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TWO STEREOISOMERS OF β-HYDROXY-γ-METHYL-GLUTAMIC ACID FROM SEEDS OF *GYMNOCLADUS DIOICUS*

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Abstract—Two new amino acids have been isolated from seeds of the legume *Gymnocladus dioicus*. These amino acids are stereoisomeric forms of β-hydroxy-γ-methylglutamic acid. The presence of other 'non-protein' amino acids in the plant is reported.

INTRODUCTION

THE GENUS *Gymnocladus* is represented by two species, *G. dioicus* (L) Koch (the Kentucky coffee tree) which grows in a limited area of North America and *G. chinensis* Baill. which is a native of China. *G. dioicus* is a large tree growing to 80 ft, and its timber has proved valuable for stakes and fencing posts because of its durability in the soil.

The tree is not attacked by insects and indeed its leaves have been used in the manufacture of insecticidal preparations.[1] The leaves and pods are also reported to be toxic to sheep, cattle and man.[2] The presence of 'non-protein' amino acids such as α,γ-diaminobutyric acid[3] and indospicine[4] render the plants which contain them toxic to mammals, and it seemed possible that such a compound might be responsible for the poisonous properties of *G. dioicus*.

When extracts of leaves, pods and seeds were analysed by paper chromatography and high-voltage electrophoresis no known toxic amino acids were detected. The extracts, however, contained major concentrations of two ninhydrin-reacting compounds with R_f values and ionic mobilities similar to, but not identical with those of glutamic acid and aspartic acid. Derivatives of both glutamic acid and aspartic acid are widespread in living systems and it is known that γ-methylglutamic acid,[5,6] γ-methyleneglutamic acid and its amide,[7,8] γ-ethylideneglutamic acid,[9,10] two stereoisomeric forms of γ-hydroxy-γ-methyl-

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glutamic acid,[11,12] γ-hydroxyglutamic acid,[13–15] dihydroxyglutamic acid[16] and β-hydroxyaspartic acid[17] occur in higher plants. The separation of these compounds on paper by a combination of electrophoresis and chromatography has been described by Peterson.[18] The R_fs and ionic mobilities of the two compounds in the extracts of *G. dioicus* did not correspond to those of any of the above compounds however.

Preliminary investigations[19] suggested that the two compounds were isomeric forms of β-hydroxy-γ-methylglutamic acid, an amino acid with three centres of asymmetry. Further work has confirmed the original findings and the present paper describes the isolation of the compounds, their chemical and physical properties, and evidence of their molecular structure. The presence of other 'non-protein' amino acids in the plant is reported.

RESULTS AND DISCUSSION

When aqueous ethanolic (50%) extracts of the seeds of *G. dioicus* were subjected to 2D chromatography on paper using butanol/acetic acid and phenol/NH_3 as solvents, major concentrations of two unidentified ninhydrin-reacting compounds were detected. These

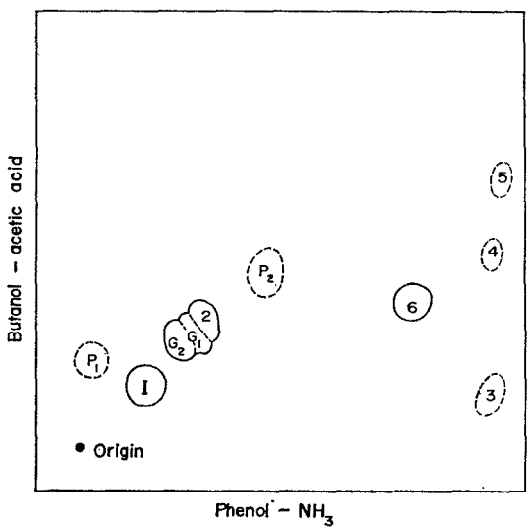

FIG. 1.

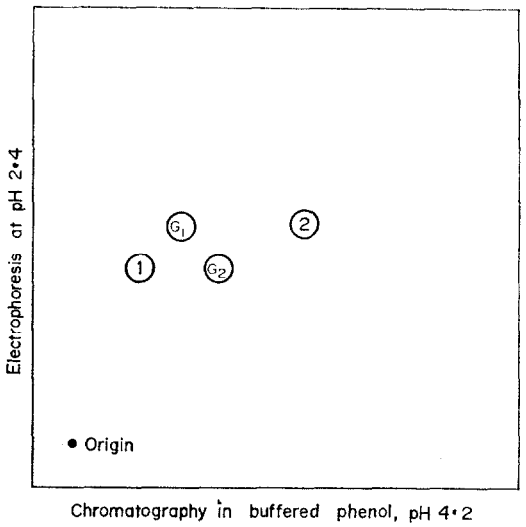

FIG. 2.

