Phytochemistry, 1966, Vol. 5, pp. 881 to 886. Pergamon Press Ltd. Printed in England

CHARACTERIZATION OF δ-ACETYL-L-ORNITHINE ISOLATED FROM *ONOBRYCHIS VICIIFOLIA* SCOP.

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(*Received* 19 *January* 1966)

Abstract—Material isolated from *Onobrychis viciifolia* Scop. (sainfoin) was fully characterized as δ-acetyl-L-ornithine. Previous reports regarding this compound did not adequately characterize either the position of acetylation or the optical isomer of ornithine involved. Procedures for discriminating between the chromatographically-similar δ-acetylornithine, α-acetylornithine and γ-acetamido-α-aminobutyric acid (γ-acetyldiaminobutyric acid) are described. A survey of leguminous seed suggested (*a*) that acetylornithine was not confined to a particular taxonomic group and (*b*) the occurrence of acetyldiaminobutyric acid in the Leguminosae.

INTRODUCTION

DURING a paper chromatographic survey of leguminous seeds, *Onobrychis viciifolia* Scop. (sainfoin) was found to contain high concentrations of material later identified as δ-acetyl-L-ornithine. This compound was isolated first from *Corydalis ochotensis* by Manske[1] and subsequently reported in other members of the Fumariaceae[2, 3] and closely related Papaveraceae,[2] as well as in species of *Asplenium*,[3] members of the grass tribe Festuceae, and in *Brachypodium sylvaticum*.[4]

In the original report,[1] δ-acetyl substitution was inferred from the sweet taste and positive ninhydrin reaction of the material! Yet all subsequent characterizations have rested solely upon an identity with material isolated originally by Manske. The need for more rigorous identification is also stressed by the fact that α-acetylornithine, which *is* ninhydrin positive, has been reported in extracts of *Escherichia coli* and closely related species.[5] In addition, Liss[6] and Schütte and Schütz[7] have confirmed that γ-acetyldiaminobutyric acid is present in the latex of *Euphorbia pulcherrima* and have shown that the compound is inseparable from δ-acetylornithine by paper chromatography using seven solvents.

RESULTS AND DISCUSSION

Two-dimensional paper chromatography of the amino acid fraction from sainfoin seeds showed the presence of an acid-labile compound, with an orthodox ninhydrin chromophore, running slightly slower than proline in both solvents (Spot 1, Fig. 1). Fractionation of a 75% (v/v) ethanol extract of ground sainfoin seed (25 kg) on cation exchange resin columns

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[1] R. H. F. MANSKE, *Can. J. Res.* **15B**, 84 (1937).
[2] G. REUTER, *Flora* **145**, 326 (1957).
[3] A. I. VIRTANEN and P. LINKO, *Acta Chem. Scand.* **9**, 531 (1955).
[4] L. FOWDEN, *Nature* **182**, 406 (1958).
[5] H. J. VOGEL and D. M. BONNER, *J. Biol. Chem.* **218**, 97 (1956).
[6] I. LISS, *Phytochem.* **1**, 87 (1962).
[7] H. R. SCHÜTTE and W. SCHÜTZ, *Ann. Chem. Liebigs.* **665**, 203 (1963).

gave a pure sample of this compound. Hydrolysis with 6 N HCl produced equimolar quantities of L-ornithine and acetic acid. Chromatography, electrophoresis and the use of a specific spray reagent (2% vanillin in propan-1-ol followed by 1% ethanolic KOH[8]) allowed discrimination between ornithine and α,γ-diaminobutyric acid.

Paper chromatography and electrophoresis were used to separate δ-acetylornithine, α-acetylornithine and γ-acetyldiaminobutyric acid. Material isolated from *Corydalis ochotensis* or sainfoin was inseparable from synthetic δ-acetylornithine (prepared by the method of Neuberger and Sanger[9]) in all solvents tested (Table 1). The structure of the isolated material was confirmed as δ- and not α-acetylornithine by comparison of the i.r.

