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STEREOCHEMISTRY OF 2-AMINOPIMELIC ACID AND RELATED AMINO ACIDS IN THREE SPECIES OF *ASPLENIUM*

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Key Word Index—*Asplenium unilaterale*; *A. prolongatum*; *A. wilfordii*; Aspleniaceae; 2-aminopimelic acid; *trans*-3,4-dehydro-2-aminopimelic acid; 4-hydroxy-2-aminopimelic acid; D-amino acids; non-protein amino acids; stereochemistry.

Abstract—We previously reported two free D-amino acids, D-2-aminopimelic acid (D-APA) and *trans*-3,4-dehydro-D-2-aminopimelic acid (D-Δ-APA), from *Asplenium unilaterale*. In the present work we isolated 4-hydroxy-2-aminopimelic acid (OH-APA) from the same plant and determined it to be the α-L-form. We also investigated the configurations of these amino acids isolated from *A. prolongatum* and *A. wilfordii* which are morphologically distinct from *A. unilaterale*. In *A. prolongatum*, APA was the D- and OH-APA was the L-isomer. In contrast, APA from *A. wilfordii* was partially racemized and the degree of racemization was significantly different in plant material collected in July and November, L:D = 3:2 and 3:7, respectively. In *A. wilfordii* OH-APA was almost pure L- and Δ-APA was mostly the D-isomer.

INTRODUCTION

We previously reported two free D-amino acids, D-2-aminopimelic acid (D-APA) and *trans*-3,4-dehydro-D-2-aminopimelic acid (D-Δ-APA), from *Asplenium unilaterale* [1]. There are only a few reports showing, with certainty, the existence of free D-amino acids in higher plants. The D-amino acids so far reported all occur as components of partially racemized forms. D-(3-Carboxy-4-hydroxyphenyl)-glycine accompanied by its L-form and racemic 3-carboxyphenylglycine were isolated and characterized from *Reseda luteola* seeds by Kjaer and Larsen [2]. According to Gray and Fowden [3, 4], the seeds of *Eriobotrya japonica* contain 4-methyleneproline and *trans*-4-hydroxymethylproline. Although the former was a racemate, the latter was composed predominantly of the D-isomer. Furthermore, Kristensen *et al.* [5] reported that pipecolic acid in the seeds of *Fagus silvatica* was a partially racemized D-form based on its CD curve. It is worth noting, therefore, that the APA in *A. unilaterale* was nearly pure D-amino acid. As we reported before, 4-hydroxy-2-aminopimelic acid (OH-APA) was also detected in *A. unilaterale* by 2D-PC [1]. Earlier, Meier and Sørensen [6] reported the occurrence of OH-APA in *A. nidus*, *A. septentrionale*, *A. trichomanes* and *A. bulbiferum* and they determined its configuration at the α-carbons to be the L-form by using L-amino acid oxidase.

In the present work we isolated OH-APA first from *A. unilaterale* and studied its configuration. In contrast with D-APA and D-Δ-APA, OH-APA in the same fern proved to be the L-form. In order to examine if such a relationship also exists in other species which contain these amino acids, we isolated the above amino acids from a further two species, *A. prolongatum* and *A. wilfordii*, and examined their configurations.

RESULTS AND DISCUSSION

OH-APA was isolated as its 7,4-lactone from the amino acid fraction of *A. unilaterale*, from which earlier we had isolated D-APA and D-Δ-APA, and was identified by 400 MHz ^{1}H NMR. The configuration was examined using snake venom L-amino acid oxidase (see Experimental). Prior to this experiment, the enzyme preparation was tested to confirm that it completely and selectively oxidized the L-isomers of some amino acids. As much as 99.3% of OH-APA-7,4-lactone from *A. unilaterale* was decomposed by the enzyme. Partial reduction of OH-APA-7,4-lactone was then effected by catalytic hydrogenation at *ca* 70° to yield a small amount of APA. The ORD value of the obtained APA, $[\alpha]_D$ +18.9° (5 M HCl) was similar to that of the L-isomer, $[\alpha]_D$ +21.5° (5 M HCl) reported by Wade *et al.* [7]. We concluded, therefore, that OH-APA in *A. unilaterale* is the L-isomer with respect to its α-carbon.

