Tetrahedron Vol. 32, pp 2799 to 2804 Pergamon Press 1976. Printed in Great Britain

TWO DIASTEOISOMERS OF 5-HYDROXY-6-METHYLPIPECOLIC ACID FROM SEEDS OF *FAGUS SILVATICA* L.†

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(*Received in UK* 3 *June* 1976; *Accepted for publication* 29 *June* 1976)

Abstract—Two diastereoisomers of 5-hydroxy-6-methylpipecolic acid have been isolated from beechnuts. The configurations of the two amino acids have been assigned as (2*S*, 5*S*, 6*S*) (**1**) and (2*S*, 5*R*, 6*S*) (**2**). The determination of structure and configuration for the two new amino acids is mainly based on spectroscopic and rotatory evidence. The ^{13}C-NMR and ^{1}H-NMR spectra of the three ionic forms of the two amino acids and of *S*-pipecolic acid and (2*S*, 5*R*)-5-hydroxypipecolic acid and the CD-curves of the cations and amfoions of **1**, **2**, and (2*S*, 5*R*)-5-hydroxypipecolic acid have been recorded.

In the course of a study of the free amino acids and peptides in seeds of *Fagus silvatica* L. (beechnuts) a spot was observed on two-dimensional paper chromatograms which could not be ascribed to any known amino acid.[1] The present paper describes the separation and isolation of the two amino acids (**1** and **2**) responsible for this spot and their identification as (2*S*, 5*S*, 6*S*) - 5 - hydroxy - 6 - methylpipecolic acid and (2*S*, 5*R*, 6*S*) - 5 - hydroxy - 6 - methylpipecolic acid. A preliminary account of part of this work has been given previously.[2]

METHODS AND RESULTS

The partial purification of **1** and **2** has been described.[1] Final purification was accomplished by preparative paper chromatography to give a sample, which gave only one spot in the standard paper chromatographic systems. However, the ^{1}H-NMR spectrum showed that it was a mixture of two closely related compounds and accordingly it could be separated by paper chromatography in special solvents (see below).

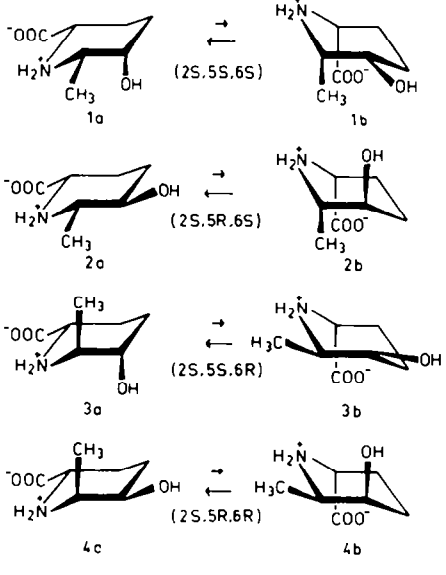
Compound **1** was obtained in a chromatographically pure state from the mixture by crystallization. Recrystallization could be performed in water–ethanol–ether. **2** was obtained from the mother liquor from this crystallization by preparative paper chromatography, using the upper phase of t-amyl alcohol:acetic acid:water (20:1:20) as solvent and development of the chromatograms for 10 days. It was obtained as a chromatographically pure, semicrystalline residue after concentration of an aqueous solution. A crystalline hydrochloride was obtained from water–ethanol–ether.

Elementary analysis of **1** indicated the composition $C_7H_{13}NO_3$ with a small amount of water, and the hydrochloride of **2** showed the composition $C_7H_{14}ClNO_3$. The IR spectra of **1** and **2** did not indicate the presence of double bonds. The spectra are distinctly different, especially in the fingerprint region. Both **1** and **2** are stable in 6 N HCl for 24 hr at 100°. With ninhydrin on paper both compounds develop a blue–purple colour similar to that exhibited by pipecolic acid.[3] Furthermore, by preservation of the chromatograms with cupric ions after development with ninhydrin[4] the blue colour persists, whereas the normal purple ninhydrin colours turn into red by this treatment. This behaviour is characteristic of pipecolic acid derivatives. The colour reaction with ninhydrin on paper chromatograms is slow and takes place only after heating. About 5 min at 100° are necessary to fully develop the colour, and even then the colour yield is lower than for normal amino acids.