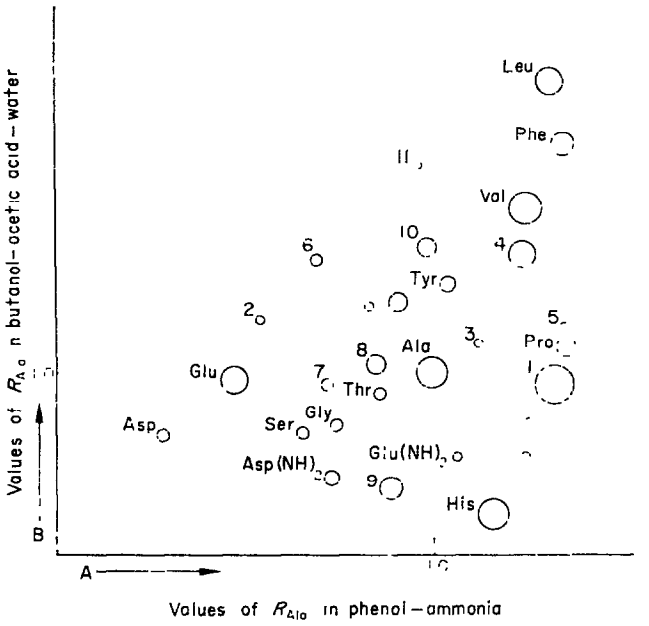

FIG. 1. DIAGRAMMATIC REPRESENTATION OF A TWO-DIMENSIONAL PAPER CHROMATOGRAM OF A SEED EXTRACT OF *Onobrychis viciifolia* Scop.

Solvent A: 75% (w/w) phenol in the presence of ammonia; solvent B: butan-1-ol–acetic acid–water (90:10:29, by vol.). The non-protein amino acids identified are: (1) δ-acetylornithine, (2) α-aminoadipic acid, (3) β-alanine, (4) γ-aminobutyric acid, (5) ethanolamine. γ-Glutamyl peptides present are those of: (6) tyrosine, (7) β-alanine, (8) β-aminoisobutyric acid, (9) *S*-methylcysteine sulphoxide, (10) phenylalanine, and (11) leucine. Unlabelled spots represent unidentified non-protein amino acids.

spectra of natural and synthetic products and by the nature of the ninhydrin reaction following copper complexion.[10]

Semi-quantitative chromatography indicated that dormant seeds of sainfoin contained about 500 μg δ-acetylornithine/g dry wt. No appreciable change in the concentration of this compound was noted after 7 days germination. The stems, leaves, roots and open flowers from mature plants were found also to contain between 470 and 500 μg δ-acetylornithine g dry wt. Free ornithine was not detected in the seeds by electrophoresis at pH 6·5 or by use of the vanillin–KOH reagent, although the equivalent of about 1 g ornithine/25 kg sainfoin

[8] G. CURZON and L. GILTROW, *Nature* **172**, 356 (1953).
[9] A. NEUBERGER and F. SANGER, *Biochem. J.* **37**, 515 (1943).
[10] P. O. LARSEN and A. KJAER, *Biochim. Biophys. Acta* **38**, 148 (1960).

seed was detected after fractionation on Dowex 50. In part, this free ornithine may have arisen by breakdown of a proportion of the δ-acetylornithine during prolonged exposure to H^+ released by cation exchange.

A number of leguminous seeds were tested for the presence of acetylornithine (Table 2): chromatograms were prepared from extracts representing 0·4 g each seed. Chromatographic position and the production of ornithine by acid hydrolysis (checked by the vanillin–KOH

TABLE 1. THE R_{ala} VALUES DETERMINED FOR DIAMINO ACIDS AND THEIR ACETYL DERIVATIVES IN FIVE SOLVENT SYSTEMS*

Amino acids	Solvents				
	A	B	C	D	E
Ornithine	1·25	0·20	0·16	0·64	0·87
α,γ-Diaminobutyric acid	+	0·25	0·61	0·57	0·98
Lysine	1·34	0·25	0·16	0·75	0·89
δ-Acetylornithine	1·28	1·04	1·05	1·02	1·03
α-Acetylornithine	–	0·90	0·66	–	1·02
γ-Acetyldiaminobutyric acid	1·27	0·98	1·07	0·94	1·03

* See Experimental section for compositions of solvents.
+, Compound streaked; –, R_{ala} not determined.

TABLE 2. THE GROUPING OF LEGUME SPECIES ACCORDING TO THEIR CONTENT OF AN ACID-LABILE COMPOUND RESEMBLING δ-ACETYLORNITHINE

Group A	Group B	Group C
Astragalus alopecuroides L.	*Anthyllis vulneraria* L.	*Arachis hypogaea* L.
Glycyrrhiza foetida Desf.	*Colutea arborescens* L.	*Astragalus baeticus* L.
Lespedeza Buergeri Mig.	*Desmodium canadense* DC.	*A. chinensis* L.
Onobrychis caput-galli Lam.	*Hedysarum coronarium* L.	*A. cicer* L.
O. viciifolia Scop.	*H. flavescens* Regel & Schmalh.	*A. frigidus* A. Gray
Vicia sativa L.	*Laburnum anagyroides* Medic.	*A. glycyphyllos* L.
	Lathyrus pratensis L.	*A. sulcatus* L.
	Lotus corniculatus L.	*Coronilla scorpiodes* Koch
	Medicago lupulina L.	*Hedysarum esculentum* Ledeb.
	Melilotus altissima Thuill.	*Onobrychis squarrosa* Viv.
	Phaseolus aureus Roxb.	*Robinia pseudoacacia* L.
	P. multiflorus Lam.	*Scorpiurus sulcatus* L.
	P. vulgaris L.	*S. vermiculatus* L.

