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γ-GLUTAMYLWILLARDIINE AND γ-GLUTAMYLPHENYLALANYLWILLARDIINE FROM SEEDS OF *FAGUS SILVATICA**

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Abstract—γ-L-Glutamyl-L-willardiine [γ-L-glutamyl-3-(1-uracil)-L-alanine] and γ-glutamylphenylalanylwillardiine have been isolated from seeds of *Fagus silvatica* The structures have been established by spectroscopy, hydrolysis to give the constituent amino acids, and for the tripeptide end-group determination and partial hydrolysis to give the two constituent dipeptides

INTRODUCTION

IN THE course of a study of the free amino acids and peptides in seeds of *Fagus silvatica* L. (beechnuts) two new acid peptides were obtained.[1,2] The present paper describes the identification of these peptides as γ-L-glutamyl-L-willardiine (γ-L-glutamyl-(3-(1-uracil)-L-alanine)) and γ-glutamylphenylalanylwillardiine.

METHODS AND RESULTS

The partial isolation of the peptides has been described in the previous communication.[2] γ-Glutamylwillardiine was obtained analytically pure by crystallization from the appropriate fractions after ion exchange chromatography on a basic resin in the acetate form. The elemental composition was established by analysis. The UV spectrum was identical with that exhibited by willardiine [3-(1-uracil)alanine] (Table 1). The PMR-spectrum in D_2O exhibited the same two vinylic protons at δ 7·62 and 5·84 (d, J 7·5 Hz) as those found in willardiine.[2] In addition a triplet at δ 3·75 (α-proton in glutamic acid), a multiplet between δ 1·9 and 2·7 (four methylene protons in glutamic acid) and a multiplet at δ 4–5 (three protons in the side chain of willardiine) were observed. After hydrolysis with hydrochloric acid L-glutamic acid and L-willardiine could be isolated and identified by comparison with authentic material. The γ-linkage was established by PMR spectroscopy at different pH-values corresponding to different ionization states by a method recently described.[4]

* Taken in part from the thesis of I Kristensen, Copenhagen (1973) [1]

[1] KRISTENSEN, I (1973) Free Amino Acids and γ-Glutamyl Peptides in *Fagus silvatica* L, Thesis, The Royal Veterinary and Agricultural University, Copenhagen, Denmark

[2] KRISTENSEN, I, LARSEN, P O and SØRENSEN, H (1974) *Phytochemistry*, **13**, 2803

[3] SHUGAR, D and FOX, J J (1952) *Biochim Biophys Acta* **9**, 199

[4] KRISTENSEN, I and LARSEN, P O (1973) *Acta Chem Scand* **27**, 3123

TABLE 1 UV SPECTRA FOR WILLARDIINE AND WILLARDIINE-CONTAINING PEPTIDES

	Solvent					
	0·2 N HCl		H_2O		0·2 N NaOH	
	λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}
Willardiine*	262	9300	263	9400	266	7200
γ-Glutamyl-willardiine	265	9600	268	9900	266	7200
γ-Glutamyl-phenylalanyl-willardiine	265	9400	267	9500	266	6900
1-Methyluracil†			268	9700	265	7000

* Reference 2
† Reference 3

γ-Glutamylphenylalanylwillardiine was obtained in chromatographically homogeneous condition by preparative paper chromatography of the appropriate fractions after ion exchange chromatography. The UV spectrum was identical with that of willardiine The absorption expected from the phenylalanine moiety is too weak relative to that of willardiine to influence the spectrum (Table 1) The PMR-spectrum in D_2O showed the two vinylic protons in willardiine at δ 7·62 and 5·85 (*d*, *J* 7·5 Hz), the five aromatic protons in phenylalanine at δ 7·43 (*s*), four protons as a multiplet between δ 4 and 5 (the three protons in the side chain of willardiine and the α-proton in phenylalanine), the α-proton in glutamic acid as a triplet at δ 3·75, the benzylic protons in phenylalanine as a multiplet at δ 2·9–3·2 and the four methylene protons in glutamic acid as a multiplet between δ 1·8 and 2·7.

