Phytochemistry, Vol. 20, No. 8, pp. 1853–1855, 1981.
Printed in Great Britain.

0031-9422/81/081853-03 $02.00/0

2,4-DIAMINO-3-METHYLBUTANOIC ACID, A NOVEL AMINO ACID IN ROOT NODULE HYDROLYSATES FROM *LOTUS TENUIS*

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(*Received* 28 *November* 1980)

Key Word Index—*Lotus tenuis*; Leguminosae; *Rhizobium* species; 2,4-diamino-3-methylbutanoic acid; GC/MS; synthesis; non-protein amino acid.

Abstract—The novel amino acid 2,4-diamino-3-methylbutanoic acid has been identified in nodules formed by two strains of *Rhizobium* bacteria on *Lotus tenuis* roots. Retention time measurements on a Chirasil-Val capillary column suggest it is present as the (2*R*,3*S*)-enantiomer. Several isomeric diamino acids were synthesized for comparative studies.

INTRODUCTION

It has been known for some time that the amino acid composition of nodules on the roots of *Lotus* species is determined by the *Rhizobium* strain rather than by the host plant [1, 2]. Besides the occurrence of the common 'protein' amino acids, the accumulation of ninhydrin-positive compounds having unusual R_f values is also evident in some cases.

During a re-investigation using GC and GC/MS techniques, we analysed the amino acid compositions in root nodules formed by *Rhizobium* strains NZP2227 and NZP2238/1 on the host plant *Lotus tenuis*. We now report the structural elucidation and suggest the configuration of the previously unobserved amino acid 2,4-diamino-3-methylbutanoic acid.

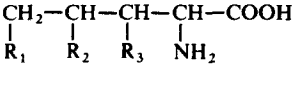
RESULTS AND DISCUSSION

Mature legume root nodules of *Lotus tenuis* inoculated, in separate experiments, with *Rhizobium* strains NZP2227 and NZP2238/1 were isolated and extracted with 80% EtOH. Following acid hydrolysis and chromatography on Amberlite IR-120, the amino acid fraction of each sample was derivatized to give a mixture of TAB (*N*-trifluoroacetyl *n*-butyl) esters [3]. Subsequent GC/EIMS analysis on a mixed phase OV-17/OV-210 column revealed the presence of a major component with a RR_t (aspartate) of 0.86. In neither case was this component observed in unhydrolysed extracts.

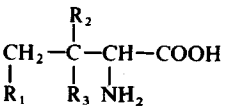
A comparison with the electron-impact mass spectra and GC relative retention times of the TAB derivatives of amino acid standards [4] suggested that the unknown was an uncommon amino acid. The high resolution mass spectrum gave a very weak molecular ion at *m/z* 380 corresponding to the formula $C_{13}H_{18}O_4N_2F_6$, which is isomeric with the TAB derivative of ornithine (**1**). The MW was confirmed by GC/CIMS (methane) in which an intense protonated molecular ion $(M + H)^+$ was observed at *m/z* 381 together with the other associated adduct ions at *m/z* 409 $(M + C_2H_5)^+$ and 421 $(M + C_3H_5)^+$. Other diagnostic ions in the EIMS of particular interest were found at *m/z* 306 (M − 74, $C_9H_8O_3N_2F_6$), 279 (M − 101, $C_8H_9O_2N_2F_6$) and 166 (M − 214, $C_6H_7ONF_3$) and are characteristic of the TAB derivatives of basic aliphatic amino acids [4, 5]. The fragment ion at *m/z* 227 ($C_8H_{12}O_3NF_3$) results from a McLafferty rearrangement involving the oxygen atom of the ester carbonyl with a γ-hydrogen atom and is indicative of an α-amino acid.

Analysis of the mass spectral fragmentation pattern provided insufficient evidence for unequivocal structure determination and it was necessary to synthesize, by established methods, the four ornithine isomers **2**–**5** for direct comparison. Each was readily distinguishable on the basis of its mass spectrum as shown in Table 1 and chromatographic properties (Table 2). A comparison of these data showed the unknown amino acid from the nodule hydrolysates to be identical in all respects to 2,4-diamino-3-methylbutanoic acid (**5**).

