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ISOLATION OF 4-METHYLENE-DL-PROLINE FROM *ERIOBOTRYA JAPONICA*

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Abstract—An amino acid has been isolated from the seeds of the angiosperm tree, *Eriobotrya japonica* (loquat) and characterised as 4-methylene-DL-proline.

INTRODUCTION

APPROXIMATELY 20 free non-protein imino acids have been isolated from higher plant tissues. Some, such as pipecolic acid[1] and baikiain[2] contain a six-membered ring system, while azetidine-2-carboxylic acid[3] has a four-membered ring. Most, however, are derivatives of proline. The five-membered proline ring may be substituted at the 3 position, as in 3-hydroxyproline[4] or, more commonly, in the 4 position, as in kainic acid,[5] 4-hydroxyproline,[6] 4-methylproline[7] and 4-hydroxymethylproline.[8] This series of 4-substituted proline derivatives has now been augmented by the discovery of 4-methyleneproline, described in this paper.[9]

RESULTS

4-Methyleneproline runs close to γ-aminobutyric acid on two dimensional paper chromatograms.[9] Other chromatographic data are given in Table 1. The new compound shows some unusual colour reactions on paper. It bleaches isatin, like azetidine-2-carboxylic acid and 3,4-methanoproline. It gives no colour reaction with *p*-dimethylaminobenzaldehyde directly, but an intense red colouration is produced if this reagent is applied after isatin. This test, once considered to be specific for 4-hydroxyproline,[10] is also given by dehydroproline.

Isolation of Methyleneproline

The amino and imino acids extracted from sun-dried loquat seeds (12·9 kg) were fractionated by cation exchange. Methyleneproline (13·35 g) was obtained from the fractions

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[10] J. B. JEPSON and I. SMITH, *Nature, Lond.* **172**, 1100 (1953).

TABLE 1. PROPERTIES OF 4-METHYLENEPROLINE

Reagent	Colour reaction given by 4-methyleneproline	Minimum quantity (μg) detectable on paper chromatograms run in solvent D
Ninhydrin	Orange-brown (at 60°) Lemon-yellow (at 100°)	5–10
Isatin	Bleaches reagent (at 105°)	20
Isatin followed by Ehrlich's reagent	Pink changing to purple (20°)	1

4-Methyleneproline runs at R_f 0·86 ± 0·04 in solvent L and at $R_{Alanine}$ 1·52 ± 0·06 in solvent D.

partly by recrystallization and partly by countercurrent separation. Samples (200 μg), chromatographed on paper in solvents D and L*, showed that amino acids were absent, the only detectable impurities being 0·5% proline and 0·2% methylproline. Elementary analysis (see next section) confirmed that the product was pure. Allowing for a 20% loss during isolation, methyleneproline represented about 40% (w/w) of the amino and imino acid fraction of loquat seeds, being present at a concentration of 1·2 g/kg.

Characterization of the Isolate as 4-Methyleneproline

The isolate gave a blue colouration with alkaline nitroprusside in the presence of acetaldehyde and thus contained an imino group. This test[11] was used on paper chromatograms as recommended by Schenk and Schütte.[12] The isolate was readily reduced, suggesting the presence of one or more double bonds. The chief product, identified as *cis*-4-methylproline, was inseparable from this compound by paper chromatography in all the solvents tested (A, C, D, L and M) and reacted with ninhydrin, isatin and Ehrlich's reagent in the same way. Only 10–20% of *trans*-4-methylproline was formed as was demonstrated by paper chromatography in solvent C and also by high voltage paper electrophoresis,[13] kindly performed by Professor C. J. O. R. Morris. Hence the isolate, presumed to be an unsaturated derivative of methylproline, was reduced stereospecifically; the reason for this has been discussed by Bethell and Kenner.[14]

Vigorous catalytic hydrogenation of the isolate gave traces of another product, which ran with authentic leucine on paper chromatograms in solvents A, C, D and M, and was clearly separable from isoleucine and norleucine in the first two solvents. Leucine also resulted when 4-methylproline was hydrogenated under similar conditions, so it must arise by reductive cleavage of the ring between the N and C-5 atoms. This confirmed that the isolate was a 4-substituted proline. Reductive ring cleavage of 3-methylene- and 5-methyleneprolines would have given isoleucine and norleucine respectively.

Elementary analysis indicated one double bond. (Found: C, 56·6%; H, 7·1%; N, 11·2%; Calc. for $C_6H_9NO_2$: C, 56·7%; H, 7·1%; N, 11·0%.) The position of this bond was determined by oxidizing with acidic permanganate. The products included aspartic acid and

* Details of chromatographic solvents are given in the Experimental.

