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4-HYDROXYISOLEUCINE FROM SEED OF *TRIGONELLA FOENUM-GRAECUM*

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Key Word Index—*Trigonella foenum-graecum*; Leguminosae; fenugreek; 4-hydroxyisoleucines; biosynthesis from isoleucine.

Abstract—The principal free amino acid present in seed of *Trigonella foenum-graecum* has been isolated and identified as (2*S*, 3*R*, 4*R*)-4-hydroxyisoleucine. This compound has not been reported previously as a constituent of higher plants, but it is a component of the toxic peptide, γ-amanitin, produced by *Amanita phalloides*. The (2*S*, 3*R*, 4*R*)-isomer lactonizes readily under acidic conditions, whilst strong acid causes partial epimerization. The (2*R*, 3*R*, 4*R*)-isomer forms a minor component of *Trigonella* seed. The 4-hydroxyisoleucine content of fenugreek increases during the growth of seedlings and plants, and ^{14}C-isoleucine was used effectively as a biosynthetic precursor.

INTRODUCTION

Trigonella foenum-graecum L. (fenugreek) (Leguminosae) is an annual, herbaceous plant widely distributed in areas of Asia, Africa and Europe. The seed has a considerable culinary use, and a potential economic value as a source of sapogenins for the steroid industry.[1] Hutchinson[2] groups the genus *Trigonella* with several other agriculturally-important legumes (e.g. the genera *Melilotus*, *Medicago* and *Trifolium*) in the tribe Trifolieae. Although cursory reports of the amino acid content of *Medicago* and *Trifolium* species exist, no information is available concerning these constituents in *Trigonella* species. We now report the results of a large-scale extraction of seed of fenugreek that led to the isolation and identification of an isomer of 4-hydroxyisoleucine as the principal unbound amino acid of this species. The hydroxyamino acid represents 30–50% of the total free amino acid complex of dry seeds.

RESULTS AND DISCUSSION

The application of paper chromatographic and amino acid autoanalytical methods to aqueous-ethanolic extracts of seed of *T. foenum-graecum* revealed an 'unidentified' compound(s) as the major seed constituent. Although the compound was mainly superimposed on the γ-aminobutyrate area of paper chromatograms (phenol-NH_3 followed by BuOH–AcOH–H_2O), it was recognized as an 'unusual' compound by a characteristic forward streaking of the spot in the second solvent. The elution profile of amino acids from the Technicon autoanalyser, operating under standard conditions,[3] showed two important, unidentified peaks in the basic region (peaks A and B, Fig. 1). These peaks had similar, very unsymmetrical shapes, that were explicable ultimately on the basis of interconversions between open-chain and lactone forms of γ-hydroxyamino acids.

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[1] FAZLI, F. R. Y. and HARDMAN, R. (1971) *Phytochemistry* **10**, 2497.

[2] HUTCHINSON, J. (1964) *The Genera of Flowering Plants*, Vol. I, p. 445, Oxford University Press, Oxford.

[3] *Assembly and Operating Instructions: Amino Acid Analyser* (1967) Instruction manual AAA-1, Technicon Corporation, New York.

Fractionation of the amino acids present in a large-scale extract of seed meal (14 kg) by conventional cation-exchange chromatographic procedures (Zeokarb 225 and Dowex 50) gave 13 g of a pure amino acid, which behaved as peak A (Fig. 1) when subjected to Technicon autoanalysis. A second compound corresponding to peak B, which was concentrated in later fractions from the Dowex-50 column, was isolated in small quantity (about 200 mg) by a modified ion-exchange procedure, in which a Dowex 50 column in the NH_4^+-form was used to retain the two new compounds (and other basic amino acids) after conversion to lactones. The lactone corresponding to peak A was almost completely removed from the column by thorough washing over a period of about 1 week; presumably the lactone was slowly reconverted to the original open-chain amino acid and released from the resin. The lactone corresponding to peak B was retained during this washing, but was released readily as the free amino when 0·2 N NH_3 was applied to the column.

