Phytochemistry, 1968, Vol. 7, pp. 2061 to 2063 Pergamon Press. Printed in England

SHORT COMMUNICATION

# *TRANS*-3-HYDROXY-L-PROLINE: A CONSTITUENT OF *DELONIX REGIA*

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(*Received* 3 *May* 1968)

**Abstract**—*Trans*-3-hydroxy-L-proline was isolated from seed of *Delonix regia* and shown to inhibit the growth of mung bean seedlings. *Delonix* seedlings also contain γ-methyleneglutamic acid and γ-methyleneglutamine.

## INTRODUCTION

KNOWLEDGE concerning the natural occurrence of hydroxyproline isomers has been extended considerably in recent years. Initially, collagen was considered to contain only the *trans*-4-L isomer, but now conclusive evidence for the presence of a small proportion of the *trans*-3-L-form has been presented by independent groups of workers.[1–3] In addition, *trans*-3-hydroxy-L-proline has been identified in the acid hydrolysate of a Mediterranean sponge.[4] The same group of workers showed the latter isomer occurs together with *cis*-3-hydroxy-L-proline in telomycin,[5] and so extended the earlier observation of Sheehan and Whitney[6] on the constituents of this antibiotic. The protein (elastin) associated with the cell walls of higher plants, which has some of the characteristics of collagen, also contains *trans*-4-hydroxy-L-proline (see Lamport[7]). The diastereoisomer, *cis*(*allo*)-4-hydroxy-L-proline, is present in leaves of *Santalum album*.[8] A detailed account of the chemistry and known distributions of these isomers is given by Mauger and Witkop.[9]

## RESULTS AND DISCUSSION

In this paper we report the occurrence of *trans*-3-hydroxy-L-proline in an unbound form as a major component of the free amino acid pool of seeds and vegetative tissues of the tropical legume, *Delonix regia*. The imino acid was isolated from *Delonix* seed using ion-exchange resin chromatographic techniques. It was characterized as the *trans*-3-L isomer by optical rotation measurement and by comparison with authentic *trans*-3-DL racemate by chromatographic and NMR methods. In contrast, chromatographic analysis of an acid hydrolysate

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of cell-wall material from radicles of young *Delonix* seedlings indicated only the presence of 4-hydroxyproline. A simple distinction can be made between 3- and 4-hydroxyproline isomers based upon differences in their colour reactions with isatin, followed by treatment with Ehrlich's reagent (see Experimental section).

When *Delonix* seeds were germinated, 3-hydroxyproline was present as a major constituent of the soluble nitrogen fraction, not only of cotyledons, but also of roots, hypocotyls and leaves. The quantities present suggested the synthesis of additional imino acid during the early stages of seedling growth. *Delonix* seedlings also contained high concentrations of $\gamma$-methyleneglutamic acid and $\gamma$-methyleneglutamine, although neither of these substances were present in the dormant seed. In this behaviour, *Delonix* closely resembled another legume, *Arachis hypogaea*,[10, 11]

The ability of *trans*-3-hydroxy-L-proline to inhibit the growth of mung bean seedlings was measured by the procedure described earlier.[12] Radicle growth was inhibited about 46 and 68 per cent when 1 and 4 mg *trans*-3-hydroxy-L-proline respectively were supplied to each batch of twenty seedlings. The inhibition caused by the higher concentration was largely reversed when 2 mg proline were provided together with the 3-hydroxyproline. Although 3-hydroxyproline then shows some of the properties expected of a proline analogue, Peterson and Fowden[13] have shown previously that no hydroxyproline isomer is activated by the prolyl-sRNA synthetase of mung bean seed. The growth-inhibitory properties associated with *trans*-3-hydroxy-L-proline then may be due to its effect in reducing the formation of cell-wall, protein-bound 4-hydroxyproline from proline in growing tissues, i.e. by a mechanism similar to that suggested to explain inhibition of *Avena* coleoptile growth by *trans*-4-hydroxyproline.[14]

## EXPERIMENTAL

### *Chromatography of Plant Extracts*

Seed material or vegetative tissues were extracted by shaking with 75% (v/v) ethanol and the amino acid fraction separated by absorption upon and subsequent elution from small Zeokarb 225 cation-exchange resin columns.[15]

