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4-CARBOXY-4-HYDROXY-2-AMINOADIPIC ACID AND OTHER ACIDIC AMINO ACIDS IN *CAYLUSEA ABYSSINICA*

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Key Word Index—*Caylusea abyssinica*; Resedaceae; non-protein amino acids; 3-(3-carboxyphenyl)alanine; (3-carboxyphenyl)glycine; 3-(3-carboxy-4-hydroxyphenyl)alanine; (3-carboxy-4-hydroxyphenyl)glycine; 4-hydroxy-4-methylglutamic acid; 4-carboxy-4-hydroxy-2-aminoadipic acid; 2-aminoadipic acid; saccharopine; γ-glutamyl peptides.

Abstract—Two diastereoisomers of 4-carboxy-4-hydroxy-2-aminoadipic acid have been isolated from leaves and inflorescences of *Caylusea abyssinica*. Green parts of the plant also contain appreciable amounts of the two diastereoisomers of 4-hydroxy-4-methylglutamic acid, 3-(3-carboxyphenyl)alanine, (3-carboxyphenyl)glycine, 3-(3-carboxy-4-hydroxyphenyl)alanine, (3-carboxy-4-hydroxyphenyl)glycine and in low concentration 2-aminoadipic acid, saccharopine [(2*S*, 2′*S*)-N^6-(2-glutaryl)lysine] and some γ-glutamyl peptides. The acidic amino acids were separated from other amino acids on an Ecteola ion exchange column with M pyridine as eluant.

INTRODUCTION

The present work is a continuation of previous studies on 4-substituted acidic amino acids in ferns [1] and acidic amino acids in Resedaceae [2, 3]. The work is also related to previous studies of 3- and/or 4-substituted glutamic acid derivatives in Leguminosae [4].

Investigations of leaves and inflorescences of *Caylusea abyssinica* (Fresen) Fisch. et Mey (Resedaceae) revealed the presence of appreciable amounts of 3-(3-carboxyphenyl)alanine (**1**), (3-carboxyphenyl)glycine (**2**), 3-(3-carboxy-4-hydroxyphenyl)alanine (**3**), and (3-carboxy-4-hydroxyphenyl)-glycine (**4**), compounds found in other Resedaceae [2]. Furthermore, the plant contained substantial amounts of other acidic amino acids among which were two hitherto unknown acidic amino acids and the two diastereoisomeric forms of 4-hydroxy-4-methylglutamic acid recently isolated from *Reseda luteola* L. These two diastereoisomers were separated into one with a high pK_{a_2} value (**5**) and another with a low pK_{a_2} value (**6**).

The two new acidic amino acids have been identified as the diastereoisomeric forms of 4-carboxy-4-hydroxy-2-aminoadipic acid. They were separated by high voltage electrophoresis (HVE) at pH 3.6 as **7** and **8**, the first examples of tricarboxylic amino acids in higher plants apart from peptides and the amino acid saccharopine [5, 6].

RESULTS AND DISCUSSION

The methods used for isolation and identification of the acidic amino acids in *C. abyssinica* were the same as those used previously [1, 2], except for the preceding separation of the pool of acidic amino acids from other amino acids. This separation is performed on an Ecteola ion-exchange column, the charge of the resin being removed by using M pyridine as eluant [7]. Thereby the strongly acidic compounds are easily isolated; even compounds which adsorb strongly to the ion-exchangers traditionally used are easily eluted [2]. Furthermore, strongly acidic and alkaline conditions which cause degradation and/or cyclization of some 4-substituted acidic amino acids [2] are avoided.

R_f values from PC, ionic mobilities by HVE and some spectroscopic properties have been previously reported for compounds **1**–**6**, saccharopine, and 2-aminoadipic acid [2, 5]. However, **5**, **6**, reduced glutathione (GSH, **9**), oxidized glutathione (GSSG, **10**), γ-glutamylglutamic acid (**11**), and α-glutamylglutamic acid (**12**) have PC and HVE properties close to those found for **7** and **8**, and these compounds are therefore included in Table 1 for comparison. The HVE mobilities at pH 6.5 for **7** and **8** show that they behave as compounds with net charges and molecular sizes corresponding to **11** and **12**.

