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TRANS-4-HYDROXYMETHYL-D-PROLINE FROM *ERIOBOTRYA JAPONICA*

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Abstract—An amino acid from the seeds of *Eriobotrya japonica* (loquat) has been characterised as predominantly the D-isomer of *trans*-4-hydroxymethylproline. The *cis*-4-hydroxymethylproline present in the same tissue had the normal L configuration.

INTRODUCTION

4-HYDROXYMETHYLPROLINE (HMPro), characteristic of rosaceous species, was first isolated from apple peel by Hulme.[1] An identical preparation[2] was obtained from apple twigs by Urbach[3] and from perry by Burroughs.[4,5] The primary structure of the isolate was established by nuclear magnetic resonance,[5] mass spectrometry[6] and synthesis[7] but the configuration was initially uncertain, some evidence favouring the *cis*-formulation[5] and some the *trans*.[6] However, the stereospecific synthesis of Bethell *et al.*[8] suggested that the compound isolated was *cis*-4-hydroxymethyl-L-proline and this designation was conclusively confirmed by Untch and Gibbon.[9]

No further isolations of HMPro have been reported, so when this work began only one of the four possible stereoisomers was known as a natural product. This paper describes a solvent able to separate the *cis* and *trans* isomers by paper chromatography and its use to establish that loquat seeds contain not only the 'normal' *cis*-L-isomer but also smaller amounts of the *trans*-D- and *trans*-L-forms.

RESULTS

Isolation of cis- *and* trans-*HMPro from Loquat*

HMPro was first detected when the amino acids from 13 kg loquat seeds were fractionated on Dowex 50.[10] Fractions containing HMPro were pooled, and, after removal of glutamic acid, gave a residue containing approximately 10% HMPro. *Cis*- and *trans*-HMPro were purified from 17% of this crude material by paper chromatography, first in solvent F and then in solvent D (cf.[10]).

[1] A. C. HULME, *Nature, Lond.* **174,** 1055 (1954).
[2] A. C. HULME and F. C. STEWARD, *Nature, Lond.* **175,** 171 (1955).
[3] G. URBACH, *Nature, Lond.* **175,** 170 (1955).
[4] L. F. BURROUGHS, Private communication (1970).
[5] R. J. ABRAHAM, K. A. MCLAUCHLAN, S. DALBY, G. W. KENNER, R. C. SHEPPARD and L.F. BURROUGHS, *Nature, Lond.* **192,** 1150 (1961).
[6] K. BIEMANN, G. G. DEFFNER and F. C. STEWARD, *Nature, Lond.* **191,** 380 (1961).
[7] A. W. BURGSTAHLER and C. E. AIMAN, *Chem. & Ind.* 1430 (1962).
[8] M. BETHELL, D. B. BIGLEY and G. W. KENNER, *Chem. & Ind.* 653 (1963).
[9] K. G. UNTCH and G. A. GIBBON, *Tetrahedron Letters* 3259 (1964).
[10] D. O. GRAY and L. FOWDEN, *Phytochem.* **11,** 745 (1972).

The *trans*-HMPro obtained after desalting (6·0 mg) was analysed by paper chromatography in solvents B, D, F and L. The only contaminant detected (with either isatin or ninhydrin) in 100 μg samples was an estimated 3% *cis*-HMPro. The *cis*-HMPro was desalted to give 10·6 mg product and analysed as before; the only detectable contaminant was 1% *trans*-HMPro (but see Experimental).

Dried loquat seeds then contain 7·5 mg HMPro/kg. Urbach[3] obtained 0·2 mg HMPro/kg from apple twigs, using less quantitative methods. Thus in comparison with other imino and amino acids, HMPro is a minor component of these tissues as it is in all the species from which it has been reported.[11]

Characterization of the Isolates

Initially, the isolates were identified by chromatographic comparison with standards. Authentic *cis*-4-hydroxymethyl-L-proline was readily available. *Trans*-HMPro was prepared by racemising the *cis*-isomer with barium hydroxide. The racemisation product contained a new imino acid, migrating more slowly than the starting material in solvent F, and this must have been *trans*-4-hydroxymethyl-DL-proline from its method of preparation.

