therein], and the configuration at C-4 in **4** and **4′** is unknown.

All *Phyllitis* species that were studied contained appreciable amounts of both the diastereoisomers **3** and **3′** in a ratio of *ca* 3 to 1. All of these species contained **1** as the dominating acidic amino acid and no trace of **1′** could be detected by the methods used. Most of the species contained **2** as well as an unknown acidic amino acid which produced a characteristic yellow colour with ninhydrin. This unknown amino acid appeared to be unstable especially in neutral and basic solutions, but a small amount was isolated by prep-HVE in buffer system 1. The compound had R_f values on PC close to but significantly less than those obtained for **2**; HVE mobility in buffer system 1 was 0.6 × Mob_{Glu}, in system 2, 2 × Mob_{Glu} and in system 3, 0.9 × Mob_{Glu}.

Some of the 4-substituted acidic amino acids were also present in substantial amounts in all of the Aspidiaceae investigated except for *Polystichum setosum* but the concentrations were lower than those found for glutamic acid. It is remarkable that **3′** and **4′**, but not the diastereoisomers **3** and **4**, were found to co-occur in the Aspidiaceae studied. In contrast, Aspleniaceae contained **1** and **3** as the major acidic amino acids except for *Asplenium septentrionale* which contained **3′** and **4**, but no trace of **1**, **3** or **4′** could be detected.

Both diastereoisomeric pairs **3** and **3′** and **4** and **4′** do in fact appear to co-occur in nature since the pure isomers were not interconverted under the conditions used for isolation of the compounds.

Ferns in various developmental stages have been analysed. The results presented in Table 2 demonstrate that the concentrations of **3** and **3′** in the leaves vary with developmental stage of the plants. In contrast, there were no marked changes in the concentrations of **1** and **4** + **4′** during the growing cycle of the fern species. The observations concerning the changes in the concentrations of **3** and **3′** are in agreement with previous reports about these amino acids [3, 15], indicating that the biosynthesis of these compounds is closely associated with the parts of the plant which are actively growing such as leaf primordia. Since different fern species accumulate different 4-substituted acidic amino acids and different diastereoisomers, the biosynthetic pathway involved may also show differences in detail between species. Detailed investigations of plants containing these acids, as is the case for some fern species, may ultimately provide a good example of biochemical taxonomy.

Table 2. Seasonal variation of 4-substituted acidic amino acids in leaves of ferns*

	4-Hydroxy-4-methylglutamic acid (3)			(3′)			4-Methylglutamic acid (1)			4-Hydroxy-2-aminopimelic acid (4 + 4′)		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
Plant	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn	Spring	Summer	Autumn
Asplenium nidus L.	+ +	+ +	(—)	+	+	(—)	+ +	+ +	+ +	+ + + +	+ +	+ + + +
P. scolopendrium vulgare Newm.	+ + + +	+ + + +	(—)	+ +	+ +	(—)	+ + + +	+ + + +	+ + + +	(—)	(—)	(—)
P. scolopendrium undulatum Newm.	+ + +	+ + +	(—)	+	+	(—)	+ + + +	+ + + +	+ + + +	(—)	(—)	(—)
P. scolopendrium A. scolopendrium Newm.	+ + + +	+ + + +	(—)	+ +	+ +	(—)	+ + + +	+ + + +	+ + + +	(—)	(—)	(—)
Polystichum acrostichoides Schott.	(—)	(—)	(—)	+ +	+ +	(—)	(—)	(—)	(—)	+	+	+

* Symbols as in Table 1.

(a) Young leaves harvested in April, (b) leaves harvested in July, (c) leaves harvested in October.

EXPERIMENTAL

Plant material. The fern species listed in Tables 1 and 2 were collected in the Botanical Garden of the University of Copenhagen and the Botanical Garden of the Royal Veterinary and Agricultural University, Copenhagen. The plants, grown outdoors, were harvested in April, July and October, 1977 and stored at −20° until extractions were carried out.

General methods and instrumentation. ^{1}H NMR spectra were determined in M DO^-Na^+/D_2O (pH 12) soln at 60 MHz. PC was performed in *n*-BuOH–HOAc–H_2O (12:3:5) (solvent 1), PhOH–H_2O–conc NH_3 (120:30:1) (w/v/v) (solvent 2) and *iso*PrOH–H_2O–conc NH_3 (8:1:1) (solvent 3) by the descending technique on Whatman No. 1 paper. HVE was carried out on Whatman 3 MM paper using a flat-plate unit in the following systems: (1) buffer pH 1.9 (HOAc–HCO_2H–H_2O) (4:1:45), 2 hr at 3.2 kV and 90 mA; (2) buffer pH 3.6 (Py–HOAc–H_2O) (1:10:200), 2 hr at 3 kV and 90 mA; (3) buffer pH 6.5 (Py–HOAc–H_2O) (25:1:500), 50 min at 5 kV and 90 mA.

