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2(*S*),3(*S*)-3-HYDROXY-4-METHYLENEGLUTAMIC ACID FROM *GLEDITSIA CASPICA*

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Key Word Index—*Gleditsia caspica*; Leguminosae; non-protein amino acids; 2(*S*),3(*S*)-3-hydroxy-4-methyleneglutamic acid; 2(*S*),3(*S*),4(*S*)-3-hydroxy-4-methylglutamic acid; 2(*S*),3(*S*),4(*R*)-3-hydroxy-4-methylglutamic acid; 2(*S*),4(*R*),-4-methylglutamic acid; 2(*S*),4(*S*)-4-methylglutamic acid; 2(*S*),3(*R*)-3-hydroxyaspartic acid.

Abstract—A new natural product, 2(*S*),3(*S*)-3-hydroxy-4-methyleneglutamic acid (G3) has been isolated from seeds of *Gleditsia caspica*. The structure has been established by chemical and spectroscopic methods. Catalytic reduction of G3 yields 2(*S*),4(*S*)-4-methylglutamic acid and a new amino acid, 2(*S*),3(*S*),4(*S*)-3-hydroxy-4-methylglutamic acid. Ozonolysis of G3 followed by oxidation gives 2(*S*),3(*R*)-3-hydroxyaspartic acid. The *S*- (or L-) configurations at C_2 in G3 and in 2(*S*),3(*S*),4(*S*)-3-hydroxy-4-methyglutamic acid and the *S*-configurations at C_3 for G3 and 2(*S*),3(*S*),4(*S*)-3-hydroxy-4-methylglutamic acid and at C_4 for 2(*S*),3(*S*),4(*S*)-3-hydroxy-4-methylglutamic acid are inferred from the configurations at C_2 in 2(*S*),4(*S*)-4-methylglutamic acid and at C_2 and C_3 in 2(*S*),3(*R*)-3-hydroxyaspartic acid. The seeds also contain appreciable quantities of 2(*S*),3(*S*),4(*R*)-3-hydroxy-4-methylglutamic acid (G1) and 2(*S*),4(*R*)-4-methylglutamic acid.

INTRODUCTION

THE PRESENT work is a continuation of previous studies of the distribution of free amino acids in *Gymnocladus*[1–4] and in the closely related genus *Gleditsia*.[5,6] The work is also related to studies of four-substituted glutamic acid derivatives in the Resedaceae.[7] *Gleditsia caspica* (Caesalpinaceae) is a leguminous tree, which is common in the regions south and west to the Caspian Sea in Iran and Russia. Investigations on seeds of this plant have revealed the presence of 2(*S*),4(*R*)-4-methylglutamic acid (**3**), of 2(*S*),3(*S*),4(*R*)-3-hydroxy-4-methylglutamic acid (G1) and of a new acidic amino acid. The new amino acid has been identified as 2(*S*),3(*S*)-3-hydroxy-4-methyleneglutamic acid (G3) on the basis of chemical and spectroscopic studies of the amino acid and its degradation products.

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RESULTS AND DISCUSSION

Paper chromatographic (Table 1) and electrophoretic (Table 2) studies of a crude extract of the seeds of *Gleditsia caspica* revealed the presence of three non-protein acidic amino acids. Two of these could be identified as 2(*S*),4(*R*)-4-methylglutamic acid (**3**) and 2(*S*),3(*S*),4(*R*)-3-hydroxy-4-methylglutamic acid (G1) whereas the third was different from a number of naturally occurring glutamic acid derivatives. The amino acids were isolated by established methods, including ion-exchange chromatography. **3** and G1 were finally identified by comparison of IR- and PMR-spectra (see Table 3) with those of authentic samples and by measurement of optical rotation (see Table 4 and Experimental).