As stated above, APA and Δ-APA in *A. unilaterale* are D-amino acids, but OH-APA in the same fern is the L-amino acid. It seemed interesting to us to examine whether such a relationship also exists in other species which contain APA and its related amino acids. *Asplenium prolongatum* and *A. wilfordii* are both relatively distinct in morphology from *A. unilaterale*.

APA and OH-APA-7,4-lactone were isolated in the same way as from *A. unilaterale*. They were identified by TLC, automated amino acid analysis (AA) and 400 MHz ^{1}H NMR. Δ-APA was not detected in *A. prolongatum* even by AA. Their configurations were examined by the snake venom L-amino acid oxidase. Synthetic DL-APA was decomposed to nearly 50% and only a small portion of APA from *A. prolongatum* was decomposed by the enzyme. APA from *A. unilaterale*, which had been con-

sidered to be the D-isomer from its $[\alpha]_D$ value, was not significantly oxidized. On the other hand, OH-APA-7,4-lactone from *A. prolongatum* was decomposed almost completely like OH-APA-7,4-lactone from *A. unilaterale* (Table 1). From these results it could be concluded that, in *A. prolongatum*, APA occurs as the D- and OH-APA as the L-amino acid.

APA, Δ-APA and OH-APA-7,4-lactone were isolated from *A. wilfordii* collected in July in Yaku Island and identified unequivocally by TLC, AA and ^{1}H NMR and their configurations investigated. Since L-Δ-APA itself was hardly attacked by the enzyme, Δ-APA was reduced to APA by catalytic hydrogenation prior to the enzyme experiment. *Ca* 60% of APA from *A. wilfordii* was decomposed and the same result was obtained in repeat experiments. Therefore, APA from *A. wilfordii* proved to be a partially racemized form containing *ca* 60% of the L-isomer, although APA from *A. unilaterale* and *A. prolongatum* were the relatively pure D-form. The OH-APA-7,4-lactone from *A. wilfodii* was decomposed almost completely and *ca* 23% of the hydrogenation product of Δ-APA was decomposed. Therefore, OH-APA of *A. wilfordii* was the L-amino acid and the majority of Δ-APA from the fern was the D-amino acid (Table 2). The results for OH-APA and Δ-APA in *A. wilfordii* are, thus, similar to those from *A. unilaterale*. The same amino acids were isolated from *A. wilfordii* collected in November in Yaku Island and their configurations were examined. Again, OH-APA was almost pure L- and Δ-APA was mostly the D-amino acid. However, *ca* 30% of APA was decomposed by the enzyme, indicating that APA from *A. wilfordii* in November was partially racemized D-APA containing *ca* 30% of the L-isomer. Although it is difficult to conclude at present that the above difference is attributable to seasonal variation, it is worth noting that the ratios of the two optical isomers of APA of *A. wilfordii* are significantly different in July (L:D = 3:2) and in November (L:D = 3:7). Even though racemization might take place, the ratio of the L- and D-forms of APA could not be reversed. These results strongly suggest that D- and L-APA are biosynthesized separately and/or metabolized in other pathways in the fern. It is necessary to investigate the biochemical relationships between D- and L-APA and other related amino acids coexisting in these species. Δ-APA was the D-isomer for the most part (75–85%), but the origin of the small L-component is unknown. It may have been produced during isolation and/or during catalytic hydrogenation. It is also possible that Δ-APA in these ferns may occur originally as a partially racemized compound like APA in *A. wilfordii*. Further studies are needed to clarify these problems.

EXPERIMENTAL

General. Chromatographic solvents were *n*-BuOH–HOAc–H_2O (63:10:27) (solvent A), PhOH–H_2O (25:8) in the presence of NH_3 (solvent B) and MeOH–pyridine–H_2O (5:5:1) (solvent C). Cellulose powder for CC and TLC was 'Avicel' (Funakoshi Pharmaceutical Co.). AA was performed using MCI 835-PF-KIT buffer. The 400 MHz ^{1}H NMR spectra were measured in D_2O and chemical shifts are reported in δ units relative to DSS ($\delta = 0$).