Pretreatment of the paper chromatograms with cupric ions resulted in a masked ninhydrin reaction of **7** and **8** indicating that the amino group is at C-2 [8]. Confirmation of the α-amino acid structures was obtained by the HVE mobilities at pH 1.9 which reflect the pK_{a_1} values. From the HVE mobilities at pH 3.6 it is concluded that the pK_{a_2} values for **7** and **8** are close to the pK_{a_2} values for **5** and **6**; this corresponds to α-hydroxy substituted carboxylic acids [1, 2, 4]. The HVE mobilities for **7** and **8**, the elution from the

Table 1. R_f values and ionic mobilities of acidic amino acids from *C. abyssinica*

Amino acids	R_f values in solvent*			Distance in cm obtained by HVE*		
	1	2	3	pH 1.9	pH 3.6	pH 6.5
Aspartic acid	0.22	0.16	0.12	22.6	9.6	22.5
Glutamic acid	0.28	0.26	0.15	25.0	2.6	20.0
2-Aminoadipic acid	0.31	0.30	0.17	28.8	0.8	17.0
Saccharopine	0.14	0.60	0.08	27.3	2.3	12.0
5 (2*S*,4*S*)-4-Hydroxy-4-methylglutamic acid†	0.23	0.22	0.18	19.8	15.4	18.2
6 (2*S*,4*R*)-4-Hydroxy-4-methylglutamic acid†	0.24	0.22	0.19	19.3	16.8	19.5
7 4-Carboxy-4-hydroxy-2-aminoadipic acid†	0.24	0.09	0.05	12.8	16.9	28.0
8 4-Carboxy-4-hydroxy-2-aminoadipic acid†	0.24	0.09	0.05	12.8	19.4	28.6
9 Reduced glutathione (GSH)	0.28	0.036	0.06	19.1	9.1	12.3
10 Oxidized glutathione (GSSG)	0.09	0.10	0.06	21.2	9.6	16.2
11 γ-Glutamylglutamic acid	0.29	0.11	0.10	19.3	10.4	26.0
12 α-Glutamylglutamic acid	0.35	0.15	0.11	27.1	0.1	26.0

* For solvent and buffer systems, see Experimental.

† For discussion on the stereochemistry, see text.

ion-exchange resins, and the R_f values in solvents 1–3 are in agreement with the structures proposed for these compounds (Table 1).

It is assumed that the configuration is *S* at C-2 for both **7** and **8**, as is known to be the case for other 4-substituted acidic amino acids isolated from plants [1, 2]. The configuration at C-4 for **7** and **8** has not yet been assigned. Recent work has raised doubt concerning assignment of absolute configuration to this type of amino acid on the basis of ^{1}H NMR data [9]. The configurations (2*S*, 4*S*) and (2*S*, 4*R*) assigned to **5** and. **6**, respectively, have also been severely questioned [2].

Figure 1 shows the ^{1}H and ^{13}C NMR spectra of the new amino acids **7** and **8** together with the formulae and an interpretation of the spectra. Table 2 presents the ^{13}C NMR results obtained for **5–8** and some reference compounds. The chemical shifts are in full agreement with the proposed structures. The off-resonance decoupled spectra of **7** and **8** showed triplets for the two methylene carbon atoms, a doublet for the C-2 carbon, and singlets for the four other carbon atoms. Assignment of shift values to the different carbon atoms are based on observations of the known pH effects on chemical shifts [2, 3, 10, 11]. However, the shift values assigned to C-1, C-6, and C-1′ in **7** and **8** are perhaps interchanged but show that the compounds contain three carboxylate carbon atoms. These facts, combined with the above-mentioned results and the evidence from the ^{1}H NMR spectra (see Fig. 1) confirm that **7** and **8** are the diastereoisomers of 4-carboxy-4-hydroxy-2-aminoadipic acid.