TABLE 1. $R_{Glutamic\ acid}$ VALUES OF ACIDIC AMINO ACIDS

Compounds	$R_{Glutamic\ acid}$ in solvents* 1	2
Aspartic acid	0·77	0·55
Glutamic acid	1·00	1·00
2(*S*),4(*S*)-4-methylglutamic acid (**1**)	1·36	1·40
2(*S*),4(*R*)-4-methylglutamic acid (**3**)	1·36	1·40
2(*S*),3(*S*),4(*R*)-3-hydroxy-4-methylglutamic acid (G1)	0·96	0·68
2(*S*),3(*R*),4(*S*)-3-hydroxy-4-methylglutamic acid (G2)	1·00	0·75
2(*S*),3(*S*),4(*S*)-3-hydroxy-4-methylglutamic acid (**2**)	1·11	0·55
2(*S*),3(*S*)-3-hydroxy-4-methyleneglutamic acid (G3)	1·00	0·65
2(*S*),4(*S*)-4-methyl-4-hydroxyglutamic acid	1·00	0·72
2(*S*),4(*R*)-4-methyl-4-hydroxyglutamic acid	0·96	0·79
2(*S*)-4-methyleneglutamic acid	1·10	1·19
2(*S*),4(*R*)-4-hydroxy-4-isobutylglutamic acid	2·03	1·72
2(*S*),3(*R*)-3-hydroxyaspartic acid (**4**)	0·38	0·15

* (1) *n*-BuOH–HOAc–H_2O (12:3:5); (2) PhOH–H_2O–conc. NH_3 (120:30:1) (w/v/v); descending technique, Whatman No. 1.

TABLE 2. IONIC MOBILITIES OF ACIDIC AMINO ACIDS

Compound*	Migration of amino acid / Migration of glutamic acid
Aspartic acid	2·59
Glutamic acid	—
1	0·78
3	1·09
G1	1·18
G2	1·55
2	0·59
G3	3·18
2(*S*),4(*S*)-4-methyl-4-hydroxyglutamic acid	3·01
2(*S*),4(*R*)-4-methyl-4-hydroxyglutamic acid	3·28
2(*S*)-4-methyleneglutamic acid	1·54
4	3·25

HVE pH 3·6; HOAc–C_5H_5N–H_2O (5:0·5:95), 70 V/cm 3 hr; Whatman No. 3MM; the mobility of glutamic acid was found to be 83 mm.

* For designation of compounds (see Table 1).

The new amino acid (G3) had the elemental composition $C_6H_9NO_5$. It developed, like 4-methyleneglutamic acid, a brown colour with ninhydrin on paper chromatograms. The

ninhydrin reaction was masked by pretreatment of the paper chromatogram with cupric ions indicating that the amino group is α to a carboxyl group.[10] The positive reaction of G3 with periodic acid[11] indicated the presence of vicinal hydroxy groups or a hydroxy group vicinal to an amino group. The positive reaction with acid permanganate and with Br_2 indicated the presence of a double bond. These facts, combined with the evidence from PMR-spectroscopy (*vide infra*), suggested that G3 was one of the previously unknown diastereoisomeric 3-hydroxy-4-methyleneglutamic acids.

TABLE 3. PMR CHEMICAL SHIFTS AND COUPLING CONSTANTS*

	G3	G1	G2	**2**	**1**	**3**
H(2)	4·12(*d*)	3·98(*d*)	3·92(*d*)	3·95(*d*)	3·75(*t*)	3·80(*dd*)
	(*J* 4·5)	(*J* 3·0)	(*J* 3·0)	(*J* 3·0)	(*J* 7·5)	(*J* 8·2, 6·0)
H(3)	4·95(*d*)	4·15(*dd*)	4·22(*dd*)	4·13(*dd*)	2·00(*t*)	2·05(*m*)
	(*J* 4·5)	(*J* 8·2, 3·0)	(*J* 6·0, 3·5)	(*J* 8·2, 3·0)	(*J* 7·5)	
H(4)		2·98(*m*)	2·80(*m*)	2·94(*m*)	2·50(*m*)	2·60(*m*)
H(1′)	5·90(*d*), 6·28(*d*)	1·25(*d*)	1·25(*d*)	1·25(*d*)	1·25(*d*)	1·25(*d*)
	(*J* 1·0)	(*J* 7·5)	(*J* 7·5)	(*J* 7·5)	(*J* 7·5)	(*J* 7·0)

