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THE CO-OCCURRENCE OF TWO DIASTEREOISOMERS OF 2, 3-DIAMINOBUTANOIC ACID IN ROOT NODULE HYDROLYSATES OF *LOTUS TENUIS*

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Key Word Index—*Lotus tenuis*; Leguminosae; *Rhizobium* species; 2, 3-diaminobutanoic acid; non-protein amino acid; synthesis.

Abstract—The non-protein amino acid 2, 3-diaminobutanoic acid has been identified in the root nodules of *Lotus tenuis* inoculated with *Rhizobium* strain NZP2213. The co-occurrence of this compound in two diastereoisomeric forms was established by comparative GC retention time measurements on a Chirasil-Val capillary column.

INTRODUCTION

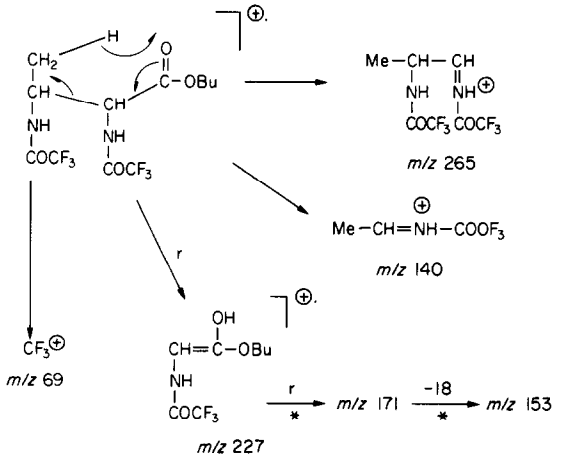
Two surveys [1, 2] of free and bound amino acids in the root nodules of *Lotus* species inoculated with various *Rhizobium* strains have established the occurrence of ninhydrin-positive compounds having chromatographic properties different from the 'protein' amino acids. A recent reinvestigation [3] of the amino acids in the root nodules of *L. tenuis* inoculated with *Rhizobium* strains NZP2227 and NZP2238/1 using GC and GC/MS led to the characterization of the novel amino acid 2, 4-diamino-3-methylbutanoic acid.

Our continued examination of another *Rhizobium* strain on the same host has resulted in the identification of a further bound non-protein amino acid. In this paper we report on the structural elucidation, configuration and chemical synthesis of 2, 3-diaminobutanoic acid (2, 3-DAB) found in the root nodules of *L. tenuis* inoculated with *Rhizobium* strain NZP2213.

RESULTS

The amino acids from a partially purified *L. tenuis* root nodule hydrolysate were resolved on a mixed phase OV-17/OV-210 GC column as their *N*-trifluoracetyl amino acid *n*-butyl (TAB) esters [4]. A comparison of gas chromatograms recorded for the root nodule sample and for a mixture of amino acid standards revealed the presence of two additional and closely eluting peaks at R_t 18.1 and 18.5 min. Neither component was evident in the gas chromatogram of the unhydrolysed root nodule extract. The resolution electron impact (EI) mass spectra taken at the apex of each GC peak gave identical fragmentation patterns with major ions at *m/z* 265, 227, 171, 153, 140, 69, 51 and 41. Under these conditions no molecular ion was detected; however, using GC/CIMS (methane) both peaks gave an intense protonated molecular ion $(M+H)^+$ at *m/z* 367 with associated adduct ions at *m/z* 395 $(M+C_2H_5)^+$ and *m/z* 407 $(M+C_3H_5)^+$.

Interpretation of the EI spectrum was made by comparison with other available mass spectral data [5, 6] and suggested that the unknown was 2, 3-DAB. The base peak at *m/z* 140 corresponded to the TFA-minimum ion (see Scheme 1) resulting from an α-cleavage to the β-amide nitrogen. The alternative α-cleavage to the α-amide nitrogen with concomitant loss of the butoxycarbonyl radical gave rise to the ion at *m/z* 265. The odd-electron ion at *m/z* 227 due to a McLafferty rearrangement [7] (β-cleavage) gave rise to *m/z* 171 probably by a second rearrangement to the enolic oxygen as evidenced by a metastable ion at *m/z* 128.8. A further metastable ion at *m/z* 136.8 supported the proposal that *m/z* 153 was the daughter ion of *m/z* 171.



Scheme 1. Proposed mass spectral fragmentation pathway for 2, 3-DAB (TAB).

Table 1. Chiral separation of TAB amino acids on Chirasil–Val

Compound	r	α
(2R, 3S)-threonine	7.92	1.05
(2S, 3R)-threonine	8.34	
(2R, 3R)-threonine	11.07	1.05
(2S, 3S)-threonine	11.59	
(2R, 3S)-2, 3–DAB	20.79	1.10
(2S, 3R)-2, 3–DAB	21.02	
(2R, 3R)-2, 3–DAB	23.48	1.02
(2S, 3S)-2, 3–DAB	23.95	
Root nodule extract	20.82	
	23.97	

r—Corrected retention time (min).
α—Resolution factor = ratio of the corrected retention time of the enantiomer eluting last over that of the enantiomer elutin first.

These data were consistent with the unknown being 2, 3–DAB and that this compound was present in the root nodule in at least two diastereoisomeric forms. The correctness of this structural identification was confirmed by a chromatographic and mass spectral comparison with authentic 2, 3–DAB synthesized from 2, 3–dibromobutanoic acid [8].