In Table 1 are recorded the molecular rotations of **1** and **2** in water and acid. For comparison rotation values from the literature for (*S*)-pipecolic acid (**5**), (2*S*, 5*R*)-5-hydroxypipecolic acid (**6**), (2*S*, 5*S*)-5-hydroxypipecolic acid (**7**), (*S*)-2-methylpiperidinium chloride (**8**), (1*S*, 2*R*)-2-methylcyclohexanol (**9**) and (1*R*, 2*R*)-2-methylcyclohexanol (**10**) are included in Table 1.

In Table 2 are recorded CD-data for **1**, **2** and **6**. For comparison, values for **5** from the literature are included in Table 2.

In Table 3 are recorded ^{1}H-NMR chemical shifts and an interpretation of the coupling data observed for the different ionic forms of **1**, **2**, **5** and **6**. The couplings assumed for the amfoions of **1** and **2** were confirmed by decoupling experiments. Further details of the interpretation of the spectra are given in the Discussion.



†Taken in part from the thesis of I. Kristensen, Copenhagen (1973).[2]

Table 1. Molecular rotations for **1**, **2**, and **5–10**

Compound	H_2O $[M]_D$	Conc.	Acid $[M]_D$	Conc.	Reference
1	−17°	1.3	+2°	2.2 in 2 N HCl	-
2*	−74°	2.8	−54°	2.8 in 2 N HCl	-
5	−33°	2.2	−14°	1.6 in 5 N HCl	5
6	−34°	1.0	−20°	0.7, 1 equiv. of HCl	6
7	−45°	1.0	−17°	0.6, 1 equiv. of HBr	6
8			−5°	2.4 in H_2O	7,8
9	+28°	1.0 in MeOH			9
10	−49°	1.0 in MeOH			9

For designation of compounds see text and Formula Chart.

*The numerical values are probably too small because of water content in the samples.

Table 2. CD-data for **1**, **2**, **5** and **6**

Compound	H_2O		0.1 N HCl	
	λ_{max} (nm)	$\Delta\varepsilon$	λ_{max} (nm)	$\Delta\varepsilon$
1	208	+0.43	210	+0.77
2	210	+0.48	210	+0.85
5[10]	208	+0.46	208	+0.76
6	208	+0.55	208	+0.89

For designation of compounds see text and Formula Chart.

The CD-curves were recorded on a Roussel-Jouan CD 185 Dichrograph using 1- or 2-mm cells and concentrations of approximately 0.5–1 mg/ml.

In Table 4 are recorded ^{13}C-NMR chemical shifts for the different ionic forms of **1**, **2**, **5** and **6**. The number of H atoms on each C atom in the amfoion of **1** and **2** was determined by use of partially decoupled spectra. Further details of the assignment of signals to the individual C atoms are given in the Discussion.

DISCUSSION

The isolation procedure and chemical properties for **1** and **2** demonstrate that both compounds are neutral amino acids containing one carboxyl group and one amino group. This conclusion is in full agreement with the spectroscopic properties of the compounds as discussed below.

The ^{13}C-NMR spectra of **1** and **2** indicate the presence of one CH_3-, two CH_2- and three CH-groups in both compounds. The ^{1}H spectra of **1** and **2** and especially the changes in the chemical shifts occurring by changing the ionization states indicate for both compounds that one CH-group is in α-position to both the carboxyl group and the amino group. A second CH-group and the CH_3-groups are close to the amino group; the chemical shift of the protons in these groups is not influenced by the ionization state of the carboxyl group. The spectrum of **2** demonstrates that the chemical shift of the proton in the