The PMR spectra were determined in D_2O soln at 60 MHz. The chemical shifts are in ppm downfield from sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate. Coupling constants are in Hz; the symbols *d*, *t*, *q*, *m* and *dd* represent doublet, triplet, quartet, multiplets and two sets of doublets respectively.

* For designation of compounds (see Table 1).

TABLE 4. $[\alpha]_D^{20}$ VALUES OF ACIDIC AMINO ACIDS

Compounds*	$[\alpha]_D$ values		Reference	
	H_2O	N HCl	H_2O	N HCl
G1 (*Gymnocladus dioicus*)	+7·1 (c, 1)	+24·7 (c, 1)		
G2 (*Gymnocladus dioicus*)	−2·2 (c, 1)	−15·7 (c, 1)		
G3 (*Gleditsia caspica*)	+14·8 (c, 1)	+35·6 (c, 1)		
2 (reduction of G3)	+41·4 (c, 0.97)	+43·5 (c, 0·83)		
1 (reduction of G3)	+20·0 (c, 0·25)	+37·4 (c, 0·23)	+17·7 (c, 0·29)†	+30·2 (c, 0·23)†§
3 (*Gleditsia caspica*)	−1·5 (c, 0·53)	+19·0 (c, 0·48)	−2·03 (c, 1·32)†	+22·2 (c, 0·66)†§
4 (ozonization of G3)	+42·3 (c, 0·74)	+52·6 (c, 0·67)	+41·4 (c, 2·43)‡	+53·2 (c, 2·46)‡

* For designation of compounds (see Table 1).
† Ref. 8.
‡ Ref. 9.
§ 5 N HCl.

Catalytic hydrogenation of G3 resulted in complete conversion into two acid amino acids, **1** and **2**. **1** and **2** were separated and isolated by ion-exchange chromatography. **1** was identified as 2(*S*),4(*S*)-4-methylglutamic acid by measurement of the optical rotation (Table 4) and by comparison with a sample consisting of a mixture of 2(*S*),4(*S*) and 2(*R*),4(*S*)-4-methylglutamic acid by use of paper chromatography (Table 1), electrophoresis (Table 2) and PMR-spectroscopy (Table 3). The absolute configuration of the isomeric 4-methylglutamic acids have previously been established.[12]

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2 had the elemental composition $C_6H_{11}NO_5$. PMR-spectroscopy (Table 3) indicated that it was one of the diastereoisomeric 3-hydroxy-4-methylglutamic acids. Two of these, G1 and G2, have previously been isolated from *Gymnocladus dioicus*.[1-3] G1 has the configuration 2(*S*),3(*S*),4(*R*) as originally proposed and confirmed by X-ray studies.[13] G2 has the configuration 2(*S*),3(*R*),4(*S*) as established by X-ray studies.[4] The configuration at C_4 in G2 was originally proposed to be 4(*R*).[3] A new isolation in large quantities of G1 and G2 have been performed and after several recrystallizations from water new values for optical rotations have been obtained (Table 4).[13] Calculations of the contributions of each optical center to the total optical activity on basis of the new rotation values indicate that the 2(*S*)-center in both G1 and G2 now conforms to the Clough–Lutz–Jirgensons rule even if the total rotation of G2 shows a large negative shift from water to HCl. The situation is comparable to that found for β-phenylserine.[14]

2 is different from G1 and G2 as demonstrated by electrophoresis (Table 2) and PMR-spectroscopy (Table 3). The configuration at C_2 in both G3 and **2** must be *S* (or L) as demonstrated by the production of **1** and of 2(*S*),3(*R*)-3-hydroxyaspartic acid (**6**) (*vide infra*) from G3. The configuration at C_3 in **2** must be *S* as demonstrated by the production of **4** from G3. Since **2** is different from G1, the configuration at C_4 in **2** must likewise be *S*. This implies that the reduction of G3 is highly stereospecific and giving the same configuration at C_4 in both **1** and **2**. No trace could be found in the hydrogenation mixture of **3** or of other isomers of 3-hydroxy-4-methylglutamic acid.