FIG. 1. A DIAGRAMATIC REPRESENTATION OF THE POSITIONS OCCUPIED BY G_1, G_2 AND THE OTHER PRINCIPLE NINHYDRIN-REACTING COMPOUNDS DETECTED IN DORMANT SEEDS OF *Gymnocladus dioicus*. (1) Aspartic acid; (2) glutamic acid; (3) arginine; (4) proline; (5) pipecolic acid; (6) 5-hydroxypipecolic acid; P_1 and P_2, unidentified peptides.

FIG. 2. A DIAGRAMATIC REPRESENTATION OF THE SEPARATION OF G_1 AND G_2 FROM ASPARTIC ACID AND GLUTAMIC ACID EFFECTED BY COMBINED CHROMATOGRAPHY AND ELECTROPHORESIS.

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[16] A. I. VIRTANEN and J. ETTALA, *Acta Chem. Scand.* **11**, 182 (1957).
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compounds were only partially resolved from each other and appeared as a double spot which occupied a position between aspartic acid and glutamic acid on the chromatogram. In addition to various protein amino acids, 5-hydroxypipecolic acid, pipecolic acid and two other unidentified ninhydrin-reacting compounds were detected (Fig. 1). The two unknown compounds found in highest concentration (designated G_1 and G_2) gave a brown-purple colour with ninhydrin and a distinctive grey-blue when 1% of 2,4,6-collidine was added to the ninhydrin reagent before use. Better resolution of G_1 and G_2, and their complete separation from glutamic acid and aspartic acid, was obtained by combining electrophoresis at 8 V/cm for 5 hr at pH 2·4 (1 N HOAc) in one dimension with prolonged chromatography (48 hr) in phenol buffered at pH 4·2 in the other (Fig. 2). On electrophoresis at pH 3·6 G_1 moved with glutamic acid and G_2 moved between glutamic acid and aspartic acid; at pH 1·9 G_1 moved between glutamic acid and aspartic acid while G_2 moved more slowly than aspartic acid, as if at this pH G_2 were the stronger acid. The two compounds were isolated by ion-exchange chromatography and elementary analysis showed them to be isomers with a common molecular formula of $C_6H_{11}O_5N$; both compounds crystallised from aqueous solvents with one water of hydration. The R_fs and the ionic mobilities of the two compounds did not correspond to those of any known amino acid. Both compounds formed chelates with cupric ions indicating the presence of a free amino group in the α-position.[20] They also underwent decomposition when treated with periodic acid in a manner characteristic of compounds containing vicinal hydroxyl groups or vicinal hydroxyl and amino groups.[21] On reduction with hydriodic acid in the presence of red phosphorous, G_1 and G_2 gave a mixture of amino acids which could not be separated from authentic *erythro* and *threo*-γ-methylglutamic acids by chromatography or electrophoresis. The same compounds were formed together with glycine when G_1 and G_2 were heated for 20 hr in 5 N NaOH. Alkaline degradation of this type, which is characteristic of β-hydroxy amino acids,[22] provided additional evidence that G_1 and G_2 were isomeric forms of β-hydroxy-γ-methylglutamic acid. The NMR spectra of the compounds (which will be discussed fully in the following paper) were consistent with this conclusion.

EXPERIMENTAL

Paper chromatography. Upward chromatography was carried out on Whatman No. 1 or 3 MM paper. Solvents used were (I) *n*-BuOH–HOAc–H_2O (12:3:5, by vol.); (2) PhOH–H_2O (4:1, w/v) in the presence of the vapour of aq. NH_3 (sp. gr. 0·88); (3) phenol saturated with buffer solution of pH 4·2 (0·642% citric acid, 0·649% $Na_2HPO_4.2H_2O$ in H_2O) and (4) *n*-BuOH–HCO_2H–H_2O (75:15:10 by vol.)

Paper electrophoresis. Low voltage electrophoresis was carried out on Schleicher–Schull 2043 b. paper using a Pleuger apparatus (Wijnegem, Belgium), IN HOAc (pH 2·4) and a potential difference of 8 v/cm. High voltage electrophoresis was carried out on Whatman 3 MM paper using a flat-plate unit (FP-3AA, Savant Instruments, Inc. Hicksville, New York), buffer solutions of pH 1·9 and 3·6[23] and a potential difference of 62 v/cm.

Combined chromatography and electrophoresis. Combined 2D chromatography and electrophoresis was carried out on Schleicher–Schull 2043 b. or Whatman 3 MM paper using various systems of buffers and solvents. Low voltage electrophoresis at pH 2·4 was combined with chromatography in *n*-BuOH–HCO_2H–H_2O, and high-voltage electrophoresis at pH 3·6 and 1·9 was combined with chromatography in *n*-BuOH–HOAc–H_2O and PhOH/NH_3.