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glycine, inseparable from the authentic compounds on paper in solvents D, L and M. The presence of a fragment as large as aspartic acid showed that, in the original imino acid, the double bond, the initial point of attack, must have been either between C-4 and the exocyclic carbon atom or between C-4 and C-5.

The former alternative was favoured by the identification of 4-oxoproline amongst the oxidation products. This substance gave a yellow-brown colour with ninhydrin, a brown colour with isatin, and was relatively unstable, being found on chromatograms run in solvent D but not on those run in solvents L or M. It was shown to be 4-oxoproline, a compound unstable at alkaline pHs,[15] by reducing it to *cis*-4-hydroxyproline and proline, identified by chromatographic comparison with standards on paper in solvents D and L. *Cis*-4-hydroxyproline is the normal reduction product of 4-oxoproline;[16] this reaction is stereospecific like the reduction of 4-methyleneproline. Under the conditions used, most of the hydroxyproline was further reduced to proline.

Thus the isolate had an exocyclic double bond and must be 4-methyleneproline. This structure was supported by the IR spectrum, interpreted by Dr. L. J. Bellamy, which showed peaks at 6·1 μ and 11·2 μ corresponding to the exocyclic methylene group. The NMR spectrum of the new imino acid, kindly measured and interpreted by Dr. R. J. Abraham, agreed with the proposed structure.

The structure was finally confirmed by synthesis. Bethell, Kenner and Shepperd[17] synthesized benzyloxycarbonyl-4-methyleneproline and showed that its IR spectrum was identical to that of the benzyloxycarbonyl derivative of the isolate when both preparations were examined in chloroform in the form of their dicyclohexylammonium salts. Burgstahler, Trollope and Aiman[18] synthesized free 4-methyleneproline and demonstrated that its NMR and IR spectra were identical to those of the isolate. The synthetic and natural preparations were inseparable by paper chromatography, had identical crystallisation characteristics, gave undepressed mixed m.ps and gave the same product on catalytic hydrogenation.

4-Methyleneproline is stable to boiling aqueous formic and acetic acids, but is decomposed by 6 N HCl at 100° within 20 hr. The product was probably 4-chloromethylproline, formed by the addition of HCl across the double bond.

Configuration of the 4-Methyleneproline Isolated from Loquat Seeds

The presence of 4-methylene-D-proline was shown with D-amino acid oxidase. The isolate (2·55 and 5·1 mg) supported the uptake of 60 μl O_2 in 75 min and 90 μl in 45 min, corresponding to 27% and 20% oxidation respectively.

The isolate had no detectable optical rotation; $[\alpha]_D^{20} + 0{\cdot}5 \pm 0{\cdot}6°$ (c 3·1 in water); $[\alpha]_D^{21} + 0{\cdot}1 \pm 0{\cdot}6°$ (c 3·9 in 5 N-hydrochloric acid). This suggested that the preparation was DL-.

The 4-methylproline formed by reducing the isolate in 5% acetic acid also had no detectable optical rotation; $[\alpha]_D < 1{\cdot}9 \pm 1{\cdot}9°$ (c 2 in water). Under these conditions, the $[\alpha]_D$ values of *cis* and *trans*-4-methyl-L-proline are $-85°$ and $-57°$ respectively.[19] Thus the reduction product of the isolate and therefore the isolate itself was racemic. The same

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[19] J. S. DALBY, G. W. KENNER and R. C. SHEPPARD, *J. Chem. Soc.* 4387 (1962).

experiment was independently performed by Bethell, Kenner and Shepperd,[17] who calculated that less than 1% of the original preparation could have been optically active.

The racemic nature of the imino acid was confirmed by comparison with synthetic preparations. The 4-methyleneproline synthesised by Burgstahler *et al.*,[18] which was identical to the isolate in every way, was racemic. The benzyloxycarbonyl-4-methyleneproline synthesised by Bethell *et al.*[17] had the L configuration. It was not quite identical with the benzyloxycarbonyl derivative of the isolate, since the two preparations, examined in the form of their dicyclohexylammonium salts, had different m.ps and different IR spectra in the solid state (nujol mulls). The synthetic material had $[\alpha]_D^{19}$ −5·5° (c 2 in $CHCl_3$) while the derivative of the isolate was optically inactive under the same conditions.

Distribution of 4-Methyleneproline in Plant Tissues

4-Methyleneproline was detected in all tissues of *Eriobotrya japonica* tested, being most prominent in the seeds. Estimated concentrations in mg/kg fr. wt. were: seeds, 100; flesh of fruit, 6; mature green leaves, 4 (detected in only 1 of 2 samples). Methyleneproline was also detected in the seeds of the related rosaceous plant, *Raphiolepis indica*.