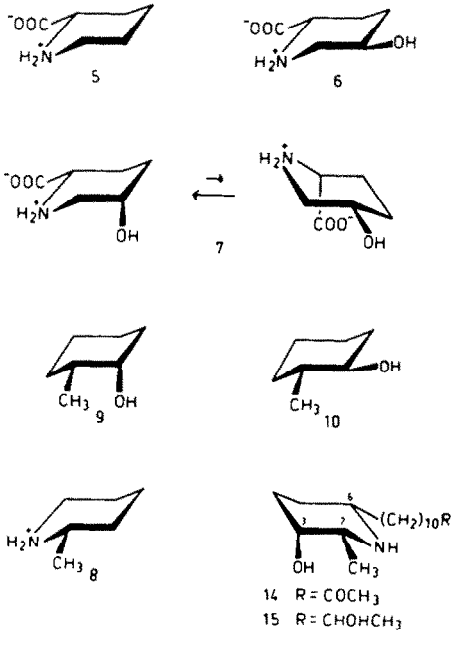


Table 3. Chemical shifts and coupling patterns in the ^{1}H-NMR spectra of **1**, **2**, **5** and **6**

	1			**2**			**5**			**6**		
Hydrogen atom	Cation	Amfoion	Anion	Cation	Amfoion	Anion	Cation	Amfoion	Anion	Cation	Amfoion	Anion
H-2	4.07	3.63	3.16	4.05	3.62	3.15	3.97	3.6	ca. 3.1	4.07	3.67	3.1
	m	two d, J 4 and 12 Hz		two d, J 3.5 and 12 Hz		m	two d, J 3.5 and 10.5 Hz			two d, J 4 and 10.5 Hz		
H-3 and H-4	1.7-2.4	1.7-2.3	1.3-2.2	1.6-2 (ax)	1.5-2.5	1.25-1.7 (ax)				1.45-2.6	1.4-2.5	1.2-1.75 (ax) broad t, splitting 9 Hz
		m		broad t, splitting 12 Hz	m	broad t, splitting 10 Hz					m	
				2.07-2.6 (eq)		1.95-2.2 (eq)	1.5-2.45	1.5-2.35	1.2-2.05			1.9-2.25 (eq) broad d, splitting 7 Hz
								m				
H-5, ax	-	-	-	3.62	3.60	3.2				4.03	4.00	3.68
				two t, J 4.5 and 10 Hz		m				two d, J 4.5 and 9 Hz		Broad m, width 32 Hz
H-5, eq	4.01	3.99	3.7	-	-	-				-	-	-
	broad s											
H-6, ax	3.45	3.36	2.72	3.12	3.04	2.48	ca. 3.07	ca. 3.02	ca. 2.58	2.93	2.87	2.4
	two q, J 1.5 and 7 Hz			two q, J 6.5 and 10 Hz			broad t, width 30-35 Hz, splitting ca. 10 Hz			two d, J 9.5 and 12 Hz		
H-6, eq	-	-	-	-	-	-	ca. 3.5	ca. 3.45	ca. 3.0	3.55	3.5	3.18
							broad d, width 23 Hz splitting 13 Hz			J 1 (6e,4e), 4.5 and 12 Hz		
CH_3	1.34	1.35	1.07	1.44	1.42	1.16	-	-	-	-	-	-
	d, J 7 Hz			d, J 6.5 Hz								

The spectra were recorded in D_2O + DCl, in D_2O and in D_2O + NaOD at 90 MHz on a Bruker HX 90E instrument, using the pulse technique and Fourier transformation. Chemical Shifts are in ppm downfield from sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl) propionate. For designation of compounds see text and Formula Chart. s singlet, d doublet, q quartet, m multiplet. For the spectrum of **6** compare ref. 11.