These HMPro standards were inseparable from the isolates by paper chromatography in all the solvents tested (A, B, D, F, J and L). In solvent F, the only one able to separate the two isomers, authentic *cis*-HMPro ran precisely with the imino acid isolated in greatest quantity and *trans*-HMPro with the imino acid isolated in smaller amount.

The structures of the isolates were confirmed by NMR spectroscopy in D_2O using a Varian Associates A-60 spectrometer. Due to the presence of water, the spectra were distorted in the 5·0–6·2 τ region. However, in the 6·2–9·2 τ region, the spectra were not only consistent with the structures postulated, but were identical with the 60 mHz spectra published by Untch and Gibbon[9] for synthetic *cis* and *trans*-HMPro. NMR spectra were also obtained at 100 mHz.

Finally, IR spectra were measured in KCl discs. The spectrum of the newly-isolated *cis*-HMPro was identical to that reported for *cis*-4-hydroxymethyl-L-proline,[2] except for the presence of a small peak at 5·8 μ, indicative of a minor impurity. The IR spectrum of *trans*-HMPro has never been published.

Configuration of the Isolates

The *trans*-HMPro isolated had $[\alpha]_D^{27}$ + 11·0 ± 1·0° (c 0·23 in water). Allowing for impurities, including non-amino acids, the true $[\alpha]_D$ must be + 13·9°. *Trans*-4-hydroxymethyl-L-proline has an $[\alpha]_D$ −48° in water.[9] Thus the isolate contained 64% *trans*-4-hydroxymethyl-D-proline and 36% of the corresponding *trans*-L-isomer by calculation. The presence of *trans*-HMPro having the D-configuration was confirmed with D-amino acid oxidase, 0·1 mg of the isolate supporting an oxygen uptake of 0·50 μl/hr and 1·0 mg a rate of 1·43 μl/hr.

The *cis*-HMPro from loquat had $[\alpha]_D^{25}$ −73·8 ± 1·9° (c 0·4 in water). Allowing for impurities, the true $[\alpha]_D$ was −75·4°. This is in excellent agreement with the value of −75·6° (in water) reported for synthetic *cis*-4-hydroxymethyl-L-proline.[12] Thus the whole of the *cis*-HMPro from loquat appeared to have the L-configuration.

When this preparation was assayed with D-amino acid oxidase, 1·0 mg samples supported a significant rate of oxygen uptake (0·10 μl/hr). However, assuming Michaelis kinetics, and taking initial rates, this uptake would be caused by the presence of 13 μg

[11] L. F. BURROUGHS, *J. Sci. Food Agric.* **11**, 14 (1960).
[12] M. BETHELL and G. W. KENNER, *J. Chem. Soc.* 3850 (1965).

(1·3%) *trans*-HMPro in the preparation, so there is no need to postulate the presence of the *cis*-D-isomer.

Evidence that the trans-*HMPro Isolated was not an Artifact*

The isolation methods used are normally considered to be non-racemizing but the original plant material was sun-dried in a sub-tropical climate and the amino acids were absorbed on Dowex 50, and thus exposed to a low pH, for 30 days at 17–25°. Part or all the *trans*-HMPro might have been formed by racemization during these procedures. This would explain why the *trans*-HMPro isolated was partially racemic and why it had not previously been obtained from plant tissues. Two experiments were therefore undertaken to show that *trans*-HMPro was really a natural product.

First, the compound was demonstrated in loquat seeds using very mild methods. The amino acids isolated from fresh loquat seeds by cation exchange (exposure to resin 4 hr) were fractionated by paper chromatography in solvent D. The crude HMPro obtained was approximately 40% *trans*. The HMPro originally isolated was 36% *trans*, so the two results were in excellent agreement.

In the second experiment, amino and imino acids were removed from an extract of loquat by cation exchange, and *cis*-HMPro was added. This was re-isolated and analysed as in the previous experiment. Quantitative isatin assays showed that the added HMPro originally contained 4 ± 3% of the *trans*-isomer, whereas the percentage after re-isolation was 6 ± 3%. If these results are significantly different, the additional *trans*-HMPro was probably derived from the deaminated plant extract, which still contained imino acids. However at worst, the isolation technique cannot cause more than 8% racemisation so at least 80% of the *trans*-isomer found in loquat HMPro must have been present in the original extract.