DISCUSSION

4-Methyleneproline completes the series of naturally-occurring proline derivatives in which the ring is substituted with a single C atom at position 4 (see Introduction). The unsaturation of the side chain, an uncommon feature, is also found in kainic[5] and domoic acids,[20] which have been isolated from brown algae.

4-Methyleneproline, like 4-methylproline and 4-hydroxymethylproline, is characteristic of the Rosaceae and may be a useful taxonomic marker. Hutchinson[21] considers that *Eriobotrya* and *Raphiolepis* are closely related, being separated only by the genera *Chaenomeles*, *Pseudocydonia*, *Docynia* and *Cydonia*. These relationships may be clarified by further studies on the distribution of the imino acid.

The imino acid may function as a soluble N reserve but, even in fresh loquat seeds, it only accounts for approximately 0·5% of the total N present. Alternatively it may act or have acted as an antimetabolite towards potential parasites attacking the seed and/or plant. Selection pressure may have favoured 4-methylene-D-proline because it was a more effective inhibitor than the L-isomer. Several naturally-occurring imino acids are antimetabolites. Azetidine-2-carboxylic acid inhibits growth in *E. coli* and several higher plants,[23,24] while kainic and domoic acids are anthelminthics.[5,20] 4-Methylene-DL-proline, itself, inhibits the growth of *Phaseolus aureus* radicles by 30% at a concentration of 1 g/kg dry wt. tissue,[24] its approximate concentration in dry loquat seeds. Other tissues could be more sensitive to the compound. Azetidine-2-carboxylic acid acts as an antimetabolite by being incorporated in place of proline to give biologically inactive protein.[24] 4-Methyleneproline is probably not incorporated into protein,[25] so it may reduce growth by inhibiting and/or repressing one or more of the enzymes involved in proline biosynthesis.

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EXPERIMENTAL

Paper chromatography. Chromatograms (descending) were run on Whatman 3MM paper, prewashed in 5% HOAc if used for preparative purposes. The solvents were: A, Upper phase *t*-AmOH–HOAc–H_2O (20:1:20, by vol.); B, *t*-AmOH–MeCOEt–H_2O (6:2:2, by vol.); C, Upper phase *n*-BuOH–H_2O (1:1, v/v) in presence of 3% (w/v) ammonia (Separation of *cis* and *trans*-4-methylproline required 120 hr at 2°); D, *n*-BuOH–HOAc–H_2O (90:10:29, by vol.); E, *n*-BuOH–Me_2CO–0·88 NH_4OH–H_2O (8:1:1:6, by vol.); F, *n*-BuOH–Me COEt–0·88 NH_4OH–H_2O (4:4:1:1, by vol.); G, Upper phase *n*-BuOH–0·88 NH_4OH–H_2O (2:1:1, by vol.); H, *i*-BuOH–*t*-BuOH–MeCOEt–H_2O (4:4:8:5, by vol.), mixed with 0·5% (v/v) $HNEt_2$; I, *t*-BuOH–MeCOEt–$NHEt_2$–H_2O (10:5:1:10 by vol.); J, *t*-BuOH–MeOH–H_2O (4:5:1, by vol.); K, Me_2CO–MeCOEt–H_2O (1:4:1, by vol.) in presence of 3% (w/v) ammonia; L, Phenol–H_2O (3:1, w/v); M, Upper phase of pyridine-EtOAc–H_2O (1:2:2, by vol.).

Two dimensional chromatograms were first developed in solvent L and then in solvent D. Amino acids were detected by dipping the chromatograms through 0·1% (w/v) ninhydrin in EtOH and heating at 70°. Imino acids were located by dipping the papers through 0·2% (w/v) isatin in acetone–HOAc (19:1, v/v) and heating at 105°. Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) was used as recommended by Jepson and Smith.[10] Crude plant extracts were not chromatographed directly; the amino acid fraction was first isolated by cation exchange.

Purification of amino and imino acids by cation exchange. A washed and activated column (10 cm long × 1 cm dia.) of either Dowex 50W-X8, 20–50 U.S. mesh or Zeo-Karb 225, 4% D.V.B., 14–52 mesh, was used to absorb cationic compounds. After washing the resin with 2 × 100 ml H_2O, amino and imino acids were eluted from it with 50 ml 2N-ammonia.

Isolation of 4-methyleneproline from loquat seeds. Powdered dry seed (12·9 kg) was extracted successively with 29·5 l. and 24 l. 70% (v/v) EtOH. The extract was passed through a 6·2 l. column of Zeo-Karb 215 (H^+ form) to absorb amino and imino acids, which were then eluted with 0·5 N ammonia.