Development of colours. Chromatograms and electrophoresis papers were dipped in ninhydrin (0·2%, v/v) dissolved either in 95% (v/v) acetone or in 95% (v/v) acetone to which had been added 1% (v/v) of 2,4,6-collidine.

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The isolation of G_1 and G_2. Seeds of *G. dioicus* were crushed and the dense dark brown seed coats removed by differential flotation in a mixture of CCl_4 and light petroleum of appropriate specific gravity. The remainder of the seed was ground to a fine powder (139 g) and stirred with CCl_4 (2 × 500 ml) to remove lipids and pigments. The defatted seed was then extracted three times at room temp. with 50% EtOH (1000 ml, then 2 × 500 ml). The combined extracts were concentrated to 500 ml under reduced pressure and passed through a column (35 × 4 cm) of Amberlite CG 120 (100–200 mesh) resin in the H^+ form. After washing with water (2000 ml) the amino acids were displaced with 1 N NH_4OH and the alkaline solution taken to dryness under reduced pressure to give 8·5 g of crude mixed amino acids. After combining the products of this and subsequent separations, 12·2 g of the amino acid mixture was redissolved in the minimum volume of IN HOAc and passed through a column (107 × 4·2 cm) of Amberlite CG 400 (100–200 mesh) in the acetate form. The amino acids were eluted with IN HOAc. After the basic and neutral amino acids had been removed, glutamic acid was eluted followed by a mixture of G_1 and G_2 and finally by aspartic acid. The fractions containing G_1 and G_2 were combined and taken to dryness under reduced pressure (9 g mixed acids). The mixture of G_1 and G_2 was then redissolved in buffer solution of pH 3·2 (pyridine–HOAc–H_2O, 16:250:734 by vol.) and applied to a column (100 × 4 cm) of Amberlite CG 120 (100–200 mesh) in the Na^+ form, equilibrated with the buffer solution of pH 3·2. The same buffer solution was used for elution, and complete separation of G_1 and G_2 was obtained. The fractions containing the two separated amino acids were taken to dryness under reduced pressure. The amino acids, which were contaminated with NaOAc, were redissolved in water and applied to separate columns (40 × 5 cm) of Amberlite CG 400 (100–200 mesh) resin in the acetate form. After washing with water, the amino acids were eluted from the columns with IN HOAc, evaporated to dryness under reduced pressure and recrystallized (G_1 from the minimum volume of hot water, G_2 from 50% EtOH). G_1: yield 2·9 g, m.p. 168·5–169 Fd. C, 36·91; H, 6·56; N, 6·86%; $C_6H_{11}O_5$ N. H_2O requires: C, 36·92, H, 6·71; N, 7·18%, $[\alpha]_D^{20}$ + 3·0 (C. 1·177, H_2O); + 23·4 (C. 1·1199, 5 N HCl). G_2: yield 320 mg, m.p. 193–194° Fd. C, 36·85; H, 6·53; N, 6·84%. $[\alpha]_D^{20}$ − 5·2 (C. 0·949, H_2O); − 28·1 (C. 1·180, 5 N HCl).

Dehydration of G_1 and G_2. After heating repeatedly at 100° under reduced pressure (2 mmHg) in the presence of P_2O_5, the crystalline products G_1 and G_2 each lost the equivalent of one water of crystallization (8·85% for G_1; 8·49% for G_2, calc: 9·23%) G_1 (anhydrous) Fd. C, 40·56; H, 6·12; N, 8·03%. G_2 (anhydrous) Fd. C, 40·55; H, 6·29; N, 8·07%. Calc. for $C_6H_{11}O_5N$: C, 40·68; H, 6·26; N, 7·91%.

Chelation with cupric ions. When treated successively with cupric nitrate and ninhydrin on paper both G_1 and G_2 reacted as amino acids containing unsubstituted α-amino groups.

Reduction with red phosphorus and HI. A few mg of G_1 and G_2 were heated with red P and HI in sealed tubes for 16 hr at 140°. In each case a mixture of *erythro*-γ-methyl-DL-glutamic acid and *threo*-γ-methyl-DL-glutamic acid was formed. These amino acids were identified by electrophoresis in pH 4·4 (Przybylska and Strong, 1968) and co-chromatography (Blake and Fowden, 1964) using authentic samples, kindly provided by Prof. L. Fowden, as 'markers'.

Alkaline decomposition of G_1 and G_2. On heating in alkaline solution (5 N NaOH) for 20 hr at 105° both compounds decomposed and gave rise to the same mixture of *erythro* and *threo*-γ-methyl-DL-glutamic acids and also glycine.

Decomposition with periodic acid. When treated with 4% periodic acid on paper both G_1 and G_2 decomposed to give NH_3 which was detected with Nessler's reagent.

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Key Word Index—Gymnocladus dioicus; Leguminosae; toxic amino acids; β-hydroxy-γ-methylglutamic acid; stereoisomers; γ-methylene glutamic acid.