Phytochemistry, 1974, Vol 13, pp 1515 to 1517 Pergamon Press Printed in England

γ-L-GLUTAMYL-L-PIPECOLIC ACID IN *GLEDITSIA CASPICA*

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(*Received* 30 *November* 1973)

Key Word Index—*Gleditsia caspica*, Leguminosae, non-protein amino acid, γ-glutamyl imino acid, γ-L-glutamyl-L-pipecolic acid

Abstract—γ-L-Glutamyl-L-pipecolic acid has been isolated from seeds of *Gleditsia caspica* (L) Desf Proof of its structure was obtained by chromatographic and spectroscopic examination of the natural product and its hydrolytic products The new compound is the first example of a naturally occurring γ-glutamyl imino acid

INTRODUCTION

THE PRESENT work is a continuation of previous studies of the distribution of amino acids in *Gymnocladus dioicus* (L) Koch.[1–3] and the closely related genus *Gleditsia*.[4] From various plants, a great number of γ-L-glutamyl derivatives of amino acids and amines have been isolated and their number is still increasing.[5] The configuration at the C(2) of the second amino acid in most of the isolated γ-glutamyl-amino acids has been established as L- (or S) but amino acids with D- (or R) configuration at C(2) have also been found in naturally occurring γ-glutamyl compounds.[6] This paper records the isolation and identification of the new compound γ-L-glutamyl-L-pipecolic acid from *G. caspica*.

RESULTS AND DISCUSSION

Investigations of crude seed extracts of *G. caspica* by PC and by high voltage electrophoresis revealed the presence of an unknown compound with high R_f values and a normal purple ninhydrin colour. The mobility of the compound during high voltage electrophoresis at pH 3·6 is almost the same as that of aspartic acid. The compound was isolated by ion-exchange chromatography and recrystallization from H_2O.

Elementary analysis and examination of the products obtained after mild acid hydrolysis showed that the natural product was a peptide of glutamic acid and pipecolic acid. The

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natural compound did not react with isatin, whereas the pipecolic acid liberated by hydrolysis gave a strong reaction.[7 8] The identity of the hydrolytic products was fully confirmed by isolation and comparison with authentic material using IR, NMR, electrophoretic and chromatographic techniques. Dinitrophenylation and subsequent hydrolysis gave the dinitrophenyl derivative of glutamic acid and free pipecolic acid The molecular rotations of the isolated glutamic acid and pipecolic acid (see Experimental) established the L- (or S) configuration at C(2) for both of them

The lack of reaction of the natural product with isatin, the behaviour on ion-exchange resins, the electrophoretic mobility, the easy cleavage in acid hydrolysis and the result from dinitrophenylation suggest that it is a γ-glutamyl peptide Definite evidence for the γ-glutamyl structure was obtained from NMR spectra of the peptide and its hydrolysis products The NMR spectra of the peptide showed a complex pattern for the methylene protons, however in full agreement with that expected for a γ-glutamyl pipecolic acid derivative The δ-values for the α-protons in both the pipecolic acid and glutamic acid part and especially pH-dependent chemical shifts for these protons (see Table 1) confirm that the compound is γ-glutamyl pipecolic acid and not α-glutamyl pipecolic acid or pipecolyl glutamic acid [9 11]

TABLE 1 CHEMICAL SHIFTS FOR THE α-PROTONS OF THE AMINO ACIDS IN VARIOUS IONIZATION STATES

	pH < 1*	D_2O†	pH 7‡	pH > 11§
α-Proton in glutamic acid	4.2 (t)	3.8	3.8	3.5
α-Proton in pipecolic acid	4	3.6	3.6	3.2
γ-L-Glutamyl-L-pipecolic acid				
α-Proton in glutamic acid moiety	4.2 (t)	3.8	3.8	3.3
α-Proton in pipecolic acid moiety	5.2	5.1		5

* Solutions of the amino acid in D_2O + trifluoroacetic acid
† Solutions of the amino acids in D_2O
‡ Solutions of the amino acids in D_2O + excess K_2HPO_4
§ Solutions of the amino acids in D_2O + NaOH
‖ Hidden in HOD band, approximately 5.0 ppm
(t) triplet

The role of γ-glutamyl derivatives in higher plants is presently unknown. Some γ-glutamyl-α-amino acids can be produced in transglutamylation reactions.[12] However studies on the specificity of an enzyme preparation from kidney bean fruit catalyzing such transglutamylation reactions showed that proline could not take part in such reactions [12] Therefore the occurrence of γ-glutamylpipecolic acid as the first γ-glutamyl imino acid from natural source and especially from legume tissues where the transpeptidase activity is relatively high[12] indicates that other biosynthetic pathways or enzyme systems with broader specificity must exist

EXPERIMENTAL

Isolation of γ-L-*glutamyl*-L-*pipecolic acid* Finely ground seeds (2 kg) were extracted with 75% EtOH (30 l) at 25°. The combined extracts were applied to a strongly acidic ion-exchange resin (Amberlite