Attempts to Detect trans-*HMPro in other Plant Tissues*

The fruits of several rosaceous plants, including those of *Pyrus communis* (pear) and *Malus pumila* (apple) contain HMPro.[11] The compound is restricted to the peel, at least in some varieties of apple.[1] Despite these reports two dimensional paper chromatography showed that many pear and apple fruit peels contained no detectable HMPro, less than 2 μg/g fr. wt.

Some samples from two types of pear (Conference and William's Bon Chrétien) did contain HMPro, which was isolated from the peel of the latter variety as from fresh loquat seeds. The product was analysed in solvent F. No *trans*-isomer was detected and, if present, it must have represented less than 10% of the total HMPro in the sample.

The only apples tested which contained HMPro were Cox's Orange Pippin and Worcester Pearmain. Starting with peel from the latter the imino acid was isolated and analysed as before. Quantitative isatin estimations showed that 7·5 g fr. wt. tissue gave 19 ± 1·0 μg *cis*-HMPro and 0·8 ± 1·0 μg *trans*-HMPro. Visual inspection of the chromatogram supported the presence of traces of the *trans*-isomer but this cannot be regarded as proven. The HMPro obtained from Worcester Pearmain peel by Hulme[1] would thus have contained very little if any of the *trans*-form.

Other Imino Acids Present in Sun-dried Loquat Seeds

4-Methylproline (4 mg/kg tissue) and 4-hydroxyproline (1 mg/kg tissue) were tentatively

identified in the original extract from their positions on two dimensional chromatograms and their colour reactions with ninhydrin, isatin and isatin/Ehrlich's reagent.

Isolation of Threonine

Two of the unusual imino acids isolated from loquat seeds had the abnormal D-configuration so it was important to examine the configuration of the protein amino acids present. The only one then available, threonine, was isolated: a sample (1·4 mg) gave no significant oxygen uptake with D-amino acid oxidase (F) and had $[\alpha]_{225}$ +1465° ± 20° (c 0·16 in 0·5N-HCl), after allowing for impurities. Pure L-threonine, measured under the same conditions, gave $[\alpha]_{225}$ +1436° ± 20°,[13] so the isolate was at least 99% L.

DISCUSSION

If *trans*-HMPro is an artifact, it could only have been formed by the racemisation of *cis*-HMPro. *Cis*-HMPro was not detectably racemized during reisolation from a deaminated loquat extract, but might have been racemised when the resin was used in the different ionic environment of the original extract. If such a reaction is to account for the observations, it would have to be rapid, since the proportion of the *trans*-isomer obtained from loquat extracts was the same whether HMPro had been exposed to the resin for 4 hr or 700 hr. It would have to be catalysed by a rare ionic species not present in apple or pear extracts and would give a mixture of all the possible isomers of HMPro except the *cis*-D-isomer. It seems impossible that such a stereospecific reaction can exist, so racemisation can be rejected as a source of *trans*-HMPro.

Amino acid enantiomers can be separated by paper chromatography[14] and partial resolution of *trans*-HMPro in solvent F during its isolation is theoretically possible. The relevant zone was cut slightly asymmetrically and this may have enriched the preparation in one of the enantiomers by up to 10%. Despite this, loquat *trans*-HMPro must always have contained an excess of the D-isomer; it cannot have been initially racemic.

Natural 4-methyleneproline is probably genuinely DL- since it was isolated by non-racemising methods from an extract containing a chemically related, partially racemic compound.

The three early isolations of D-amino acids from higher plants are suspect.[15] The more recent isolations are those of *N*-malonyl-D-tryptophan from *Caragana arborescens* seeds and *Malus pumila* fruits,[16] racemic (3-carboxyphenyl)-glycine from *Iris tingitana* bulbs and *Reseda luteola* seeds, and racemic (3-carboxy-4-hydroxyphenyl)-glycine enriched in the D-isomer from the same *R. luteola* seeds.[17] The latter compound, was relatively stable to acid hydrolysis, so racemization during its isolation by ion exchange was unlikely though not completely excluded. This evidence, taken with the present results, indicates that higher plants like fungi, insects and annelids,[14] can occasionally synthesise D-amino acids. It is curious that the seeds of *Reseda luteola*, like those of *Eriobotrya japonica*, contain a pair of chemically related amino acids, one racemic, and the other racemic with an excess of the D-enantiomer.