Hydrolysis in 6 N HCl for 20 hr at 100° in a sealed ampoule resulted into complete conversion into glutamic acid, phenylalanine, and willardiine as revealed by PC PC analysis of material subjected to partial hydrolysis (see Experimental) revealed the presence of unchanged starting material, of γ-glutamylphenylalanine, of glutamic acid, phenylalanine, and willardiine, and of a new ninhydrin-reactive compound. The new compound produced willardiine and phenylalanine on acid hydrolysis. Hence phenylalanine is the second amino acid in the tripeptide, being connected both to glutamic acid and to willardiine. End-group determination with dinitrofluorobenzene[5] established glutamic acid as the *N*-terminus PMR-spectra at different pHs supported the γ-linkage, which was established by the identification of γ-glutamylphenylalanine as a hydrolysis product The new compound is therefore unequivocally identified as γ-glutamylphenylalanylwillardiine. The configuration of the constituent amino acids has not been determined

The possibility has been considered that the new tripeptide is an artefact. γ-Glutamylphenylalanine and willardiine are the two dominating ninhydrin-reactive components in the beechnuts, and γ-glutamylwillardiine is also present in substantial quantities. The production of the tripeptide from γ-glutamylphenylalanine and either γ-glutamylwillardiine or willardiine was attempted in a number of experiments, but with no success (see Experimental) This indicates that the tripeptide is not produced during the isolation procedure It can however not be totally excluded that the tripeptide is produced in the seeds during storage The seeds must contain γ-glutamylphenylalanine and willardiine together in relatively high concentrations and have a relatively low water content

[5] SANGER, F (1945) *Biochem J* **39**, 507

DISCUSSION

Neither of the peptides has been identified previously in natural material. γ-Glutamylwillardiine is an example of the many naturally occurring γ-glutamyl derivatives of non-protein amino acids. The new compound occurs in *F. silvatica* together with a number of γ-glutamyl derivatives of protein amino acids.[2] The seeds of *F. silvatica* also contain substantial amounts of free L-willardiine.

Tripeptides have only rarely been identified in plants. Glutathione is supposed to occur in all living cells[6] and has been isolated from the beechnuts [2] γ-Glutamylcysteinyl-β-alanine (homoglutathione) has been isolated from *Phaseolus aureus.*[7] γ-Glutamylvalylglutamic acid has been isolated from *Juncus conglomeratus.*[8] γ-Glutamyl-*S*-(2-carboxypropyl)cysteinylglycine and γ-glutamyl-*S*-(prop-1-enyl)cysteinyl-*S*-(prop-1-enyl)-cysteine sulfoxide have been isolated from *Allium* species [9,10] γ-Glutamyl-γ-glutamylmethionine has been isolated from *Phaseolus aureus.*[11]

The occurrence of γ-glutamylphenylalanylwillardiine is rather surprising, especially because it contains a normal peptide bond to a non-protein amino acid. However, it cannot be completely excluded that the compound is produced by non-enzymatic processes.

EXPERIMENTAL

General methods and instrumentation have been described in the previous communication [2] Microanalyses were performed by Mr G Cornali and his staff

γ-L-Glutamyl-L-willardiine This compound was isolated from fraction (1 4 1) (130 mg) described in the previous communication [2] The residue was dissolved in excess aqueous ammonia (4 ml), the solution filtered, adjusted to pH 2 8 with 4 N HCl, and cooled to 10° and the precipitate collected by filtration (50 mg) Recrystallization from H_2O (3 2 ml) afforded an analytically pure sample (42 mg) (Found C, 41 19, H, 5 31, N, 16 14 $C_{12}H_{16}N_4O_7$, H_2O required C, 41·62, H, 5 24, N, 16·18% The contents of H_2O of crystallization was determined by drying over P_2O_5 and re-equilibration) $[\alpha]_D^{31}$ − 55 0° (*c* 1, 1 N HCl) M p decomp above 204° IR_{KBr} ν_{max} 3550 cm^{-1} (strong), 3480 (*s*), 3340 (*s*), 3040 (*s*), 2920 (medium), 2640 (*w*), 1710 (*s*), 1650 (*s*), 1610 (*m*), 1520 (*m*), 1490 (*s*), 1475 (*s*), 1440 (weak), 1415 (*s*), 1390 (*w*), 1370 (*m*), 1345 (*w*), 1335 (*m*), 1315 (*s*), 1295 (*w*), 1285 (*m*), 1255 (*s*), 1215 (*w*), 1205 (*w*), 1200 (*m*), 1160 (*m*), 1135 (*w*), 1110 (*w*), 1080 (*m*), 1030 (*m*), 995 (*m*), 925 (*w*), 850 (*m*), 830 (*w*), 810 (*m*), 780 (*w*), 765 (*w*), 725 (*m*), 715 (*w*), 670 (*w*), 620 (*w*), 585 (*w*), 555 (*s*), 520 (*m*), 470 (*m*), 455 (*m*), 435 (*w*), 405 (*w*), 375 (*w*), 360 (*m*), 305 (*m*) UV see Table 1 For PMR data in D_2O see Methods and Results PMR in 6% trifluoroacetic acid in D_2O δ 4 13 (1 H, *t*, α-CH in glutamic acid), in 0 4 NaOD in D_2O. δ 3 22 (1 H, triplet, α-CH in glutamic acid) R_f in solvent 1 (reference 2) 0 11, in solvent 2 0 30 10 mg Of the isolate was treated with 1 N HCl for 6 hr at 110° in a sealed ampoule After concentration to dryness and removal of excess HCl by repeated evaporations with H_2O, the residue was dissolved in H_2O and applied to Dowex 1 × 8 resin (0 5 × 5 cm, 200–400 mesh, AcO^-) The column was washed with H_2O and 0 05 N AcOH and eluted with 1 N AcOH The combined H_2O and 0 05 N AcOH effluents after concentration to dryness and recrystallization from H_2O yielded L-willardiine (3 8 mg), $[\alpha]_D^{23}$ − 18° (*c* 0 3, 1 N HCl) [Lit [12] $[\alpha]_D^{20}$ − 20° (*c* 2 0, 1 N HCl)] The 1 N AcOH eluate was concentrated to dryness, and recrystallization from EtOH–H_2O yielded L-glutamic acid (1 2 mg), $[\alpha]_D^{22}$ + 31 4° (*c* 0 08, 6 N HCl) [Lit [13] $[\alpha]_D^{25}$ + 31 8° (*c* 2, 5 N HCl)] The IR-spectra of the two amino acids were identical with those of authentic samples