Isolation of acidic amino acids. Leaves (500 g) were homogenized in H_2O (800 ml) and filtered. The filtrate was taken to dryness by lyophilization and isolation was then performed using ion-exchange chromatography as described previously [16], except that all fractions with the acidic amino acids were taken to dryness by lyophilization. The compounds **1**, **4** and **4′** were isolated from fraction (1.2.) [16]: **4** and **4′** appeared in the fractions immediately before glutamic acid and **1** appeared in the fractions immediately after glutamic acid [11]. Compound **2** was isolated from fractions between **1** and aspartic acid whereas **3** and **3′** appeared in the fractions immediately after aspartic acid. Prep-PC in solvent 1 of the fractions containing **1** and **2**, respectively, followed by chromatography on Dowex 50 w (× 8, 200–400 mesh, H^+, 0.7 × 10 cm) afforded chromatographically pure **1** and **2**. Prep-HVE in buffer system 3 for 1 hr of the fractions containing **4** and **4′**, followed by preparative HVE in buffer system 2 for 4 hr, and chromatography on Dowex 50 w as described for **1** afforded chromatographically pure **4** and **4′**. Prep-HVE in buffer system 2 for 3 hr of the fractions containing **3** and **3′**, followed by chromatography on Dowex 50 w as described for **1**, afforded chromatographically pure **3** and **3′**. The compounds thus isolated were identified by comparison with the corresponding synthetic material (unpublished results and [11]) by co-chromatography using PC in solvents 1, 2 and 3, by HVE in buffer systems 1, 2 and 3, and by ^{1}H NMR [10, 11 and refs. cited therein]. Investigation of seasonal variation of the acidic amino acids in fern leaves (25 g portions) was performed as described previously [16].

L-*Amino acid oxidase* (EC 1.4.3.2) from snake venom (*Crotalus terr. terr.*) was used to establish the configuration at C-2 in the isolated acidic amino acids. The rates of oxidation of the isolated acidic amino acids were equal to or higher than that of the rates found for synthetic reference compounds (racemic amino acids) due to some inhibition of the D-amino acids. These investigations are part of unpublished results.

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REFERENCES

1. Panvisavas, R., Worthen, L. R. and Bohm, B. A. (1968) *Lloydia* **31**, 63.
2. Virtanen, A. I. and Berg, A.-M. (1955) *Acta Chem. Scand.* **9**, 553.
3. Steward, F. C., Wetmore, R. H. and Pollard, J. K. (1955) *Am. J. Botany* **42**, 946.
4. Grobbelaar, N., Pollard, J. K. and Steward, F. C. (1955) *Nature* **175**, 703.
5. Soeder, R. W. (1973) *Phytochemistry* **12**, 2297.
6. Tanaka, M., Nakamura, S., Nisizawa, K. and Miwa, T. (1971) *Bot. Mag. Tokyo* **84**, 41.
7. Virtanen, A. I., Uksila, E. and Matikkala, E. J. (1954) *Acta Chem. Scand.* **8**, 1091.
8. Blake, J. and Fowden, L. (1964) *Biochem. J.* **92**, 136.
9. Peterson, P. J. (1972) *N.Z. J. Botany* **10**, 3.
10. Kaas, K. and Sørensen, H. (1977) *Acta Chem. Scand.* A **31**, 364.
11. Dardenne, G., Casimir, J. and Sørensen, H. (1974) *Phytochemistry* **13**, 2195.
12. Larsen, P. O., Sørensen, H., Cochran, D. W., Hagaman, E. W. and Wenkert, E. (1973) *Phytochemistry* **12**, 1713.
13. Meier, L. K., Olsen, O. and Sørensen, H. (1979) in preparation.
14. Greenstein, J. P. and Winitz, M. (1961) *Chemistry of the Amino Acids*, p. 85. Wiley, New York.
15. Bramesfeld, B. and Virtanen, A. I. (1956) *Acta Chem. Scand.* **10**, 688.
16. Sørensen, H. (1976) *Phytochemistry* **15**, 1527.