Plant material. Fronds and rhizomes of *A. prolongatum* Hook. (2.5 kg) were collected in August 1982 in Koza, Wakayama Pref. and those of *A. wilfordii* Mett. ex Kuhn were collected in July

Table 1. Oxidation of APA, Δ-APA and OH-APA from *A. unilaterale* and *A. prolongatum* by L-amino acid oxidase

Sample	Amount of amino acid*		Amount of decomposed amino acid (L-isomer) (%)
	Immediately after addition of enzyme	After incubation for 48 hr	
L-Valine	1278	0	100.0
D-Valine	1239	1238	0.1
L-Leucine	1369	0	100.0
D-Leucine	1297	1236	4.7
L-Norleucine	1248	0	100.0
D-Norleucine	1516	1475	2.7
Synthetic			
DL-APA	1072	563	47.5
	1112	569	48.9
A. unilaterale			
APA	903	852	5.6
	933	877	6.0
Δ-APA	1092	822	24.7
OH-APA	899	6	99.3
A. prolongatum			
APA	893	805	9.8
	895	831	7.2
OH-APA	945	13	98.7

*Amounts are expressed as ratios of the peak area in an automatic amino acid analyser. For details, see text and Experimental.

Table 2. Oxidation of APA, Δ-APA and OH-APA from *A. wilfordii* by L-amino acid oxidase

Sample	Amount of amino acid*		Amount of decomposed amino acid (L-isomer) (%)
	Immediately after addition of enzyme	After incubation for 48 hr	
A. wilfordii (July)			
APA	1033	427	58.6
	2054	874	57.4
Δ-APA	1311	1008	23.1
OH-APA	481	23	95.2
Synthetic DL-APA (control)	1161	581	50.0
A. wilfordii (November)			
APA	380	258	31.9
Δ-APA	468	401	14.2
OH-APA	696	10	98.5
Synthetic DL-APA (control)	1040	512	50.7

*Amounts are expressed as ratios of the peak area in an automatic amino acid analyzer. For details, see text and Experimental.

(800 g) and November (780 g) 1983, respectively, from different populations in Yaku Island, Kagoshima Pref. Voucher specimens are deposited in the Herbarium of the Faculty of Science, University of Tokyo (TI).