The stereochemistry of **5** was determined by comparing the GC characteristics of the synthetically prepared amino acid derivative with that found in the root nodule hydrolysates on the chiral stationary phase, Chirasil-Val.



1 $R_1 = NH_2$, $R_2 = R_3 = H$
2 $R_1 = R_2 = H$, $R_3 = NH_2$
3 $R_1 = R_3 = H$, $R_2 = NH_2$



4 $R_1 = H$, $R_2 = Me$, $R_3 = NH_2$
5 $R_1 = NH_2$, $R_2 = H$, $R_3 = Me$

Table 1. Partial mass spectra of TAB derivatives of ornithine and selected isomers*

Ion (m/z)	1	2	3	4	5	Unknown amino acid
380	0.1	—	0.2	—	0.3	0.2
306	3.1	0.1	2.5	0.3	2.7	2.8
279	2.8	2.0	10.6	—	12.7	12.9
267	1.2	0.3	—	—	—	—
261	1.2	0.4	—	—	—	—
227	1.1	8.2	4.4	1.1	8.9	9.3
211	1.6	0.5	—	0.5	1.1	—
209	1.5	0.5	—	—	1.4	1.7
193	3.1	2.2	3.1	2.6	3.4	3.4
181	2.6	0.6	—	—	2.3	2.3
171	—†	1.4	5.6	0.9	10.9	11.0
166	100	10.8	100	7.7	100	100
154	3.1	100	4.1	100	54.6	54.5
153	7.4	15.4	5.6	—	18.2	18.1
140	6.5	—	68.8	—	—	—
139	21.3	—	—	—	—	—
126	12.3	7.7	2.2	—	27.3	27.2

*Per cent relative intensity.
† < 0.1 %.

Under the GC conditions described, the four possible enantiomers of the synthetic product were resolved with peaks being observed at R_t 24.77, 24.99, 29.82 and 30.04 min. Interpretation of these results was based upon previous amino acid analyses on Chirasil-Val by Frank *et al.* [6]. These authors observed that in the case of racemic *N*-perfluoropropyl-isoleucine and -alloisoleucine isobutyl esters the (2*R*,3*S*)- and (2*S*,3*R*)-enantiomeric pair eluted before the (2*R*,3*R*)- and (2*S*,3*S*)-enantiomers and that within each pair the (2*R*,3*S*)-, (2*R*,3*R*)-isomers eluted faster than the corresponding (2*S*,3*R*)-, (2*S*,3*S*)-isomers. The same elution pattern has been reported by Koenig *et al.* [7] for isoleucine and alloisoleucine using an *N*-trifluoroacetyl-L-phenylalanyl-L-leucine cyclohexyl ester stationary phase and by Nakaparksin *et al.* [8] for isoleucine, alloisoleucine and threonine on *N*-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester.

Table 2. Chromatographic data on TAB derivatives of ornithine and selected isomers

	RR_t*	R_f†	R_f‡
1	1.10	0.15	0.1
2	0.92	0.2	0.2
3	0.94	0.2	0.2
4	0.51	0.2	0.3
5	0.86	0.1	0.2
Unknown amino acid	0.86	0.1	0.2

*GC retention of *N*-trifluoroacetyl *n*-butyl esters relative to aspartic acid on 2% OV-17/1% OV-210.
†PC on Whatman No. 1 paper in *n*-BuOH–HOAc–H_2O (12:3:5).
‡PC on Whatman No. 1 paper in H_2O–satd PhOH.