Table 4. ^{13}C-NMR chemical shifts for **1**, **2**, **5** and **6**

	1				**2**				**5**			**6**		
Carbon atom	Cation	Amfoion	Anion	Anion calc.	Cation	Amfoion	Anion	Anion calc.	Cation	Amfoion	Anion	Cation	Amfoion	Anion
C-2	58.0	60.5	61.2	58.8	57.5	60.1	61.0	60.4	57.5	59.9	61.4	56.5	58.8	60.6
C-3	20-5	21.4	24.2	23.9	25.4	26.2	30.1	29.1	26.5	27.3	30.4	23.7	24.5	29.8
C-4	29.7	30.2	31.6	29.5	31.4	32.0	33.5	32.8	22.2	22.4	24.8	30.0	30.5	33.5
C-5	65.3	65.6	67.6	68.4	69.6	70.0	73.7	74.2		22.6	25.9	63.6	64.0	67.9
C-6	56.7	56.1	53.3	53.4	57.5	57.1	57.1	57.3	44.8	44.5	45.5	48.2	48.0	51.6
CH_3	15.5	15.6	18.2	-	15.5	15.7	18.5	-	-	-	-	-	-	-
COOH	171.6	174.9	182.2	-	171.5	174.6	181.6	-	172.3	175.4	182.7	171.6	174.4	181.9

The spectra were recorded in D_2O + HCl, in D_2O and in D_2O + NaOH at 22.63 Mhz on a Bruker WH 90 instrument, using the pulse technique with Fourier transformation. Chemical shifts are in ppm downfield from TMS. Dioxane was used as internal standard in all samples. For methods for calculation of chemical shifts for the anions of **1** and **2** see text. For designation of compounds see text and Formula

third CH-group in this compound likewise is influenced by the ionization state of the amino group. The chemical shift of the remaining CH-proton in **1** is also influenced but to a smaller degree by the ionization state of the amino group. The ionization changes observed are in good agreement with values from the literature.[12] The elementary analyses performed indicate two double bond equivalents in both **1** and **2**. The carboxyl group accounts for one and since no other double bonds are reflected in the NMR and IR spectra, both compounds must contain one ring.

The combined evidence points to the presence of a CH_3–CH–CH–CH_2–CH_2–CH–COO^--skeleton and indicates that **1** and **2** are diastereoisomeric 5 - hydroxy - 6 - methylpipecolic acids. An oxygen-containing ring could not explain the titration shifts observed in the ^{1}H-NMR spectra. A 3-membered nitrogen-containing ring can be excluded because of the stability of the compounds and also would not provide an explanation for the titration shifts. A 5-membered ring, as in 5-(1-hydroxyethyl)proline is again unlikely in view of the titration shifts observed. Also the coupling constants in the CH–CH-fragment (1.5 and 10 Hz for **1** and **2** respectively) are incompatible with this structure, since here free rotation within the fragment would be expected.

The pipecolic acid structure also explains the slow ninhydrin reactions. In analogy it has been observed that 1 - methyl - 6 - hydroxy - 1,2,3,4 - tetrahydroisoquinoline - 3 - carboxylic acid does not react with ninhydrin,[13] whereas 6,7 - dihydroxy - 1,2,3,4 - tetrahydroisoquinoline - 3 - carboxylic acid does.[14]

Both **1** and **2** supposedly have (2*S*)- (i.e. L-) configuration. Provided that the contribution to the rotation values from the asymmetric centers at C-5 and C-6 are not influenced by protonization of the carboxylate ion, this assumption is supported by the dextrorotatory shift in rotation on going from water to acid (Table 1) according to the Clough–Lutz–Jirgensons rule.[15] This rule holds for **5**, **6** and **7** (Table 1), and also for the isomeric 4 - hydroxy - pipecolic acids.[5] The CD-curves also support the (2*S*)-configurations (Table 2). The sign, size and position of the CD-maxima are nearly identical for **1**, **2**, **5** and **6**, so that the Cotton effect presumably is due to the common

carboxylate ion or carboxyl group[10] with no contribution from the centers at C-5 and C-6. The similarity between **1**, **2**, **5** and **6** may furthermore be taken as evidence for the same position (equatorial) of the carboxyl group in all four compounds. This conclusion is also supported by the signals for the C-2 protons in the ^{1}H-NMR spectra. The signal is a broad multiplet in all four compounds (Table 3). Correspondingly the axial proton (H-6) in O,O'-benzylidenedesoxoprosophylline (**11**) gives a broad signal (half height 24 Hz)[16] whereas the equatorial proton H-6 in O,O'-benzylidenedesoxoprosopinine (**12**)[17] and H-2 in the nitroso derivative of **5**[18] appear as narrow signals.