Ozonolysis of G3 followed by oxidation resulted in the production of 2(*S*),3(*R*)-3-hydroxyaspartic acid (L-erythro-3-hydroxyaspartic acid) (**4**). **4** was identified by measurement of optical rotation (Table 3), by comparison of the IR-spectrum with those published for **4** and for 2(*S*),3(*S*)-3-hydroxyaspartic acid **5**,[9] and by comparison of PMR-spectroscopic data with those reported in the literature for **4** and **5**.[15] Furthermore the identity was established by comparison of the electrophoretic mobility (Table 2) with that reported in the literature.[16]

The PMR-spectra of G3, **1**, **2**, **3**, **4**, G1, G2 and racemic samples of **1** and **3** were all measured in water in the same concentration and under the same conditions. The results obtained are listed in Table 3. The structure proposed for the various compounds are in full agreement with the PMR-spectroscopic evidence. The detailed pattern exhibited for the protons at C_2 and C_3 in G1 and **2** are very similar and different from that exhibited for the same protons in G2. This agrees with the configurational assignment in **2**.

The occurrence of 3-hydroxy-4-methyleneglutamic acid gives new evidence for the versatility in plant metabolism of substituted glutamic acids.[7] The biosynthesis of 4-methylglutamic acid has been investigated in *Gleditsia triacanthos* and a derivation from leucine proposed.[5] In this study it was suggested that both 3- and 4-hydroxy derivatives of 4-methylglutamic acid can arise from the parent amino acid. 4-Methyleneglutamine and 4-methylglutamic acid which are not present in seeds of *G. triacanthos* but which are formed during germination of these seeds were not labeled by incorporation of labeled 4-methylglutamic acid or 4-hydroxy-4-methylglutamic acid into the seedlings. The occurrence of a new 4-methyleneglutamic acid together with 4-methylglutamic acid and 3-hydroxy-4-methylglutamic acid in the seeds of *G. caspica* gives further support to a metabolic relation between 4-methyl and 4-methyleneglutamic acids.

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EXPERIMENTAL

Isolation of 2(S),3(S)-3-*hydroxy*-4-*methyleneglutamic acid* (*G3*), 2(S),4(R)-4-methylglutamic acid (**3**) *and* 2(S),3(S),4(R)-3-*hydroxy*-4-*methylglutamic acid* (*G1*). Finely ground seeds of *Gleditsia caspica* (2 kg) were extracted with 75% EtOH (30 l.) at 20°. The combined extracts were applied to a strongly acidic ion-exchange resin (Amberlite CG 120, H^+ form, 100–200 mesh, 5 × 100 cm). The resin was washed with 75% EtOH (6 l.) and then with H_2O (5 l.). The amino acids were eluted with 2 N NH_3 (4 l). The NH_3 eluate was taken to dryness under red. pres. The amino acids (26 g) were divided into two and separated by two consecutive runs on a strongly basic ion-exchange resin (Dowex 1 × 8, acetate form, 200–400 mesh, 5·6 × 45 cm). On elution with 0·5 M HOAc (20 ml fractions) glutamic acid appeared in fractions 85–120, **3** in fractions 85–114, G1 in fractions 115–159, aspartic acid in fractions 235–268 and the new compound G3 in fractions 330–365. The fractions from both separations containing G3 were taken to dryness and traces of HOAc were removed by repeated evaporation from H_2O. Yield of chromatographically pure material after crystallization from H_2O: 140 mg. (Found: C, 40·55; H, 5·15; N, 7·98. $C_6H_9NO_5$ required: C, 41·15; H, 5·19; N, 7·94%); $[\alpha]_D$ values: see Table 3. IR: ν_{max}^{KBr} 3350 cm^{-1} (w), 3200(m), 3000(w), 1712(s), 1640(s), 1585(m), 1510(s), 1415(s), 1348(w), 1340(w), 1305(w), 1255(s). For δ- and J values (see Table 3). **3** was obtained from fractions 85–114. Separation from glutamic acid was obtained by preparative PC on Whatman 3MM in *n*-BuOH–HCO_2H–H_2O (75:15:10). Recrystallization from H_2O afforded a pure sample (31 mg). For PC and electrophoretic behaviours, optical rotation and PMR data (see Tables 1–4). IR: ν_{max}^{KBr} 1685 cm^{-1}(s), 1575(s), 1510(s), 1400(s), 1355(w), 1323(w), 1287(m), 1170(m), 1075(m). G1 was obtained from fractions 115–159. Recrystallization from H_2O afforded a pure sample (1360 mg). For chromatographic and electrophoretic behaviour and PMR data (see Tables 1–3). $[\alpha]_D^{24.1}$ + 7·2° (*c* 1) H_2O (Table 4).