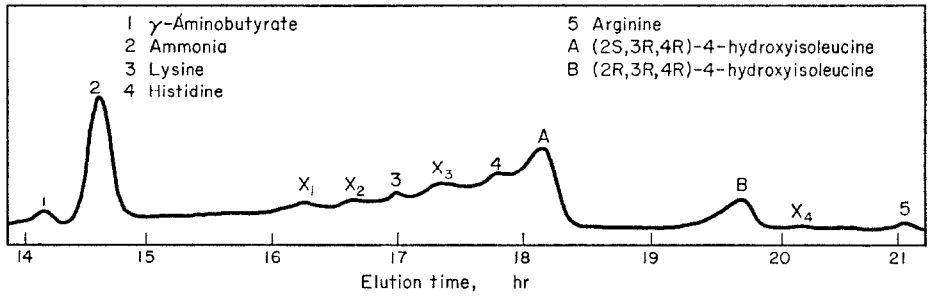
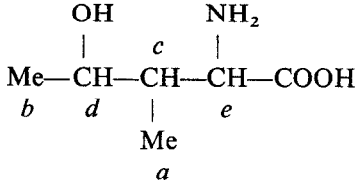


FIG. 1. A LATE SECTION OF THE TECHNICON AMINO ACID ANALYSER PROFILE OBTAINED FROM AN EXTRACT OF *Trigonella foenum-graecum* SEED.

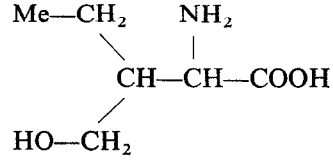
X_1, X_2, X_3, X_4 are unidentified minor components of extract. The column was run under the standard operating conditions.[3]

(2S, 3R, 4R)-4-*Hydroxyisoleucine*

The 13 g isolate of amino acid, identifiable with peak A analysed as $C_6H_{13}NO_3$. The ease of lactone formation indicated a γ-hydroxyl group. Prolonged reduction (H_2/Adam's Pt catalyst, 60–80°, 1 atmos.) gave isoleucine, and a little *allo*isoleucine (Technicon autoanalyser and PC using *tert*-amyl alcohol–AcOH–H_2O as solvent). The NMR spectrum was compatible only with the structure, 2-amino-3-methyl-4-hydroxypentanoic acid (I), and eliminated the alternative 2-amino-3-hydroxymethylpentanoic acid (II, an isomeric 4-hydroxyisoleucine).



(I)



(II)

The spectrum showed as expected two doublets (through coupling with single CH protons) at high field attributable to protons of Me groups *a* and *b*, a multiplet of the expected area in mid-field assigned to the tertiary CH*c*, and a multiplet at low field due to CH*d* and CH*e* and of area representing 2 protons. Studies of the circular dichroism (CD) exhibited by the isolate in comparison with reference compounds (see Table 1) enabled a (2*S*)-configuration (i.e. an L-isomeric form) to be assigned to the compound: invariably a (2*S*)-configuration was associated with a positive Cotton effect (+$\Delta\epsilon$ value). An *R*-configuration was assigned to C-4 because the isolate was identical (as a lactone form) with the (2*S*, 3*R*, 4*R*)-isomer of the lactone of 4-hydroxyisoleucine, characterized from hydrolysates of γ-amanitin[4] and kindly donated by Professor Th. Wieland (Heidelberg). This accumulated evidence established 2*S*, 3*R*, and 4*R* configurations respectively for the three asymmetric C atoms of the amino acid.

TABLE 1. CIRCULAR DICHROISM DATA FOR 4-HYDROXYISOLEUCINES AND OTHER REFERENCE COMPOUNDS

Compound	CD values $\Delta\epsilon$	λ (nm)
L-Isoleucine	+1·21 m	200
D-Alloisoleucine	−1·76 m	200·5
(2*S*, 3*R*, 4*R*)-4-Hydroxyisoleucine	+1·69 m	197·5
(2*R*, 3*R*, 4*R*)-4-Hydroxyisoleucine	−1·88 m	199
(2*S*, 3*R*, 4*R*)-4-Hydroxylisoleucine lactone HCl	+1·89 m	214
(2*R*, 3*R*, 4*R*)-4-Hydroxyisoleucine lactone HCl	−1·26 m	215
(2*S*, 4*R*)-4-Hydroxynorvaline	+0·63 m	199
(2*S*, 4*S*)-4-Hydroxylysine	+0·89 m	199
(2*S*)-4-Hydroxyleucine lactone HCl	+0·37 m	220