Two-dimensional paper chromatograms were developed using 75% (w/w) phenol–$NH_3$ as the first solvent, followed by butanol-1-ol–acetic acid–water (90:10:29, v/v). The *trans*-3 isomers of hydroxyproline separate as slightly faster-moving spots from the *trans*-4 isomers after prolonged development of chromatograms in the second solvent. The 3- and 4-hydroxy derivatives also reacted distinctively when chromatograms were sprayed with 0·2% isatin in acetone: on heating, both *cis*- and *trans*-4-hydroxyprolines gave a blue colour characteristic of many imino acids, but 3-hydroxyproline isomers bleached isatin and appeared as white areas against the yellow background colour of the chromatograms. If isatin-treated chromatograms were oversprayed with Ehrlich's reagent, the blue spots from 4-hydroxyprolines faded and a pink colour was gradually produced.

### *Isolation and Properties of* trans-3-*Hydroxy-L-Proline*

Ground seed of *Delonix regia* (4·7 kg) was extracted with 75% (v/v) ethanol (24 l.) for 10 days with intermittent stirring. The separated seed residue was re-extracted with more 75% ethanol, and then the two extracts were combined and concentrated *in vacuo* to 3 l. The pH was adjusted to 4·0, and the extract was treated with charcoal at 60° to decolorize and to coagulate any extracted protein. The clarified extract was applied to a column of Zeokarb 225 (× 8) ($H^+$ form, 52–100 mesh, 100 cm × 5·5 cm dia.). After washing to remove non-cationic materials, the amino acids were eluted using 0·23 N-$NH_3$. 3-Hydroxyproline was present in the first thirty-five ninhydrin-positive fractions (50 ml), together with aspartic and glutamic acids, asparagine, serine

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and threonine. These pooled fractions were concentrated to 600 ml, adjusted to pH 7·0, and applied to a Dowex-1 (× 10) column (acetate form, mesh 100–200, 130 cm × 2·5 cm dia.). Aspartic and glutamic acids were retained and the eluate at this stage gave a residue (3·6 g) consisting mainly of 3-hydroxyproline, a little asparagine and traces of serine and threonine. After three recrystallizations from 95% (v/v) ethanol, a pure sample of 3-hydroxyproline (white needles) was obtained. $[\alpha]_D^{20}$ −16·8° (*c* 2 in $H_2O$), + 21·4° (*c* 1 in 6 N-HCl); literature values[4] for *trans*-3-hydroxy-*L*-proline, $[\alpha]_D^{20}$ − 17·4° (*c* 1 in $H_2O$), + 13·3° (*c* 0·5 in N-HCl). The specific rotations determined for the *cis*-3 and *cis*- and *trans*-4 stereoisomers of hydroxyproline are significantly different from these values.[4,16] (Found: C,45·7; H,6·8; N,10·4. Calc. for $C_5H_9NO_3$: C,45·8; H,6·9; N,10·7 per cent.)

The NMR spectrum determined in $D_2O$ for the isolate was identical with that of a sample of *trans*-3-hydroxy-DL-proline and with the published spectrum[3] for *trans*-3-hydroxy-L-proline.

*Hydroxyproline of a Cell-Wall Fraction*

A cell-wall fraction was prepared by repeatedly macerating radicles from young *Delonix* seedlings in dilute tris-HCl buffer, pH 7·2, until microscopical examination indicated the absence of unbroken cells. The residue was washed successively by suspension and filtration from water (× 3), 5% (w/v) trichloroacetic acid (× 3), ethanol, acetone and ether. The dried wall material was hydrolysed with 6 N-HCl at 100° for 18 hr and, after separating the amino acid fraction from the hydrolysate using a small Zeokarb column, the individual compounds were separated on a two-dimensional paper chromatogram. 4-Hydroxyproline was identified on the basis of $R_f$ values and colour reactions, but no 3-hydroxyproline was detected.

*Acknowledgements*—We are grateful to the California Foundation for Biochemical Research for a grant to support Miss M-L Sung. The NMR spectra were kindly performed by Dr. R. C. Sheppard (Liverpool). Our thanks are due to Professor J. C. Sheehan (Mass. Inst. Technology), Dr. F. Irreverre (Bethesda) and Dr. J. D. Ogle (Cincinnati) for samples of *cis*- and *trans*-3-hydroxy-DL-prolines.

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