Compounds **1** and **2** must be two of the compounds **1**, **2**, **3** and **4**, which each may occur in two chair conformations, **a** and **b**. Since it is reasonable to assume that conformations involving an axial carboxyl group are unlikely, particularly when 1,3-diaxial to the Me group at C-6, **1b** and **2b** are very unlikely and shall not be considered. **3b** and **4b** are possible, but conformations **3a** and **4a** would be expected to predominate.

With regard to the configuration at C-5 in **1**, the narrow signal (almost a singlet) observed for H-5 indicates an equatorial position for this proton. A similar broad singlet has been observed for H-3 in carpaine **13**,[19] cassine (**14**) and carnavaline (**15**).[20] In contrast, H-5 in **6** (Table 3, cf. Ref. 11), and the axial H-3 in **11**[16] and **12**[17] give broad multiplets. Therefore **1** must have the OH-group axial as in (**a1**, **c1** or **d2**), the latter possibility is, however, excluded by the appearance of the H-2 signal.

Having established the configuration at C-2 and C-5 in **1**, that at C-6 may be determined on basis of the coupling constants between H-5 and H-6, which is only about 1.5 Hz. This shows a gauche relationship and hence structure **1a**, rather than **3a** applies. Similarly the coupling constants between H-2 and H-3 and between H-2' and H-3' in **13**[19] and azimine (**16**),[21] and between H-2' and H-3' in pseudocarpaine (**17**)[19] are small (1–3 Hz). Both interacting protons in **1a** as well as those in **13** and **16** are trans coplanar to electronegative atoms (O or N), a fact that partially accounts for the small coupling constants.[22,23]

The configurations at C-5 and C-6 in **2** appear from the large coupling constants (10 Hz) between H-5 and H-6. Both protons must be axial, and **2** must have the structure **2a**, **3b** being excluded by the appearance of H-2. Similar coupling constants have been observed between H-2 and H-3 in **12**,[16,17] and between the axial protons at C-5 and C-6 in **6** (Table 3, cf. Ref. 11). The multiplet found for H-5 in **2** is to be expected.

Also the proton chemical shifts are in agreement with the structure **1a** for **1**, and **2a** for **2**. The proton at C-6 in **1** is observed at 3.36 ppm, whereas it is found at 3.04 ppm in **2** (Table 3). This downfield shift from **2** to **1** must be due to the change of the OH-group at C-5 from equatorial to axial position.[24] The H-5 in **1** is observed at 3.99 ppm whereas it is found at 3.6 ppm in **2** (Table 4). This downfield shift is in good agreement with the change from equatorial to axial position.[24,25]

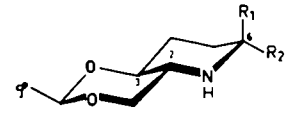
The strong levorotatory shift observed on going from **1** to **2** cannot be due to the OH-group alone because the differences in rotation between **5**, **6** and **7** are small (Table 1). The Me-group cannot either be responsible, its position being the same in **1** and **2**, and besides **8** has only a small rotation (Table 1). On the other hand a change in the Me–OH interaction seems to be capable of causing a large shift, as evident from the rotation of **9** and **10** (Table 1). **10** has the two substituents in equatorial positions, whereas **9** must predominantly occur with the OH-group axial. The sign and size in shift of rotation from **1** to **2** can thus be interpreted as support for structures **1a** and **2b**, respectively.