Table 3 shows the content of the acidic amino acids in leaves and inflorescences of *C. abyssinica*. The acidic amino acids **1–6** have previously been isolated from other Resedaceae [2] and are included for comparison. The hitherto unknown amino acids **7** and **8** are among the major acidic amino acids in this plant. 2-Aminoadipic acid and saccharopine are only present in low concentration. These two amino acids are considered to be catabolic products of lysine and therefore widely distributed in plants [5, 6]. More remarkable, however, is the occurrence of **7** and **8** in *C. abyssinica* and the co-occurrence of these amino acids with **5** and **6**. This co-occurrence indicates that they are biosynthetically related to the group of 4-substituted acidic amino acids previously described as constituents of other plants [1–4].

It is tempting to assume that a 3-carbon unit and 2-ketosuccinate (oxaloacetate) in an aldolase-type reaction may yield the 2-keto acids corresponding to **7** and **8**, which then accept amino groups by transamination to give **7** and **8**. As mentioned above, it is not suggested that 2-aminoadipic acid is biogenetically related to this group of amino acids, although this is proposed in the case of the hydroxylated higher homologues, 4-hydroxy-2-aminopimelic acids (**13** and **14**) [2]. Interestingly, 4-carboxy-4-hydroxy-2-aminopimelic acid and the 4,7-lactone thereof (**15**) have recently been isolated from a basidiomycete [11]. The 2-keto acid corresponding to these compounds may, by analogy with the above-mentioned proposals, be biosynthetically derived from a 3-carbon unit and 2-ketoglutarate.

EXPERIMENTAL

Plant material. Leaves and inflorescences of *C. abyssinica* were collected in the Botanical Garden of the University of Copenhagen and in a field plot at the Department of Plant Culture, Royal Veterinary and Agricultural University, Copenhagen. The plant material was freeze-dried and stored at −20° until extractions were carried out.

General methods. The ^{13}C NMR and ^{1}H NMR spectra were recorded as previously described [2]. PC was performed in *n*-BuOH–HOAc–H_2O (12:3:5) (solvent 1), PhOH–H_2O–12 M NH_3 (120:30:1) (w/v/v) (solvent 2) and *i*-PrOH–H_2O–12 M NH_3 (8:1:1) (solvent 3) by descent on Whatman No. 1 paper. HVE was carried out on Whatman 3 MM paper using