[13] J. P. JENNINGS, W. KLYNE and P. M. SCOPES, *J. Chem. Soc.* 294 (1965).
[14] R. BENTLEY, *Molecular Asymmetry in Biology*, Vol 1, Academic Press, New York (1969).
[15] A. NEUBERGER, *Adv. Protein Chem.* **4**, 363 (1948).
[16] M. H. ZENK and H. SCHERF, *Biochim. Biophys. Acta* **71**, 737 (1963).
[17] A. KJÆR and P. O. LARSEN, *Acta Chem. Scand.* **17**, 2397 (1963).

EXPERIMENTAL

Paper chromatography; purification of amino and imino acids by cation exchange (desalting); D-amino acid oxidase assays, see accompanying paper.[10]

Quantitative isatin estimations. Chromatograms were dipped through the isatin reagent,[10] heated to 40° for 60 min, and washed under hot (45°) running tap water to remove unreacted isatin. After drying at 30°, samples of paper were cut from the chromatogram and any blue colouration present was eluted by shaking them individually with phenol–H_2O (3:1, w/v) for 3 hr. Each eluate was made up to 5 ml and its absorbance was measured at 600 nm in a 1-cm cell, against a phenol–H_2O blank. *Cis*-HMPro (1 μmole) gave an extinction of 1·2 ± 0·1 and the *trans*-isomer gave the same colour yield to within ± 10%.

Separation of cis- *and* trans-HMPro. Paper electrophoresis. The two isomers were separated by high voltage electrophoresis in a pH 2·0 buffer (61 ml HCO_2H; 97 ml HOAc; H_2O to 2 l.). The imino acids moved 27·8 and 29 cm in 3 hr on 3MM paper under a potential gradient of 50 V/cm. This separation was considered inadequate for preparative purposes.

Paper chromatography. Solvent F (*n*-BuOH–MeCOEt–0·88 NH_4OH–H_2O, 4:4:1:1 by vol.), used conventionally, gave inconsistent results because the imino acids accelerated with time, and were often swept off the paper before they had completely separated. This is abnormal. The rate of migration of a compound normally decreases with time and then remains constant once the solvent front has reached the edge of the paper. This acceleration effect was much reduced by preventing used solvent from accumulating on the floor of the chromatography tank. In addition, papers were initially washed in 5% HOAc, pre-equilibrated to 72% R.H. for 3 days[18] and then run for 130 hr at 15–23°. Under these conditions, *trans*-HMPro ran 30 cm and *cis*-HMPro 35·1 cm.

Column chromatography. Unsuccessful attempts were made to separate the two imino acids with solvent F on an 82 cm long column of cellulose powder. A partition column of similar dimensions employing *n*-BuOH–MeCOEt–0·88 NH_4OH–H_2O (16:16:9:9, by vol.) also failed to resolve the isomers.

Isolation of cis- *and* trans-*HMPro from loquat seeds.* Fractions 11–21 from the Dowex 50 column were pooled and passed through a 150 ml Dowex 1 × 4 column in the acetate form to remove glutamic acid. The *cis*- and *trans*-HMPro present in 0·22 g of the residual mixture (1·3 g) were separated by paper chromatography in solvent F using an average loading of 0·55 mg/cm.

The crude *trans*-HMPro obtained was desalted to give 17·1 mg of a solid having $[\alpha]_D^{22}$ + 5·1° ± 15° (c 0·3 in water). This was purified on paper in solvent D. The loading was 0·08 mg/cm and all chromatograms were run for 48 hr at 20–25°. After desalting, 11·6 mg *trans*-HMPro was recovered having $[\alpha]_D^{21}$ + 10·1° ± 1·8° (c 0·3 in water). Amino acids were still present, so the preparation was re-purified with solvent D as before, except that all chromatograms were run for 72 hr. The Dowex 50 resin used for the final desalting procedure was cycled between acid and ammonia 10× before use and the ammoniacal eluate from the column was collected in 2 ml fractions. All the isatin positive fractions, Nos. 9–13, were pooled to give *trans*-4-hydroxymethyl-DL-proline; solubility in water > 60 g/l. at 20°. Aqueous solutions gave a glass on evaporation, which crystallized into clusters of needles after 2 months in vacuo in a desiccator.