The amino and imino acids were absorbed onto Dowex 50 × 4 (H^+ form, 100–200 mesh)[26] and fractionated by eluting them with 0·2 N ammonia. The eluate from the main column (54 cm long × 3·2 cm dia.) was passed directly through a smaller one (20 cm long × 1·2 cm dia.) to improve resolution.[27] The eluate was collected in 15 ml fractions, 156 of which contained amino acids. Pure methyleneproline (1·75 g) was recovered from fractions 62–101 by decolourizing them with charcoal and recrystallizing the solutes once from 75% (v/v) EtOH and once from 90% (v/v) EtOH. Glutamic acid was removed from fractions 22–61 by treatment with Dowex 1 (acetate form).

The remaining amino and imino acids from fractions 22–101 were separated by countercurrent distribution using *n*-BuOH–HOAc–H_2O (5:1:7, by vol.) which was esterified beforehand and used at 20 ± 1°. Crude methyleneproline was dissolved in the aqueous phase (20 ml) of tube 1 and the 200 tube apparatus was operated for 790 transfers. The contents of tubes 71–109 were pooled and decolourized with charcoal. Two such countercurrent separations gave 11·6 g pure methyleneproline from 19·8 g of the starting material. The solubility of 4-methylene-DL-proline in water exceeds 500 g/l. at 25°.

Catalytic hydrogenation of 4-methyleneproline and 4-methylproline. 4-Methyleneproline (0·4 mg) in 0·1 ml H_2O was reduced for 5 min at 20° in a stream of H_2 in the presence of 10 mg Adam's PtO_2 catalyst. A better yield of leucine (about 4%) was obtained by reducing the mixture for 30 min at 50° in the presence of 1 mg catalyst. 4-Methylproline (0·4 mg) in 0·1 ml H_2O was hydrogenated for 60 min at 50° in the presence of 1 mg catalyst to give a 1% yield of leucine.

Oxidation of 4-methyleneproline with acidic permanganate. Oxidation to aspartic acid and glycine. 4-Methyleneproline (1 mg) in 1·2% (w/v) H_2SO_4 (0·285 ml) was oxidized with 1·0% (w/v) $KMnO_4$ in 1·5% (w/v) H_2SO_4 (0·4 ml) at 80°, and excess acid was neutralized with 40 mg $BaCO_3$. When the pH became greater than 2·5, the supernatant was desalted by cation exchange. The resin column (5 cm long) was only washed with 3 × 5 ml H_2O, after it had absorbed the amino acids, to minimize loss of aspartic acid. 4-Methyleneproline (1 μmole) gave approximately 0·12 μmole aspartic acid and 0·1 μmole glycine, estimated by visual comparison with standards on paper chromatograms.

Oxidation to 4-oxoproline. Conditions were as before except that the 4-methyleneproline (1 mg) was oxidized with 0·3 ml acidic permanganate and, when the reaction mixture was neutralized, the pH was not allowed to rise above 4. After centrifugation, the supernatant was chromatographed directly and not desalted. The major product was 4-oxoproline, which ran at $R_{Alanine}$ 0·78 on paper in solvent D. 4-Oxoproline was identified by reducing the crude oxidation product of 0·4 mg methyleneproline, dissolved in 0·2 ml H_2O, with hydrogen for 30 min at 20° in the presence of 10 mg Adam's PtO_2 catalyst. The oxidation product of 1 μmole methyleneproline gave approximately 0·03 μmole *cis*-4-hydroxyproline and 0·14 μmole proline. *Cis*-4-hydroxyproline (1 μmole), reduced as above, gave 0·6 μmole proline.

D-*Amino acid oxidase assays.* 4-Methyleneproline was assayed with an enzyme from hog kidney, prepared

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as described by Greenstein and Winitz.[28] Oxygen uptake was measured by Warburg manometry, both the enzyme (2 ml) and the substrate (0·5 ml) being dissolved in 0·05 M potassium pyrophosphate buffer, pH 8·2.

All other assays were done in a special Barcroft manometer using hog kidney D-amino acid oxidase of activity 20 U/g (Koch-Light Laboratories). Both the enzyme (10 mg) and substrate were dissolved in a total of 0·3 ml 0·07 M sodium pyrophosphate buffer, pH 8·2. Oxygen uptake was followed for 3 hr at 25° and all rates given are averages for this period. A silicone oil (Hopkin and Williams, MS 200) was used as manometer fluid, since aqueous manometer fluids caused artifacts. The apparatus constant for oxygen was 0·403 and readings were taken to ± 0·01 cm.

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Key Word Index—Eriobotrya japonica; Rosaceae; 4-methylene-DL-proline.