Group A contain acetylornithine; group B, compounds chromatographically resembling δ-acetylornithine; group C, no or undetectable quantities of acetylornithine. See text for details of criteria used.

reaction and by electrophoresis at pH 6·5) formed the criteria used to discriminate between acetylornithine and acetyldiaminobutyric acid. Ornithine itself was not detected in any of the species before acid hydrolysis. While copper complexion before spraying with ninhydrin[10] permits discrimination between α-acetylornithine (weakly ninhydrin positive) and δ-acetylornithine (ninhydrin negative), this distinction could not always be made due to the limited quantities of seed material available.

In Table 2, group A consists of species producing identifiable quantities of ornithine on

hydrolysis: these species are considered to contain acetylornithine exclusively. Group B consists of species shown to contain acid-labile material, chromatographically resembling δ-acetylornithine, but which yielded no detectable ornithine after acid hydrolysis. The failure to detect ornithine after hydrolysis in some species results from the low concentrations of the acetylated amino acid present initially and the relative insensitivity of the vanillin–KOH reagent (nearly 10 μg ornithine are necessary for adequate recognition). However, in other species γ-acetamido-α-aminobutyric acid may occur to the exclusion of acetylornithine. Van Etten and Miller[11] have detected α,γ-diaminobutyric acid in *Colutea arborescens* and *Medicago lupulina*, and our experiments have shown that acid hydrolysis of a seed extract of *Medicago lupulina* led to the production of α,γ-diaminobutyric acid, identified by electrophoresis at pH 6·5.

Group C consists of species which failed to show the presence of compounds chromatographically similar to acetylornithine. However, although in the case of *Arachis hypogaea*, no acetylornithine was detected, fractionation of an extract of defatted seed meal (45 kg) indicated the presence of about 250 mg of acetylornithine. This example illustrates the uncertainty that must surround many statements concerning the absence of minor constituents in seeds, and the failure to detect acetylornithine in species forming group C in part may be due to the insensitivity of the techniques employed.

Presumably the environmental conditions prevailing during development and maturation may markedly affect the levels of monoacetyl amino acids present in the seeds. For instance, one sample of *Astragalus alopecuroides* seed contained about 450 μg acetylornithine g dry wt., while in another sample the compound could not be detected. Similarly, our analyses indicated that *Medicago lupulina* seed contained about 550 μg γ-acetyldiaminobutyric acid g dry wt., but Dr. E. A. Bell (private communication) failed to detect this acetyl derivative in a different batch of seed.

EXPERIMENTAL

Chromatographic and Electrophoretic Methods

Descending paper chromatography was performed on Whatman No. 4 and No. 3MM filter paper. The following solvent systems were used for one-dimensional chromatography: A, 75% (w/w) phenol in the presence of ammonia vapour; B, butan-1-ol–acetic acid–water (90:10:29, by vol.); C, ethyl acetate–pyridine–water (2:1:2, by vol.); D, methanol–pyridine–10 N-HCl–water (32:4:1:7, by vol.); E, *tert*-butanol–ethyl methyl ketone–diethylamine–water (10:5:1:10, by vol.); F, *tert*-amyl alcohol–acetic acid–water (20:1:20, by vol.); and G, butan-1-ol saturated with 3 N-ammonia.

High voltage paper electrophoresis was performed on Whatman No. 3MM filter paper, using a Locarte Co. (London) apparatus. The following buffers were used: (1) pH 2·0, formic acid (61 ml), acetic acid (97 ml) and distilled water to 2 l.; (2) pH 3·45, acetic acid–pyridine–water (10:1:190, by vol.); (3) pH 6·5, acetic acid–pyridine–water (4:100:1900, by vol.).

Isolation of δ-Acetylornithine

δ-Acetylornithine was extracted from finely ground sainfoin seed (25 kg) by steeping in 75% (v/v) ethanol (50 l.) for 2 months and isolated by absorption on and elution from cation-exchange resin columns (ZeoKarb 215, 110 × 8·5 cm, followed by Dowex 50, 50 × 5 cm).

[11] C. H. VAN ETTEN and R. W. MILLER, *Econ. Botany* **17**, 107 (1963).

After two recrystallizations from 65% (v/v) ethanol, pure δ-acetylornithine (1·0 g) was obtained (Found: C, 48·4; H, 7·90; N, 16·0. $C_7H_{14}N_2O_3$ required: C, 48·3; H, 8·05; N, 16·1%). $[\alpha]_D^{20}$ +6·1° ($C=5$, in water), +20·1° ($C=5$, in 5 N HCl), m.p. 246° (decomp.).