When this isomer is treated with 6 N HCl at 100° for several hours, the resulting mixture contains a second compound which exhibits exact coincidence with peak B when subjected to Technicon autoanalysis. When first describing this reaction, Wieland *et al.*[4] suggested that 35% epimerization about the C-4 atom occurs in 24 hr forming a (2*S*, 3*R*, 4*S*)-lactone. However, we found that a sample containing their presumed mixture of 2*S*, 3*R*, 4*R*- and 2*S*, 3*R*, 4*S*-lactones gave *allo*isoleucine on reduction. Hasan and Wieland have re-examined the products of epimerization caused by heating with 5 N $Ba(OH)_2$ or 6 N HCl and will report[5] as follows: treatment of 2*S*, 3*R*, 4*R*-isomer with base produces epimerization at C-2 to yield the minor 2*R*, 3*R*, 4*R*-isomer reported below and identified as peak B, whereas 6 N HCl causes some epimizeration about C-4 but a more extensive inversion about C-3 (perhaps via formation of a 3,4-unsaturated acid) to yeild a mixture finally containing 2*S*, 3*R*, 4*R*-, 2*S*, 3*R*, 4*S*- and 2*S*, 3*S*, 4*R*-isomers in an approximate 1 : 1 : 3 ratio. Lactones form by treatment with N-HCl (100°, 5–10 min) without any detectable epimerization.

(2R, 3R, 4R)-4-*Hydroxyisoleucine*

This minor isolate, identifiable with peak B (Fig. 1), was separable (as a lactone) from the major isomer by paper electrophoresis at pH 6·5: under these conditions, the (2*R*)-lactone

[4] WIELAND, TH., HASAN, M. and PFAENDER, P. (1968) *Ann. Chem.* **717**, 205.
[5] HASAN, M. and WIELAND, TH., to be reported at the *9th Intern. Cong. Biochem. Stockholm, July* 1973.

moved slightly faster towards the cathode. Catalytic hydrogenation gave mainly *allo*-isoleucine, with a little isoleucine, whilst CD studies established a negative Cotton effect indicative of a D-configuration (2*R*) at the C-2 atom. NMR studies of a series of structural isomers[5] have confirmed a (2*R*, 3*R*, 4*R*)-configuration for this minor isolate. During PC and amino acid autoanalysis, it was inseparable from the 2*S*, 3*S*, 4*R*-isomer.

When the elution profile of amino acids from the Technicon autoanalyser is scanned at $\lambda = 570$ nm, this 2*R*-isomer can be detected (as peak B) about 10-times more sensitively than major 2*S*, 3*R*, 4*R*-isomer (peak A). Nevertheless, our purest samples of 2*S*-isomer have been essentially free of contaminating peak B when examined under the standard 21 hr Technicon operating procedure (60°, elution buffer pH range 2·85–5·0). These conditions clearly effect lactonization without epimerization, and so the presence of peak B in the profile of simple aqueous-ethanol extracts indicates that the 2*R*-isomer is a normal seed constituent.

Distribution and synthesis of 4-*hydroxyisoleucine*

Two other *Trigonella* species, *T. caerulea* and *T. cretica*, were available as seeds for analysis. 4-Hydroxyisoleucine formed only a minor component of the free amino acid complex, and was detected with certainty only after conversion to the lactone and absorption upon Dowex 50 resin in the NH_4^+-form. The hydroxyamino acid could not be detected in seed extracts of several *Medicago* and *Trifolium* species.

The total amount of 4-hydroxyisoleucine present in a plant of *T. foenum-graecum* increases steadily during all phases of growth, and so germinating seedlings have been used for biosynthetic studies. [U-^{14}C]L-Isoleucine was supplied in water imbibed by dry seeds during 24 hr, and growth was allowed to continue in moist vermiculite at 30°. After 2 days, about 0·5% of the radioactivity supplied was present in 4-hydroxyisoleucine, whilst incorporation reached 1% after 5 days. 4-Hydroxyisoleucine was the major labelled cationic product arising from ^{14}C-isoleucine. These observations suggest that isoleucine is probably converted by a direct pathway to the 4-hydroxy derivative, and that further studies might profitably investigate the possibility of a C-4 hydroxylation mechanism.