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CG 120, 100–200 mesh, 5 × 100 cm, H^+ form in 75% EtOH) The resin was first washed with 75% EtOH (6 l) and then with H_2O (5 l) The amino acids were eluted with 2 N NH_3 (4 l.) The ammonium eluate was taken to dryness under reduced pressure (26 g) The mixture of amino acids (in two portions of 13 g) was dissolved in HOAc (0 5 N) and applied to a strongly basic ion-exchange resin (Dowex 1 × 8, 200–400 mesh, 5 6 × 45 cm, acetate form) and the amino acids were eluted with 0 5 N HOAc (20 ml fractions). Glutamic acid appeared in fractions 85–120, aspartic acid in fractions 235–268 and the new compound in fractions 175–220 The fractions from both preparations containing the new product were taken to dryness and traces of acetic acid were removed by repeated evaporations from H_2O The solid was recrystallized from H_2O Yield of chromatographically pure sample 160 mg (Found C, 50·61, H, 7·09, N, 10·87 $C_{11}H_{18}N_2O_5$ requires C, 49·25, H, 6·72, N, 10 45%) $[\alpha]_D^{20}$ −66 8° (*c*, 0 5 in H_2O), $[\alpha]_D^{20}$ −49 4 (*c*, 0 45 in 1 N HCl) IR ν_{max}^{KBr} 3050 cm^{-1} (*s*), 2910 (*s*), 1700 (*m*), 1660 (*s*), 1605 (*s*), 1570 (*w*), 1495 (*s*), 1426 (*m*), 1406 (*m*), 1342 (*s*), 1309 (*m*), 1274 (*s*), 1241 (*s*) NMR The spectra of the compound in D_2O at pH < 1, D_2O, D_2O at pH 7 and D_2O at pH > 11 (see Table 1) were in agreement with the pattern expected for the glutamyl derivative of pipecolic acid. The spectrum at pH < 1 showed the protons from the 2 methylene groups in the glutamic acid part as a complex triplet at 2 3 ppm and a complex quartet at 2 7 ppm, the protons from the methylene groups in the pipecolic acid part showed a very complex pattern as for free pipecolic acid For the protons at the C(2) in both the glutamic acid and pipecolic acid parts, see Table 1

Hydrolysis with isolation of L-*glutamic acid and* L-*pipecolic acid* A soln of the peptide (90 mg) in HCl (5 ml, 1 N) was heated 2 hr at 90° and the soln was evaporated to dryness After 3 successive dissolutions and evaporations from H_2O, the residue dissolved in H_2O was applied to an acid ion-exchange resin (Dowex 50 W × 8, H^+ form, 200–400 mesh, 0 5 × 5 cm) After washing with H_2O (10 ml), the column was eluted with aq pyridine (6 ml, 1 N) The pyridine eluate was conc. to dryness, the residue dissolved in H_2O and applied to a strongly basic ion-exchange resin (Dowex 1 × 8, acetate form, 200–400 mesh, 0 5 × 5 cm) The column was washed with H_2O (10 ml) and then eluted with HOAc (0 5 N, 20 ml) The H_2O effluent was evaporated to dryness Recrystallization from H_2O–acetone yielded a colourless sample of L-pipecolic acid (28 mg) $[\alpha]_D^{20}$ −26 2° (*c*, 0 47 in H_2O), $[\alpha]_D^{20}$ −11·9°(*c*, 0 42 in 1 N HCl) Lit. values for L-pipecolic acid $[\alpha]_D^{23}$ −24 6° (*c*, 4 3 in H_2O),[13] for L-pipecolic acid, HCl $[\alpha]_D^{23}$ −10 5° (*c*, 9 8 in H_2O) [13] IR and NMR spectra were identical with those obtained for an authentic sample The HOAc eluate was evaporated to dryness, acetic acid was removed by repeated evaporations from H_2O, and the residue was recrystallized from H_2O to give a colourless sample of L-glutamic acid (32 mg) $[\alpha]_D^{20}$ +31 3° (*c*, 0 27 in 1 N HCl) Lit value for L-glutamic acid $[\alpha]_D^{25}$ +32° (*c*, 2 in 5 N HCl) [14] IR and NMR spectra were identical with those obtained for an authentic sample

General methods and instrumentation PC was performed in *n*-BuOH–HOAc–H_2O (12:3.5)(solvent 1), PhOH–H_2O-conc NH_3 (120.30·1) (w/v/v) solvent 2) and PhOH saturated at pH 4 2 [15] The following R_f values were found by the descending technique on Whatman No 1 paper in solvents 1, 2 and 3 respectively Pipecolic acid: 0·47, 0·93, 0·91 Glutamic acid 0·24, 0·30, 0·22 γ-Glutamyl pipecolic acid 0·46, 0·60, 0·74 HVE was carried out on Whatman 3 MM paper using a flat-plate unit (Shandon Model L 24) In buffer at pH 3 6,[16] 70 V/cm, 45 min, the mobilities towards the anode were in cm Glutamic acid 1 2, Aspartic acid 2 7, γ-Glutamylpipecolic acid 3 Chromatograms and electrophoresis papers were dipped in ninhydrin (0·2% in acetone) and in isatine [7,8] Dinitrophenylation with subsequent hydrolysis was performed as previously described [17] Microanalyses were performed by Mr G Cornali, Copenhagen Optical rotations were determined on a Perkin–Elmer Model 141 photoelectric polarimeter in 1 dm tubes IR spectra were determined in KBr pellets NMR spectra were measured on a JEOL C-60HL spectrometer, chemical shifts in ppm downfield from 2,3,4,4-tetra-deuterio-3-(trimethylsilyl) propionate in D_2O

Acknowledgements—The authors express their gratitude to Dr Djazirei, Department of Agriculture and Natural Resources, Teheran, Iran for providing seeds of *Gleditsia caspica* They wish to thank Professor P O Larsen for valuable discussions during the present work Part of this work was performed in Copenhagen during a stay of one of us (G D) made possible by the Danish Ministry of Education through a grant as part of the Belgian–Danish Cultural Agreement

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