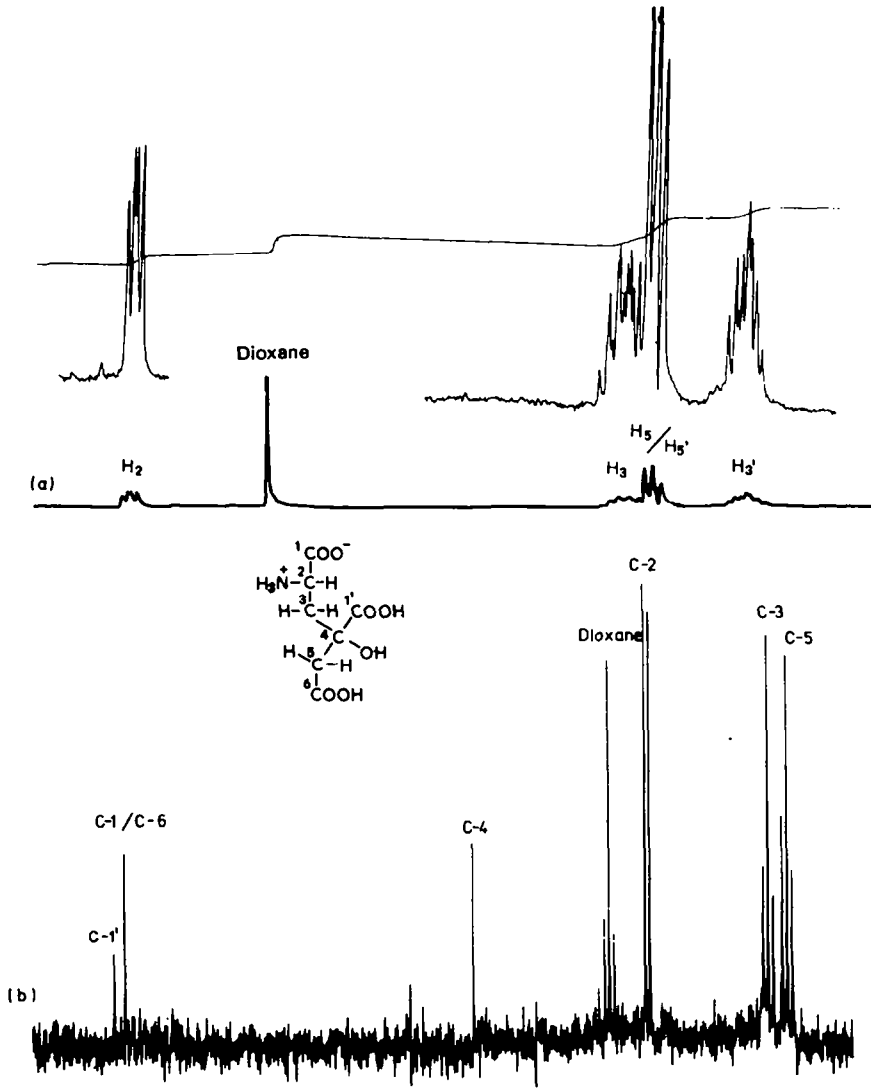

Fig. 1. ^{1}H NMR spectrum at 270 MHz (a) and the off-resonance decoupled ^{13}C NMR spectrum at 67.889 MHz (b) of the two diastereoisomers of 4-carboxy-4-hydroxy-2-aminoadipic acid isolated from *C. abyssinica* with dioxane as internal reference.

Table 2. ^{13}C NMR chemical shifts* of 4-substituted acidic amino acids

Carbon atom No.	Compound 5	6	7	8	13†	14†	15‡
C-1	183.8	182.5	180.1 (*s*)	180.1 (*s*)	183.0	183.0	176.3
C-2	53.9	55.3	58.5 (*d*)	58.5 (*d*)	54.2	55.3	51.4
C-3	45.8	43.8	30.4 (*t*)	30.3 (*t*)	41.9	41.9	35.4
C-4	75.9	77.9	99.2 (*s*)	99.2 (*s*)	69.6	70.8	87.0
C-5	26.8	27.4	25.9 (*t*)	25.8 (*t*)	34.5	34.5	27.9
C-6			180.1 (*s*)	180.1 (*s*)	34.1	34.1	38.1
C-7					183.9	183.9	171.0
C-1′	184.1	183.7	182.6 (*s*)	182.6 (*s*)			178.9

* Spectra of **5**, **6**, **13** and **14** in 1M DO^-Na^+, D_2O soln and of **7** and **8** in an ammoniacal D_2O solution at 67.889 MHz on a Bruker HX 270 instrument using pulse technique with Fourier transformation [2]. Chemical shifts are in ppm downfield from TMS [δ (TMS) = δ (dioxane) + 67.4 ppm] with dioxane as internal standard; the symbols *s*, *d* and *t* represent singlet, doublet and triplet. C-1′ is the carboxyl carbon at C-4.

† Values of the two diastereoisomers of 4-hydroxy-2-aminopimelic acid **13** and **14** from ref. [2].

‡ Values of 4-carboxy-4-hydroxy-2-aminopimelic acid 4,7-lactone (**15**) in acidic aqueous solution from ref. [10].