Based on these observations we assigned the GC peaks in order of elution as (2*R*,3*S*)-, (2*S*,3*R*)- (2*R*,3*R*)- and (2*S*,3*S*)-*N*-trifluoroacetyl-2,4-diamino-3-methylbutanoic acid *N*-butyl ester. GC analysis of each derivatized nodule hydrolysate gave a peak at R_t 24.97 min for **5** suggesting that this amino acid was present in these root nodules of *L. tenuis* as its (2*R*,3*S*)-enantiomer.

EXPERIMENTAL

Mps were determined on a Kofler hot-stage and are uncorr. Chemical shifts in the ^{1}H NMR spectra are expressed as δ values in ppm relative to TMSO. High and low resolution GC/EIMS were obtained on a double beam AEI MS30 equipped with a single stage all-glass jet separator and interfaced to a Pye 104 gas chromatograph. GC/CIMS (methane) were recorded on a Hewlett–Packard Model 5982A GC/MS. GC (FID) was performed on either a Hewlett–Packard 7620 or Hewlett–Packard 5840A instruments using a 2.5 m × 0.3 cm glass column packed with 2% OV-17/1% OV-210 on Gas Chrom Q programmed from 90° to 230° at 4°/min and held at 230° for 15 min. Separation of enantiomers was carried out on a 25 m × 0.3 mm open tubular glass capillary column coated with Chirasil-Val (Applied Science Labs, State College, Pa, U.S.A.) programmed at 90°–200° at 4°/min with a 4 min delay.

Isolation of amino acids. In separate experiments, seedlings of *Lotus tenuis* inoculated with *Rhizobium* isolates NZP2227 and NZP2238/1 were grown under controlled conditions for 2 months. Approximately 500 mg fresh nodules were harvested, macerated and extracted with hot 80% EtOH (3 × 20 ml) for 2 min and then filtered. The combined filtrates were taken to dryness, redissolved in 2 ml distilled water and clarified by centrifugation at 4000 rpm for 3 min. The supernatant was hydrolysed for 12 hr at 100° with 2 ml 6 N HCl, and then taken to dryness. The residue was dissolved in 5 ml 0.1 N HCl and placed on an ion exchange column of Amberbite IR-120 [H^+]. After washing with H_2O, the amino acid fraction was displaced from the column with 6 N NH_4OH.

Derivatization of amino acids. The basic fraction was taken to dryness and derivatized according to the procedure of ref. [3] giving a mixture of *N*-trifluoracetyl *n*-butyl esters and then analysed by GC and GC/MS.

2,3-*Dibromopentanoic acid.* 2-Pentenoic acid was prepared by the Doebner modification of the Knoevenagel reaction [9]. Bp 106–108°/20 mm (lit. [10] 108°/17 mm). ^{1}H NMR (CCl_4): δ 0.92 (3 H, *t*, J = 7 Hz, Me), 2.03 (2 H, *m*, CH_2), 5.57 (1 H, *d*, J_{trans} = 15 Hz, 2-CH), 6.86 (1 H, *dt*, J = 7, 15 Hz, 3-CH), 11.67 (1 H, *s*, COOH). To the unsaturated acid (10 g) in dry CCl_4 (15 ml) at 0° was added a soln of Br_2 (15.9 g) in CCl_4 (100 ml) and the mixture stirred overnight. Work-up gave 2,3-dibromopentanoic acid (11.1 g, 61%), mp (petrol) 52.5–53.0° (lit. [10] 57°). ^{1}H NMR (CCl_4): δ 0.95 (3 H. *t*, J = 7 Hz, Me), 2.0 (2 H, *m*, CH_2), 4.20 (2 H, *s*, 2-CH + 3-CH), 11.50 (1 H, *s*, COOH). MS *m/z* (rel. int.): 263 (1), 261 (2), 259 (1).