γ-Glutamylphenylalanylwillardiine This compound was obtained from fraction (1 5) described previously [2] Preparative PC in BuOH–AcOH–H_2O (12 3 5) on Whatman No 3MM paper yielded a fraction (120 mg) containing the tripeptide contaminated with a UV-absorbing but non-ninhydrin reactive component Final purification was accomplished on Dowex 1 × 8 resin (0 9 × 60 cm, 200–400 mesh, AcO^-, 80 ml/hr, 5-ml fractions, elution with H_2O (25 ml), 0 5 N AcOH (375 ml), and 1 N AcOH 525 ml)) Fractions 100–130 were concentrated to dry-

[6] WALEY, S G (1966) *Adv. Protein Chem* **21,** 1
[7] CARNEGIE, P R (1963) *Biochem J* **89,** 459, 471
[8] VIRTANEN, A I and ETTALA, T (1958) *Acta Chem Scand* **12,** 787
[9] VIRTANEN, A I and MATIKKALA, E J (1961) *Suom Kemistilehti* **B 34,** 53
[10] MATIKKALA, E J and VIRTANEN, A. I (1966) *Suom Kemistilehti* **B 39,** 201
[11] KASAI, T, SAKAMURA, S, INAGAKI, S and SAKAMOTO, R (1972) *Agr Biol Chem* **36,** 2621
[12] DEWAR, J H and SHAW, G (1962) *J Chem Soc* 583
[13] GREENSTEIN, J P and WINITZ, M (1961) *Chemistry of the Amino Acids*, Vol 3, p 1929, Academic Press, New York