Stereochemical configurations of naturally occurring 2, 3–DAB were determined by a comparison of GC retention time data for each of the four enantiomers in the synthetically prepared racemate with that found in the root nodule hydrolysate on a WCOT glass capillary column coated with the chiral polysiloxane phase, Chirasil–Val [9, 10].

To establish the elution order for each of the four enantiomers attempts were made to synthesize (2R, 3S)–2, 3-diaminobutanoic acid [11] for comparative purposes; however, in our hands this was unsuccessful. As an alternative we turned to the TC elution data for the four enantiomeric forms of threonine. Under the GC conditions described baseline separation of each diastereomic pair was achieved with resolution factors similar to that reported previously [9]. While the data for 2, 3–DAB revealed resolution factors lower than that found for threonine due to less chiral recognition on Chirasil–Val, baseline separation of diastereomeric pairs was possible.

These observations and the fact that isoleucine also shows the same elution order on a number of chiral phases [9, 12, 13] suggested that the enantiomers of 2, 3–DAB had an elution sequence of 2R, 3S < 2S, 3R < 2R, 3R < 2S, 3S on Chirasil–Val.

GC analysis of the derivatized hydrolysate clearly showed that the root nodules of *Lotus tenuis* inoculated with *Rhizobium* strain NZP2213 contained two diastereoisomeric forms of 2, 3–DAB, which have been identified as (2R, 3S)– and (2S, 3S)–2, 3-diaminobutanoic acid.

DISCUSSION

The natural occurrence of the non-protein amino acid 2, 3–DAB has previously been reported in the amphomycin group of peptide antibiotics. A detailed amino acid analysis [14–16] of aspartocin revealed that 2, 3–DAB was present as a mixture of (2R, 3S)– and (2R, 3R)-stereoisomers. Bodansky *et al.* have shown the co-occurrence of the same two isomers in amphomycin [17, 18]. In the case of glumamycin [19] 2, 3–DAB has been reported to be present as its (2R, 3R)-isomer while in laspartomycin the same amino acid (unknown stereochemistry) has been identified by Naganawa *et al.* [20]. Two further reports on this group of antibiotics suggest that 2, 3–DAB may also be present in crystallomycin [21] and Tsushimycin [22].

To our knowledge this is the first report suggesting the occurrence of (2S, 3S)-2, 3–DAB and the co-occurrence of (2R, 3S)- and (2S, 3S)-2, 3–DAB in nature.

EXPERIMENTAL

Materials. Samples of (2S, 3R)-, (2R, 3R)-, (2S, 3R)- and (2R, 3S)-threonine were purchased from Sigma.

Gas chromatography. A Hewlett–Packard Model 5840A microprocessor controlled gas chromatograph equipped with a flame ionization detector was used during preliminary examination of derivatized root nodule extracts. Separations were carried out on a 2.5 × 4 mm i.d. glass column packed with 2% OV–17/% OV–210 on Gas Chrom Q. The best column temperature program for analysis was from 90 to 230° at 4°/min and held at the final temp. for 15 min. Injector and detector temperatures were held at 250 and 270°, respectively. N_2 carrier gas flow rate was 40 ml/min.

Separation of enantiomers was carried out on a 25 × 0.3 mm open tubular glass capillary column coated with Chirasil–Val (Applied Science Laboratories, State College, PA) programmed at 90–200° at 4°/min. N_2 flow rate was 2 ml/min. Injector and detector temps. were identical to those described above.

GC/MS. Low-resolution GC/EIMS determinations were performed on a double beam AEI MS30 mass spectrometer interfaced via an all-glass SGE jet separator to a Pye 104 gas chromatograph. Gas chromatographic conditions were identical to those described above except He was used as carrier gas. GC/CIMS (methane) were recorded on a Hewlett–Packard Model 5982A GC/MS.

Isolation of amino acids. The following procedure is primarily that of ref. [4] with some modification to permit extraction of root nodules. *Lotus tenuis* seedlings were inoculated with *Rhizobium* strain NZP2213 and grown under controlled conditions for 3 months. *Ca* 500 mg fresh nodules were harvested, macerated and extracted with 80% EtOH (3 × 20 ml) for 2 min and then filtered through preheated Whatman No. 1 filter paper. The combined filtrates were taken to dryness, redissolved in 2 ml distilled water and clarified by centrifugation at 4000 rpm for 3 min. The resulting supernatant was hydrolysed for 12 hr at 100° with 3 ml 6 N HCl, and then taken to dryness. The residue was redissolved in 5 ml 0.1 N HCl and quantitatively transferred to an Amberlite IR–120 [H^+] ion exchange column. After washing with H_2O, the amino acid fraction was displaced from the column with 6 N NH_4OH.

Derivatization of amino acid fraction. The basic fraction was taken to dryness on a rotary evaporator and derivatized according to the procedure of ref. [4], giving a mixture of *N*-trifluoracetyl *n*-butyl esters which was analysed by GC and GC/MS.

2, 3-*Diaminobutanoic acid.* This compound was synthesized from 2, 3-dibromobutanoic acid under the con-

ditions described by Neuberg [8]. MS (TAB derivative) *m/z* (rel. int.) 265(5), 227(18), 171(23), 153(27), 152(8), 140(100), 69(13), 57(30), 56(5), 45(7), 41(18).

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