The assignment of the individual signals to the C atoms in **5** has been made on basis of the known spectra for piperidine and the piperidinium ion,[26] using shift parameters for amino acids.[27] Even if the justification for using these shifts parameters in a cyclic system may be questioned the agreement is fairly good for all three ionization states of **5** except for C-2. The titration shifts observed are in good agreement with known titration shifts for amino acids,[28,29] and for piperidine itself.[26]

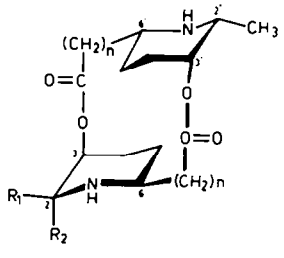
The assignment of the individual signals to the C atoms in **6** has been made on basis of the experimental values for **5** using published shift parameters for the introduction of an equatorial OH-group into a cyclohexane ring.[30] Considering the differences between a cyclohexane ring and a piperidine ring the agreement between calculated and experimental values seems satisfactory, especially for the anion where the largest difference is 2.4 ppm (for C-6). Again the titration shifts are in good agreement with those expected.[28,29]

The assignment of the individual signals to the C atoms in **1** and **2** has also been made on basis of the experimental values for **5**. First the effect of introduction of the Me-group has been calculated using published values for the introduction of an equatorial Me-group into piperidine.[31] Similar values may be found using other published values for the effect of a Me-group in a piperidine ring.[32–35] Then the effect of the OH-group is calculated using published values for the introduction of either an equatorial or axial OH-group into methylcyclohexane.[30] The agreement is again fairly good, especially for the anions. The calculated values for the anions of **1** and **2** listed in Table 4 demonstrate that the largest difference between calculated and experimental values is 2.4 ppm for **1** (for C-2) and 1 ppm for **2** (for C-3). Using the experimental values for **6** and the published values for the introduction of an equatorial Me group into piperidine[31] values for **2** are also found in close agreement with the experimental (largest deviation for the anion 1.5 ppm). The titration shifts for **1** and **2** are in good agreement with those expected.[28,29]

The ^{13}C-NMR spectra therefore also support the configurational assignments made, especially providing an



11 R_1 = H (H-6), R_2 = $(CH_2)_{11}CH_3$
12 R_1 = $(CH_2)_{11}CH_3$, R_2 = H (H-6)



13 n = 7, R_1 = CH_3, R_2 = H (H-2)
16 n = 5, R_1 = CH_3, R_2 = H (H-2)
17 n = 7, R_1 = H (H-2), R_2 = CH_3

explanation for the large difference between **1** and **2** in chemical shifts for C-3 and C-5. Similar calculations for the two other isomers, **3** and **4**, cannot be performed with confidence because of the possibility of two conformations and lack of knowledge of the influence of an axial carboxyl group. The values for the conformer **4a** have been calculated using the experimental values for **6** and the published values for introduction of an axial Me-group in a cyclohexane ring.[36] These values show large differences (up to 7.1 and 7.9 ppm) from the experimental values for both **1** and **2**. The same lack of fit is obtained by first adding the influence of an axial Me-group to the experimental values for **5**[36] and then adding the influence of an equatorial OH-group.[30]

Thus the combined evidence permits the conclusion that **1** is (2*S*, 5*S*, 6*S*) - 5 - hydroxy - 6 - methylpipecolic acid with the predominant conformation **1a**, whereas **2** is (2*S*, 5*R*, 6*S*) - 5 - hydroxy - 6 - methylpipecolic acid with the predominant conformation **2a**.

Pipecolic acid is present in varying concentrations in all plant material.[37] A number of pipecolic acid derivatives have been identified in various higher plants, including (2*S*, 4*S*) - 4 - hydroxypipecolic acid (and probably other stereoisomers),[38] (2*S*, 5*R*)-pipecolic acid, (*S*)-4,5-dehydropipecolic acid (baikiain), 4-aminopipecolic acid (of unspecified configuration),[37] and (2*S*, 4*R*, 5*S*) - 4,5 - dihydroxypipecolic acid.[39,40] Even though the two 5 - hydroxy - 6 - methylpipecolic acids co-occur with pipecolic acid in *F. silvatica*[1] a biogenetic relationship cannot be assumed. It is a general trend among non-protein plant amino acids that most variations are due to different functional groups including C–C multiple bonds and rings, whereas the basic carbon skeletons remain unchanged. Of more interest in connection with the two new amino acids may therefore be the isolation from higher plants of various piperidine alkaloids containing a carbon side chain at C-2, a Me-group at C-6, and in some cases an OH-group or a CO-group at C-5 in the piperidine ring.[37] A number of these alkaloids have been included in the discussion of the ^{1}H-NMR spectra (cf. Formula Chart). Biosynthetic studies indicate an origin from acetate units and not from lysine for at least some of these compounds.[37]

The occurrence of two diastereoisomeric amino acids in the same plant has been observed a number of times in recent years (see, e.g.[38,41]).