ness (54 mg), and recrystallization from water yielded the chromatographically pure tripeptide (35 mg) $[\alpha]_D^{22}$ −76° (*c* 1, H_2O). M.p. decomp. above 195°. IR$_{KBr}$ ν_{max}: 3310 (*s*), 3050 (*m*), 2920 (*m*), 1640–1690 (*s*), 1525 (*m*), 1455 (*w*), 1410 (*w*), 1355 (*w*), 1315 (*w*), 1240 (*m*), 1140 (*w*), 1110 (*w*), 1080 (*w*), 1030 (*w*), 990 (*w*), 870 (*w*), 820 (*m*), 775 (*w*), 750 (*w*), 700 (*m*), 665 (*w*), 550 (*w*), 520 (*w*), 475 (*w*), 415 (*w*). UV: see Table 1. For PMR-data in D_2O, see Results. PMR in 6% trifluoroacetic acid in D_2O: δ 4·03 (α-CH in glutamic acid), in 0·4 N NaOD: δ 3·15 (α-CH in glutamic acid). R_f in solvent 1 (reference 2): 0·28; in solvent 2: 0·65. Partial hydrolysis was performed with 2 N HCl on 0·1 mg samples in sealed ampoules at 50° for various periods. Excess HCl was removed by repeated evaporations with water and the reaction mixtures were analysed by two-dimensional PC. All traces of the tripeptide disappeared after 5 days of hydrolysis. γ-Glutamylphenylalanine was detected after 1 day but disappeared completely after 5 days of hydrolysis. Authentic γ-glutamylphenylalanine was also completely hydrolysed after 6 days. Phenylalanylwillardrine was detected after 1 day and had not disappeared after 7 days. A hydrolysis period of 2 days was selected to give a maximum yield of phenylalanylwillardrine. 1·3 mg of the tripeptide was therefore hydrolysed for 2 days under the conditions described above. Excess HCl was removed by repeated evaporations with water and the residue applied to Dowex 1 × 8 resin (0·5 × 6 cm, 200–400 mesh, AcO^-). Washing with water eluted willardrine. Elution with 1 N AcOH: The water effluent contained phenylalanine, willardrine, and phenylalanylwillardrine and the AcOH-eluate contained unchanged tripeptide, γ-glutamylphenylalanine, and glutamic acid as revealed by PC. After hydrolysis of the water effluent with 6 N HCl for 24 hr at 100° only phenylalanine and willardrine were present. 1 mg of the tripeptide dissolved in aq. Na_2CO_3 (0·2 ml, 0·15 M) was mixed with dinitrofluorobenzene (1 μl) in EtOH (0·2 ml). After stirring for 2 hr and removal of excess dinitrofluorobenzene by Et_2O extraction, the soln was applied to Dowex 50W × 8 resin (H^+). The effluent containing the dinitrophenyl derivative of the tripeptide was hydrolysed with 6 N HCl for 8 hr at 120° in a sealed ampoule. Excess HCl was removed by repeated evaporations with H_2O and an aq. soln of the residue applied to Dowex 50W × 8 resin (H^+). The effluent was analysed by PC on Whatman No. 1 paper in the PrOH–H_2O conc. NH_3 (8 : 1 : 1). The DNP-derivatives of glutamic acid, phenylalanine, and willardrine were synthesized for comparison. The following R_f-values were found: DNP-amino acid from the peptide 0·31, DNP-glutamic acid 0·31, DNP-phenylalanine 0·73, DNP-willardrine 0·41, 2,4-dinitrophenol 0·69, 2,4-dinitroaniline 0·79, dinitrofluorobenzene 0·80.

Attempts to produce γ-glutamylphenylalanylwillardrine from γ-glutamylphenylalanine and either γ-glutamylwillardrine or willardrine. (a) 1 mg of each of the dipeptides in 200 μl of 1 N AcOH at 50° for 11 days: no tripeptide formed. (b) Evaporation residue from a solution in water of 1 mg of each of the tripeptides and 3·5 mg of sucrose (to prevent crystallization), at 50° for 5 days: no tripeptide formed. (c) Evaporation residue from a solution in NH_3 aq. 1 M of 2 mg of γ-glutamylphenylalanine, 1 mg of willardrine, and 0·5 mg of sucrose, at 50° for 4 days: no tripeptide formed. (d) A soln of 2 mg of γ-glutamylphenylalanine + 1 mg of willardrine in 1 N AcOH was applied to a small column of Dowex 50W × 8 resin. After washing with H_2O the amino acids were left on the column at room temp. for 7 days and eluted with NH_3 aq. 1 M: no tripeptide formed (but significant amounts of glutamic acid and phenylalanine). (e) A solution of 2 mg of γ-glutamylphenylalanine + 1 mg of willardrine + 1 mg of KCl in aq. AcOH was applied to a small column of Dowex 50W × 8 resin. Without washing with H_2O the amino acids were left on the column for 7 days and eluted with NH_3 aq. 1 M: no tripeptide formed (but significant amounts of glutamic acid and phenylalanine). (f) 20 mg of γ-glutamylphenylalanine and 10 mg of willardrine refluxed in 0·5 ml of H_2O for 7 hr: no tripeptide formed (but significant amounts of glutamic acid and phenylalanine). (g) Evaporation residue from a solution of 2 mg of γ-glutamylphenylalanine, 1 mg of willardrine and 10 μl of propylene glycol (to prevent crystallization), at 50° for 7 days: no tripeptide formed. (h) A soln of 11 mg of γ-glutamylphenylalanine in 50 μl of H_2O at 50° for 7 days: no decomposition.

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