Crude *cis*-HMPro was desalted to give 23·5 mg of a brown solid having $[\alpha]_D^{21}$ −53·7° ± 3·0° (c 0·4 in water). The colouration was removed with 2 ml washed, activated charcoal (Norit OL) and the residue (18·6 mg) had $[\alpha]_D^{21}$ −65·2° ± 1·8° (c 0·34 in water). This was purified by paper chromatography in solvent D, followed by desalting (details as for *trans*-HMPro purification). The *cis*-4-hydroxymethyl-L-proline obtained was very soluble in water (solubility > 115 g/l at 20°) and crystallised from this solvent as bunches of needles.

Non-amino acid impurities in the isolates. A blank experiment was performed in which the final solvent D fractionation and desalting of *trans*-HMPro were exactly reproduced, except that no sample was streaked onto the chromatography paper. A visible ninhydrin-negative residue weighing 0·07 ± 0·03 mg was obtained which represented the non-amino acid impurity (1·2%) which must have been present in the isolated *trans*-HMPro. *Cis*-HMPro from loquat was assumed to contain 1·0% of this type of impurity, for purposes of calculation.

The product of the blank experiment, in 2·5 ml H_2O, had an optical rotation at the D-line of 0·0008° ± 0·0010° and was assumed to be optically inactive.

Preparation of trans-*HMPro. Cis*-4-hydroxymethyl-L-proline (0·6 mg) was heated with 0·3 ml H_2O and 100 mg barium hydroxide for 87 hr at 105° in a sealed tube. The product, which was filtered and desalted, contained approximately 0·1 mg *cis*-HMPro and 0·17 mg *trans*-HMPro. This mixture was used as an internal standard.

Reisolation of cis-*HMPro from a deaminated loquat extract. Cis*-HMPro (100 μg) was added to a deaminated extract of 40 g dry loquat seeds. The chromatogram run in solvent D, which formed part of the reisolation procedures, showed, in addition to *cis*-HMPro, two isatin positive components, which must have been derived from the nominally deaminated extract.

[18] A. J. TOMISEK and P. W. ALLAN, *J. Chromatog.* **14**, 232 (1964).

The quantitative isatin procedure gave the following results:

Sample	Amount of each isomer present (μg)	
	Cis-HMPro	*Trans*-HMPro
20 μg original *cis*-HMPro	19·3	0·7
20% of the reisolated HMPro	24·3	1·4

Thus the 100 μg of HMPro originally added increased to 129 μg. The extra HMPro probably came from the residual imino acids of the deaminated extract. If 29 μg of HMPro was gained thus, approximately 12 μg of it would be *trans*. In fact, only 4 μg *trans*-HMPro appeared during the experiment.

Isolation of threonine. The residue (1·3 g) which contained 10% HMPro, also contained 7% threonine. A sample (64 mg) was chromatographed on washed paper in solvent F for 18 hr at a loading of 0·15 mg/cm. The threonine zone was eluted and desalted. The identity of the product, (3·6 mg) was confirmed by chromatography in solvents D, E, F, H and L. A 30-μg sample, run in solvent E, contained no detectable allothreonine or other ninhydrin positive impurities. The preparation contained 89·5 ± 2% anhydrous threonine according to the cadmium ninhydrin estimation[19] and Folin's reaction[20] and, assuming it to be hydrated, was 96% pure.

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[19] J. HEILMANN, J. BARROLLIER and E. WATZKE, *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 219 (1957).
[20] K. BLAU and W. ROBSON, *Chem. & Ind.* 424 (1957).

Key Word Index—Eriobotrya japonica; Rosaceae; D-aminoacids; *trans*-4-hydroxymethyl-D-proline.