Hydrolysis Products of Isolated δ-Acetylornithine

The amino acid product of hydrolysis (5 N-hydrochloric acid overnight at 100°) was identified as ornithine by chromatography in solvents C, D and E (Table 1) and by electrophoresis at pH 3·45 and 6·5. Chromatograms were sprayed with 2% vanillin in propan-1-ol (heated for 10 min at 100°) followed by ethanolic 1% KOH (heated for 10 min at 100°) when ornithine gave a salmon pink chromophore after 30 sec heating.[8] Under similar conditions, yellow chromophores were produced from lysine and α,γ-diaminobutyric acid. Optical rotation measurements upon the hydrolysate gave $[\alpha]_D^{20}$ ±22·0° +0·6° ($C=5$, in 5 N-hydrochloric acid), authentic L-ornithine has $[\alpha]_D^{24-26}$ +28·4°. Natural δ-acetylornithine is then the L-isomer.

Acetic acid present in the hydrolysate was distilled into 2 N-ammonia. Chromatography in solvent G, using ammonium acetate as a standard, indicated the presence of acetate ion (spots located with 0·04% bromophenol blue in ethanol adjusted to pH 6·7). Acetic acid was confirmed by treating a similar distillate with *S*-benzylthiouronium chloride, following the method of Gmelin *et al.*[12] The identity of the *S*-benzylthiouronium acetate formed was confirmed by i.r. comparison with authentic derivatives of acetate, formate and propionate synthesized by the same method.

After hydrolysis of natural acetylornithine by treatment with glacial acetic acid (17 hr at 100°), ornithine production was assayed quantitatively by the method of Chinard[13] (reaction with ninhydrin at pH 1·0). In this way 1 mole of acetylornithine was shown to yield 1 mole of ornithine on hydrolysis.

Determination of the Position of Acetyl Substitution

Copper can complex with the α-amino, but not with other amino groups, present in diamino acids. When isolated acetylornithine was run on chromatograms developed in solvent B and these were sprayed, first with an ethanolic solution of 0·25% copper nitrate and 2% sodium acetate (heating for 2 min at 70°) and then with 1% ninhydrin in ethanol,[10] no coloured spots were revealed. Synthetic δ-acetylornithine behaved similarly, whereas the weak chromophore produced from α-acetylornithine by direct ninhydrin treatment was unchanged in intensity after copper complexing. The γ-acetyldiaminobutyric acid isolated from *Euphorbia* by Liss[6] also failed to react with ninhydrin after copper complexion, indicating γ-substitution.

Synthesis of δ-Acetylornithine

Using the principle of copper blocking of the α-amino group, δ-acetyl-L-ornithine was synthesized, on a semi-micro scale, from L-ornithine mono-HCl (complexed by copper carbonate) and acetic anhydride, by the method of Neuberger and Sanger.[9] δ-Acetyl-DL-[2-^{14}C]ornithine, used in later metabolic experiments,[14] was synthesized similarly, and after chromatography in solvent B, unchanged DL-[2-^{14}C]ornithine, δ-acetyl-DL-[2-^{14}C]ornithine and α,δ-diacetyl-DL-[2-^{14}C]ornithine (R_{ala} 3·0) were recovered in the proportions 1·0:5·6:3·4.

[12] R. Gmelin, A. Kjaer and P. O. Larsen, *Phytochem.* **1**, 233 (1962).
[13] F. P. Chinard, *J. Biol. Chem.* **199**, 91 (1952).
[14] D. H. Brown and L. Fowden, *Phytochem.* **5**, 887 (1966).

A trace of α-acetylornithine was also detected by chromatography in solvents B and F, but its concentration was not estimated.

Separation of Mono-acetyl Dibasic Amino Acids

Synthetic δ-acetylornithine and material isolated from *Corydalis* and sainfoin were inseparable in all solvents tested. Discrimination between α- and δ-acetylornithine was achieved by chromatography in solvents B and C (Table 1) and by electrophoresis at pH 2·0. Close correspondence was found between i.r. spectra of paraffin mulls of synthetic δ-acetyl-L-ornithine and the material isolated from sainfoin. Both spectra differed appreciably from the i.r. spectrum of α-acetyl-L-ornithine. γ-Acetyldiaminobutyric acid and δ-acetylornithine were separated by electrophoresis at pH 2·0 and partially by chromatography in solvents B and D. A complete separation of the two compounds was achieved by chromatography in solvent F for 10 days at 4°, when γ-acetyldiaminobutyric acid travelled 18·5 cm and δ-acetylornithine travelled 16·3 cm.

Acknowledgements—D. H. B. is indebted to the Department of Scientific and Industrial Research for a maintenance grant. We also thank the Central Research Fund of the University of London for an apparatus grant.