EXPERIMENTAL

General methods and instrumentation have been described in the previous communication.[1] Microanalyses were performed by Mr. G. Cornali and his staff.

1 and **2** were isolated from fractions (1.1.2) and (1.1.3) described in the previous communication[1] by preparative PC in solvent 1 (Ref. 1). A sample was obtained which showed only one spot in standard PC systems but which was shown to be a mixture by ^{1}H-NMR spectroscopy. The mixture was dissolved in abs. EtOH. Cooling to −18° for 14 days resulted in the precipitation of pure crystalline **1** (120 mg). The mother liquor was subjected to preparative PC with the upper phase of t-amyl alcohol–AcOH–H_2O (20:1:20) as solvent and with development for 10 days, resulting in the separation of **1** and **2**. **1** was recrystallized from water–EtOH–ether. **2** was obtained as a semicrystalline residue (90 mg) by evaporation of an aqueous solution.

Compound **1**: (Found: C, 51.31; H, 8.15; N, 8.35. Calc. for $C_7H_{13}NO_3$: C, 52.82; H, 8.23; N, 8.80. **1** IR: ν_{max}^{KBr} 3230 cm^{-1}, 2600, 1625, 1570, 1395, 400. $[\alpha]_D^{23}$ − 11.0° (*c* 1.3, H_2O). $[\alpha]_D^{23}$ + 1.1° (*c* 2.2, 2 N HCl). R_f in solvent 1 (Ref. 1) 0.39, in solvent 2 0.87. For CD-data, ^{1}H-NMR and ^{13}C-NMR data see Tables 2, 3 and 4.

Compound **2**: IR: ν_{max}^{KBr} 3290, 3060, 1635, 1395, 1050. $[\alpha]_D^{23}$ − 46.4° (*c* 2.8, H_2O), $[\alpha]_D^{23}$ − 33.8° (*c* 2.8, 2 N HCl). The contents must be assumed of small amount of water in the hygroscopic material used for the rotation determinations. R_f in solvent 1 (Ref. 1) 0.41, in solvent 2 0.85. For CD-, ^{1}H-NMR and ^{13}C-NMR data see Tables 2–4.

Compounds **1** and **2** can be separated by high voltage paper electrophoresis at pH 1.9 (HCOOH–AcOH–H_2O (33:148:1819), Whatman 3 MM, 4000 V) where **1** shows the highest mobility.

The hydrochloride of **2** was produced by evaporation of a soln in aqueous HCl and recrystallization from water–EtOH–ether. **2**, HCl (Found: C, 42.76; H, 7.16; Cl, 17.96; N, 6.99. Calc. for $C_7H_{14}ClNO_3$: C, 42.97; H, 7.21; Cl, 18.12; N, 7.16). IR: ν_{max}^{KBr} 3280, 2920, 2520, 1745, 1425, 1210, 1045, 635, 560, 470. $[\alpha]_D^{22}$ − 35.6° (*c*, 1, H_2O).

Acknowledgements—We are greatly indebted to Prof. M. G. Ettlinger, University of Copenhagen, for valuable discussions and suggestions concerning the interpretation of the spectroscopic and rotational evidence. The assistance of Dr. K. Bock in recording the NMR curves is gratefully acknowledged. The NMR measurements were made at the Institute of Organic Chemistry and the CD-Measurements at Chemical Laboratory A, both at the Technical University of Denmark, on instruments made available through grants from the Danish Natural Science Research Council.

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