Isolation. (a) OH-*APA-7,4-lactone from* A. unilaterale. In our previous work [1], we obtained fractions 64–88 in which only APA was detected by TLC; fractions 89–99, APA and OH-APA; and fractions 100–140, OH-APA, from a cellulose column. Fractions 100–140 were combined. On conc below 40° the majority of OH-APA was changed to its 7,4-lactone. The syrup obtained was applied to a Dowex 1 × 4 column (200–400 mesh, OAc^- form, 135 × 2.2 cm) and fractionation was carried out with 0.1 M HOAc (9 ml fractions). Fractions 24–25 contained a very unstable ninhydrin-positive compound which was presumably the OH-APA-1,4-lactone reported in ref. [8]; fractions 27–35, OH-APA-7,4-lactone; and fractions 190–230, OH-APA. Fractions 27–35 were combined and evaporated, resulting in crystalline OH-APA-7,4-lactone (490 mg from 4 kg plants) which gave a single spot and peak on TLC and AA, respectively. It was recrystallized twice from EtOH–H_2O, mp 209–211° (decomp.). $[\alpha]_D$ + 57.7° (5 M HCl; *c* 0.51). (Found: C, 48.26; H, 6.51; N, 8.07. Calc. for $C_7H_{11}NO_4$: C, 48.55; H, 6.40; N, 8.09%.) ^{1}H NMR (D_2O): δ1.99 (1H, *dddd*, *J* = 8.5, 9.0, 9.5, 12.5 Hz, H-5a), 2.12 (1H, *ddd*, *J* = 8.5, 10.5, 15.5 Hz, H-3a), 2.37 (1H, *ddd*, *J* = 3.0, 5.5, 15.5 Hz, H-3b), 2.49 (1H, *dddd*, *J* = 4.5, 7.0, 9.5, 12.5 Hz, H-5b), 2.62 (1H, *ddd*, *J* = 4.5, 9.0, 18.0 Hz, H-6a), 2.69 (1H, *ddd*, *J* = 9.5, 9.5, 18.0 Hz, H-6b), 3.88 (1H, *dd*, *J* = 5.5, 8.5 Hz, H-2), 4.88 (1H, *dddd*, *J* = 3.0, 8.5, 10.5, 12.5 Hz, H-4). IR ν_{max}^{KBr} cm^{-1}: 3425, 3125, 3000, 2950, 2700, 2625, 2575, 2100, 1765 (lactone), 1620, 1590, 1565, 1495, 1450, 1425, 1400, 1390, 1365, 1350, 1320, 1295, 1280, 1240, 1200, 1160, 1140, 1110, 1065, 1040, 1025, 1000, 990, 970, 940, 920, 880, 840, 810, 770, 650. (b) *APA and* OH-*APA-7,4-lactone from* A. prolongatum. Fronds and rhizomes were extracted (× 5) with 80% EtOH. The combined extract (50 l.) was passed through a column of Amberlite IR-120B (H^+ form, 600 ml) and the amino acids eluted with 2 M NH_4OH (6 l.). The eluate was concd and applied to a column of Dowex 1 × 4 (200–400 mesh, OAc^- form, 130 × 2.2 cm). Fractionation was carried out with 0.5 M HOAc (fractions 1–102, each 6 ml; fractions 103–125, each 10 ml). Fractions 85–125 which contained APA and OH-APA were combined and evaporated. The concentrate was applied to a cellulose column (125 × 2.4 cm) and fractionated with solvent A (12 ml/fraction). Fractions 71–77, APA; and fractions 86–120, OH-APA. Fractions 71–77 were applied again to a cellulose column (133 × 2.4 cm) and fractionated with solvent C, giving a pure sample of APA (10 mg). ^{1}H NMR (D_2O): δ1.41 (2H, *m*, H-4), 1.64 (2H, *tt*, *J* = 7.2, 7.2 Hz, H-5), 1.87 (2H, *m*, H-3), 2.40 (2H, *t*, *J* = 7.2 Hz, H-6), 3.74 (1H, *t*, *J* = 6.2 Hz, H-2). Fractions 86–120 were applied to a Dowex 1 × 4 column (200–400 mesh, OAc^- form, 137 × 2.4 cm) and fractionated with 0.1 M HOAc (10 ml/fraction). Fractions 27–29, which contained only OH-APA-7,4-lactone, were combined and lyophilized yielding crystals of OH-APA-7,4-lactone (92 mg). (c) *APA, Δ-APA and* OH-*APA-7,4-lactone from* A. wilfordii. Isolation was carried out using the same methods as those described for *A. unilaterale* and *A. prolongatum*. Yield: APA, 17.8 mg; Δ-APA, 113 mg; and OH-APA-7,4-lactone, 29 mg from 800 g June material. Yield: APA, 10 mg; Δ-APA, 122 mg; and OH-APA-7,4-lactone, 16.3 mg from 780 g of November material. ^{1}H NMR of Δ-APA (D_2O): δ2.42 (2H, *dt*, *J* = 6.7, 6.7 Hz, H-5), 2.53 (2H, *t*, *J* = 6.7 Hz, H-6), 4.25 (1H, *d*, *J* = 8.3 Hz, H-2), 5.65 (1H, *dd*, *J* = 8.3, 15.5 Hz, H-3), 6.01 (1H, *dt*, *J* = 6.7, 15.5 Hz, H-4).

Hydrogenation. Hydrogenation of Δ-APA was carried out over Adams' Pt catalyst at room temp. and atm pres. for 2 hr (yield: *ca* 100%). OH-APA-7,4-lactone was reduced in the same way as for 4-hydroxy-4-methylglutamic acid according to ref. [9]. H_2 was bubbled for 6 hr through a suspension of OH-APA-7,4-lactone (40 mg) and Adams' Pt catalyst (200 mg in reduced form) in H_2O (3 ml) at 70–80°, producing APA (10% yield). The APA was isolated (3.6 mg) from the mixture using a cellulose column (135 × 1.2 cm) with solvent A and identified by TLC, AA and ^{1}H NMR.

Oxidation with L-amino acid oxidase. Crude L-amino acid oxidase prepared from Habu-snake (*Trimeresurus flavoviridis*) venom [10] in 0.02 M $MeCOONH_4$ buffer, pH 7.2 (150 μl) was added to an amino acid soln (*ca* 100 μg in 50 μl H_2O) at 0°. Except for the amino acids from *A. unilaterale*, the concd pure or lyophylized samples were used without recrystallization. Immediately, 80 μl of the mixture was removed and added to

100 μl 1 M HCl. After the remaining mixture had been incubated at 37° for 48 hr with vigorous stirring, 80 μl of the mixture was added to 100 μl of 1 M HCl as above. Both samples were diluted with H_2O (820 μl) and analysed quantitatively by AA.

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