Reduction of G3 to give 2(S),4(S)-4-*methylglutamic acid* (**1**) *and* 2(S),3(S),4(S)-3-*hydroxy*-4-*methylglutamic acid* (**2**). 48 mg in H_2O (10 ml) were hydrogenated in the presence of PtO_2 (20 mg) at 20°. After 2 hr, the reduction was complete. Electrophoretic investigations showed the presence of only two products, **1** and **2**, in the ratio 1:3. After removal of the catalyst by filtration, the compounds were separated on a strongly basic ion-exchange resin (Dowex 1 × 8, acetate form, 200–400 mesh, 2·5 × 70 cm, elution with 0·2 M HOAc, 10 ml fractions). **2** was eluted in fractions 216–235 (14 mg) and **1** in fractions 256–272 (5 mg). **1**. For paper chromatographic and electrophoretic behaviour, optical rotation and PMR data (see Tables 1–4). **2**. (Found: C, 40·3; H, 6·1; N, 7·9. $C_6H_{11}O_5N$ required: C, 40·7; H, 6·3; N, 7·9%). IR: ν_{max}^{KBr} 3520 cm^{-1}(w), 3370(w), 3130(m), 1720(s), 1600(s), 1530(s), 1440(s), 1332(m), 1310(m), 1250(m), 1198(s), 1155(m), 1010(s). For PC and electrophoretic bahaviour, optical rotation and PMR data (see Tables 1–4).

Ozonolysis and oxidation of G3 to give 2(S),3(R)-3-*hydroxyaspartic acid* (**4**). A stream of O_3 was passed through a soln of G3 (20 mg) in H_2CO_2H (5 ml) for 25 min. After removal of excess O_3 in a stream of N_2. H_2O_2 (10 μl 30%) and H_2O (1 ml) were added. After 3 hr at 20° the soln was taken to dryness. The residue was dissolved in H_2O and applied to Dowex 50 W × 8 resin (H^+, 200–400 mesh, 0·5–5 cm). After washing with H_2O (10 ml) the column was eluted with aq. C_5H_5N (1 M, 2 ml). The C_5H_4N eluate was evaporated to dryness (18·8 mg). Two crystallizations of the residue from H_2O afforded **4** as a colourless crystalline solid (10·5 mg). For chromatographic and electrophoretic behaviours and for rotation values (see Tables 1, 2 and 4). PMR spectroscopic data of **4** in D_2O soln at various pHs were in agreement with the data reported for *erythro*-3-hydroxyaspartic acid.[15] IR(KBr) was identical with the spectrum published for *erythro*-3-hydroxyaspartic acid.[9]

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