EXPERIMENTAL

PC and paper electrophoretic methods were as described for a similar investigation of nitrogenous constituents in *Acacia georginae*.[6]

Isolation of 4-*hydroxyisoleucines.* Ground fenugreek seed (14 kg) was extracted ×3 with 20% EtOH (total vol. 85 l.). The amino acid fraction was separated on a Zeokarb 225 column (100 × 10 cm, H^+-form); N NH_3 was used for elution, and fractions containing 4-hydroxyisoleucine were pooled and evaporated *in vacuo*. The combined fraction was next applied to a Dowex 50 × 8 column (100 × 5 cm, H^+-form, resin mesh 100–200); this column was eluted with 0·2 N NH_3, when the required amino acids accumulated in the later fractions. Concentration and crystallization gave 5·7 g of pure (2*S*, 3*R*, 4*R*)-4-hydroxyisoleucine (Found: C, 49·2; H, 8·9; N, 9·5. $C_6H_{13}NO_3$ requires: C, 49·0; H, 8·9; N, 9·5%). $[\alpha]_{Dl}^{20}$ +31° (*c* 1, H_2O). Mother liquors were acidified (*N* HCl) and heated at 90° for 10 min to effect lactonization. After removal of residual HCl and neutralization, lactones and basic amino acids were absorbed on a Dowex 50 column (100 × 5 cm, NH_4^+-form). Non-basic components were quickly washed through the column, and then washing with H_2O (14 l.) was continued slowly for 7 days. A further 7 g of 2*S*, 3*R*, 4*R*)-isomer was recovered from these washings. Finally, the column was eluted with 0·2 N NH_3, when (2*R*, 3*R*, 4*R*)-4-hydroxyisoleucine eluted before the basic amino acids. Concentration and crystallization gave 205 mg pure material (Found: C, 48·7; H, 8·8; N, 9·4. $C_6H_{13}NO_3$ requires: C, 49·0; H, 8·9; N, 9·5%). $[\alpha]_D^{20}$ +1° (*c* 1, H_2O).

Properties of the isolates. The 2*S*, 3*R*, 4*R*-isomer ran slightly faster than the 2*R*, 3*R*, 4*R*-form of the hydroxyamino acid on PC's developed with *tert*-amyl alcohol–AcOH–H_2O. Both isolated isomers were readily converted to their γ-lactones (N HCl, 100°, 5 min). The lactones separated during paper electrophoresis at

[6] ITO, K. and FOWDEN, L. (1972) *Phytochemistry* **11**, 2541.

pH 6·5; the 2*S*, 3*R*, 4*R*- and 2*R*, 3*R*, 4*R*-isomers moved about 14 and 16·5 cm respectively towards the cathode when 100 V/cm were applied for 1 hr. When H_2 was bubbled through aqueous solutions of the 4-hydroxyisoleucines in the presence of Adam's PtO_2 catalyst, negligible reduction occurred at laboratory temperature, but little hydroxyamino acid remained after a 5 hr treatment at 80°. Isoleucine and *allo*isoleucine were identified as reduction products by co-chromatography with authentic materials, using PC (*tert*-AmOH–HOAc–H_2O, 20 : 1 : 20) and Technicon autoanalyser techniques. NMR spectroscopy was performed at 60 MHz using a 10% (w/v) solution of 2*S*, 3*R*, 4*R*-isomer in D_2O. Resonances were observed at 8·95 and 8·65 ppm (doublets, pair of $C\underline{H}_3$–), 7·95 ppm (broad multiplet, > CH*c*-proton in structure I), and 6·03 ppm (multiplet, > CH*d*- and > CH*e*-protons of I) (3-trimethylsilylpropan-1-sulphonic acid sodium salt = 10 ppm). CD determinations were made by Dr. P. M. Scopes (Westfield College, London). All compounds, including lactones, having an L-configuration at the C-2 atom (2S-forms) exhibited positive Cotton effects, whilst D-forms showed negative $\Delta\epsilon$ values. The CD maxima of γ-lactones occurred at higher wavelengths than those of open-chain amino acids (see Table 1).

^{14}C-Isoleucine incorporation experiments. *Trigonella* seeds (0·5 g samples) were supplied with 5 μCi [U-^{14}C]isoleucine (10 mCi/mmol) dissolved in a limited quantity of H_2O that was fully imbibed by the seeds during 24 hr at 30°. The seedlings were transferred to moist vermiculite and grown in a hood for further periods of 2 and 5 days. Seedling extracts were made in 70% EtOH, and 4-hydroxyisoleucine was separated as the lactone on small cation-exchange resin columns (NH_4^+-form) together with basic amino acids. The final separation was by PC using BuOH–HOAc–H_2O, 4 : 1 : 5). ^{14}C present in 4-hydroxyisoleucine was determined using a Packard radiochromatogram scanner.