Methyl 2,4-dibromopentanoate. Bromine (13.3 ml) was added dropwise to a mixture of γ-valerolactone (23.2 g) and red P (2.68 g) at 0°. The temp. was raised to 80° and a further portion of Br_2 (13.3 ml) was added and then stirred for a further 3 hr during which 2,4-dibromo-pentanyl bromide [11] formed. The reaction was cooled and excess Br_2 removed with a stream of dry N_2. Following addition of MeOH (30 ml) over 30 min at 0° the reaction was partitioned with H_2O (50 ml). The aq. layer was extracted with Et_2O (3 × 20 ml). Work-up gave the title compound (32 g, 50%), bp 50°/8 mm. ^{1}H NMR (CCl_4): δ 1.60, 1.55, (3 H, 2*d*, J = 7 Hz, Me; two diastereiosomers), 2.17 (2 H, *m*, CH_2), 3.6 (3 H, *s*, COOMe), 4.20 (2 H, *m*, 2-CH + 3-CH). MS *m/z* (rel. int.): 276 (0.1), 274 (0.2), 272 (0.1).

Methyl 2,4-dibromo-3-methylbutanoate. A soln of 3-methyl-2-butenoic acid (118 g) and NBS (237 g) in CCl_4 (1.2.1) was refluxed and irradiated (100 W incandescent tungsten bulb) for 12 hr and then cooled, filtered and concd to 300 ml. Following addition of Fe powder (40 g) the reaction was refluxed for a further 12 hr. The solvent was removed and the residue distilled to give 3-methylbut-2-enoic 1,4-lactone [12] (50 g, 43%), bp 138–141°/25 mm. ^{1}H NMR (CCl_4): δ 2.03 (3 H, *s*, Me), 4.57 (2 H, *s*, CH_2), 5.62 (1 H, *s*, CH). The unsaturated lactone (20 g) in EtOH (50 ml) was stirred under H_2 over Adams catalyst until H_2 intake ceased (12 hr). The catalyst was filtered off and the soln worked-up giving 3-methylbutanoic 1,4-lactone (18.0 g, 90%), bp 132–142°/55 mm (lit. [13] 85–88°/10 mm). ^{1}H NMR (CCl_4): δ 1.13 (1 H, *m*, 3-CH), 1.25 (3 H, *d*, J = 6 Hz, Me), 2.43 (2 H, *m*, 2-CH_2), 4.15 (2 H, *m*, 4-CH_2). The lactone was treated with Br_2 and red P, then MeOH, as described above to give methyl 2,4-dibromo-3-methylbutanoate (70%), bp 140°/54 mm. ^{1}H NMR (CCl_4): δ 1.41, 1.37 (3 H, 2*d*, J = 7 Hz, Me: two diastereoisomers), 2.60 (1 H, *m*, 3-CH), 3.58, 3.88 (2 H, 2*d*, J = 6 Hz, CH_2: two diastereoisomers), 3.97 (3 H, *s*, COOMe), 4.33, 4.77 (1 H, 2*d*, J = 5, 8 Hz, 2-CH: two diastereoisomers). MS *m/z* (rel. int.): 276 (0.15), 274 (0.3), 272 (0.15).

2,3-*Dibromo-3-methylbutanoate.* Bromination of 3-methylbut-2-enoic acid gave 2,3-dibromo-3-methylbutanoate (60%), mp (petrol) 107–108° (lit. [10] 107–108°). ^{1}H NMR (CCl_4): δ 1.95 (3 H, *s*, Me), 2.05 (3 H, *s*, Me), 4.60 (1 H, *s*, CH), 11.45 (1 H, *s*, COOH). MS *m/z* (rel. int.): 261 (0.15), 259 (0.3), 257 (0.15).

TAB derivatives of diamino acids. The dibromo acid or ester (400 mg) was heated (100 ml) for 12 hr in a sealed tube with ammonia (5 g, 0.914, 8 ml). The mixture was evapd to dryness and the residue purified by TLC (unactivated silica; *n*-BuOH–HOAc–H_2O, 12:3:5) and derivatized as previously described [3]. MS and chromatographic data are given in Tables 1 and 2.

Acknowledgements—We thank Mr. R. M. Greenwood for provision of nodules and Dr. G. Wright for chemical ionization GC/MS spectra.

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