Table 3. Acidic amino acids in leaves and inflorescences of *C. abyssinica**

Amino acids	Plants harvested in August	October
Aspartic acid	+ +	+ + +
Glutamic acid	+ + + +	+ + + +
2-Aminoadipic acid	(+)	(+)
Saccharopine	(+)	(+)
3-(3-Carboxyphenyl)alanine (**1**)	+	+ +
(3-Carboxyphenyl)glycine (**2**)	(+)	(+)
3-(3-Carboxy-4-hydroxyphenyl)alanine (**3**)	+ + +	+ + +
(3-Carboxy-4-hydroxyphenyl)glycine (**4**)	+ + +	+ + +
(2*S*,4*S*)-4-Hydroxy-4-methylglutamic acid (**5**)†	+	(+)
(2*S*,4*R*)-4-Hydroxy-4-methylglutamic acid (**6**)†	(+)	(+)
4-Carboxy-4-hydroxy-2-aminoadipic acid (**7**)†	+ +	+
4-Carboxy-4-hydroxy-2-aminoadipic acid (**8**)†	+	+
Glutathione (**9**)	+	+ +
γ-Glutamylglutamic acid (**11**)	+	+

* Relative amounts of the amino acids are observed from the intensity of the ninhydrin spots after PC and HVE or from the peaks by use of the amino acid analyser. Regarding the amount isolated from plants harvested in October, see Experimental. (+) = not detectable before concentration by ion-exchange chromatography; + = weak; + + = medium; + + + = strong; + + + + = very strong.

† For discussion on the stereochemistry at C-2 and C-4, see text.

a flat plate unit at 4° in (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. Prep. PC, prep. HVE, and amino acid analysis, were carried out as previously described [2]. The various fractions were subjected to 2D–PC and HVE in buffer systems 1, 2, and 3.

Isolation of acidic amino acids. Freeze-dried leaves and inflorescences of *C. abyssinica* (330 g harvested in October) were homogenized in boiling MeOH and neutral and acidic amino acids were isolated by ion-exchange chromatography using an Amberlite IR 120 (H^+, 10×70 cm) column as previously described [2]. The amino acid-containing fractions were pooled and concd to a semisolid residue (5.83 g) which was dissolved in H_2O (150 ml) and transferred to an Ecteola–Cellulose (AcO^-, 2.5×70 cm) column. Fractions (21 ml) were collected at 75 ml/hr. After flushing with H_2O (fractions 1–30), the column was eluted with M Py. Fractions 35–60 (1.8 g) contained acidic amino acids which were investigated by 2D–PC, HVE, and amino acid analysis.

Further separation was performed on a column of Dowex 1 (×8, 200–400 mesh, AcO^-, 2.5×90 cm). Fractions (21 ml) were collected at 150 ml/hr. After flushing with H_2O (fractions 1–30), the column was eluted with 0.5 M HOAc (fractions 31–100), 2 M HOAc (fractions 101–200) and M HCO_2H (fractions 200–500). Fractions 61–70 (250 mg) contained 2-aminoadipic acid, saccharopine, glutamic acid, and some unidentified compounds; fractions 71–80 (220 mg) contained glutamic acid; fractions 81–94 (80 mg) contained γ-glutamyl peptides of neutral amino acids; fractions 118–130 (154 mg) contained aspartic acid; fractions 131–147 (100 mg) contained **1**, **5**, and **6**; fractions 148–180 (140 mg) contained **1**, **2**, and **9**; fractions 181–190 (50 mg) contained **9** and **11**; fractions 281–285 (80 mg) contained **7** and **8**, fractions 360–500 (300 mg) contained **3** and **4**. Further purification and identification of 2-aminoadipic acid, saccharopine, **1**–**6**, and **11** were performed by methods described elsewhere [2].

The amino acids **7** and **8** were obtained as a semicrystalline evapn residue from fractions 281–285 (80 mg) in a concn ratio of 3:1 and contaminated with <5% of other acidic amino acids, as revealed from PC, HVE, and NMR spectra. PC and HVE properties of **7** and **8** are shown in Table 1, the ^{13}C NMR data are presented in Table 2. The ^1H NMR spectrum of **7** in M $NaOD/D_2O$ exhibited signals from the C-2 proton at 4.2 ppm (1H, *dd*), from the C-3 protons at 1.9–2.5 (2H, *m*) and from the C-5 protons at 2.30 (2H, *dd*). In the corresponding spectrum of **8**, the signals from the C-2 proton appeared at 4.2 ppm (1H, *dd*), from the C-3 protons at 1.9–2.5 (2H, *m*), and from the C-5 protons at 2.37 (2H, *dd*).

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