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N-γ-L-GLUTAMYL-β-D-AMINOPHENYLPROPANOIC ACID, A DIPEPTIDE FROM THE AQUATIC FERN, *AZOLLA CAROLINIANA*

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Key Word Index—*Azolla caroliniana*; Azollaceae; water fern; dipeptide; γ-glutamyl-β-aminophenylpropanoic acid; β-phenylalanine.

Abstract—A novel dipeptide, γ-L-glutamyl-β-D-phenylpropanoic acid has been isolated from extracts of the water fern, *Azolla caroliniana*.

Azola is a genus of heterosporous aquatic ferns which contain an N_2-fixing cyanobacterium (*Anabaena azollae*) as a symbiont [1]. Examination of boiling water extracts of *Azolla caroliniana* for amino acids, utilizing precolumn derivitization with *o*-phthalaldehyde/thiol reagent (OPT) and HPLC, revealed an unknown. This substance was a major constituent, and was shown to occur in the fern rather than the cyanobacterium [1]. Its abundance, along with our interest in soluble nitrogenous compounds and nitrogen metabolism in the symbiosis, prompted us to isolate and identify it.

The unknown behaved as an acidic amino acid, and was purified by ion exchange techniques. After crystallization, it analysed as $C_{14}H_{18}N_2O_5$. UV indicated the presence of an aromatic ring, but the low extinction values ruled out direct attachment of any heteroatom or carboxylate group. Rather mild acid hydrolysis yielded glutamic acid (Glu), which was identified by both PC and HPLC(OPT). That the remaining fragment was indeed $C_9H_{11}NO_2$ was confirmed by isolation and analysis. Unlike the original material, the hydrolysis product gave only a very weak ninhydrin reaction (PC) unless heated, and was therefore apparently not an α-amino acid. It behaved as a neutral amino acid, and was eventually shown to be β-aminobenzenepropanoic acid (β-Phe) by comparison to an authentic sample using PC, HPLC(OPT)† and NMR.

Thus, the unknown was a dipeptide composed of Glu and β-Phe. The ninhydrin reactivity indicated that the amino group of Glu (rather than β-Phe) was free, with the amide bond through the nitrogen of the β-Phe moiety. The relative ease of hydrolysis plus the reaction with ninhydrin to give 1 mole of CO_2 indicated that the peptide bond involved the γ-carboxyl (rather than α) of Glu (an α-glutamyl derivative would not have decarboxylated [2]. Not surprisingly, the configuration of the Glu was found to be L. However, that of the β-Phe moiety was shown to be D.

Based on the above evidence, the dipeptide is γ-L-glutamyl-β-D-phenylpropanoic acid (γ-Glu-β-Phe, **1**, HO_2C–$CH(NH_2)$–CH_2–CH_2–CO–NH–$CH(Ph)$–CH_2–CO_2H. While the isomeric form γ-Glu-Phe (derived from phenylalanine) has been reported to occur in plants such as garlic [3] and soybeans [4], the β-Phe dipeptide **1** has not previously been reported from any source. Since **1** was undetected or present in only trace amounts in other *Azolla* species [unpublished results], it is not a common constituent of the genus.

EXPERIMENTAL

For paper chromatography (descending, Whatman No. 1 paper) the solvent systems were: 1. *n*-BuOH–HOAc–H_2O (12:3:5); 2. *n*-BuOH–pyridine–H_2O (1:1:1). Amino acids were determined using the HPLC/OPT methods utilized in our earlier work [1]. All evaporations were done at < 40° using a rotary vacuum evaporator.

Isolation and purification. Ca 400 g (blotted fr. wt) of *A. caroliniana* Willd. was extracted with H_2O (1 l.) in a boiling H_2O bath for 10–15 min. The extract was filtered (0.4 μ membrane), several drops of toluene added, and stored in the cold until needed. The extract from *ca* 2.5 kg of material was put on a 4.7 × 14 cm AG-50 × 2 column (200–400 mesh, H^+ form) and washed well with H_2O (7 l.). After about half the washing was completed, the upper, dark layer of resin was stirred to break up any clumps. Amino acids were then eluted with 2 N NH_4OH and collected in 800 ml (beginning when the dark band was *ca* 2/3 down the column). The eluate was concd to 150 ml, and HPLC/OPT indicated *ca* 900 μmol of **1** present.

This concentrate was next put on a 2.5 × 24.5 cm AG-1 × 2 column (200–400 mesh, OAc^- form), and neutral amino acids washed off with H_2O (550 ml). Elution with 0.5 N HOAc yielded Glu (125–375 ml), followed by **1** (475–675 ml) as monitored by HPLC/OPT. (The latter fractions of **1** overlapped with ASP, but as long as the ASP content was less than 20 mol % of the total in the pooled fractions, it could be removed by recrystallization.)

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†The low fluorescence response (HPLC-OPT) of β-Phe resulted in this component being missed in initial hydrolytic reactions. This plus the weak ninhydrin reaction initially led us to believe that the hydrolysis product possessed a secondary rather than primary amino group.

The pooled material was evaporated to dryness and the residue recrystallized from H_2O (18 ml) to give 214 mg of **1** as off-white stout needless (mp 217–219°). Essentially all of the ASP remained in the mother liquor. An analytical sample was vacuum-dried at 100°. Found: C, 57.5; H, 6.19; N, 9.47. $C_{14}H_{18}N_2O_5$ requires: C, 57.14; H, 6.16; N, 9.52%. PC gave a single ninhydrin-positive spot at room temp., R_f 0.59 (solv. 1), 0.40 (solv. 2), and no reaction with isatin. UV $\lambda_{max}^{H_2O}$ (ε): 268 nm (sh, 110), 262 (168), 256 (220), 251 (188), 246 (sh, 140), 241 (sh, 111).

Hydrolysis. (A) Compound **1** (6.93 μmol) in 2 ml 1 N HCl was heated overnight at 110° (sealed tube, argon). PC revealed two ninhydrin positive spots (R_f 0.59, 0.40, solv. 1; 0.28, 0.22, solv. 2). The faster running spot gave little ninhydrin colour unless heated for several min (110°). The slower running component was identical to Glu (PC) and gave the same colour with isatin. HPLC(OPT) also showed a peak corresponding to Glu (0.94 mol/mol of **1**). A subsequent hydrolysis showed that very little **1** remained under even milder conditions (0.5 N HCl, 7 hr, 110°).

(B) A preparative scale hydrolysis was run on 100 mg of **1** (5 ml 1 N HCl). The crude product was dried over NaOH, and chromatographed on AG-1 × 4 (0.5 × 10 cm, OAc^- form) to remove Glu. The H_2O eluate (9 ml) contained the desired hydrolysis product, which was further purified on AG-50 × 2 (0.5 × 9.5 cm, H^+ form). Elution with 2 N NH_4OH (12 ml) gave 47 mg (84%). Crystallization from H_2O (1 ml) gave 34 mg (mp 223.5–224.5°, dec.).

An analytical sample was vacuum dried at 100°. Found: C, 65.04; H, 6.69; N, 8.39. Calc. for $C_9H_{11}NO_2$: C, 65.4; H, 6.71; N, 8.51%. This material was indistinguishable from authentic β-Phe (Aldrich Chemical Co.) by PC (solvents 1 and 2), by ^1H NMR (50% TFA–D_2O solvent), and by HPLC(OPT) at both pH 5 and 7. (β-Phe and Phe effectively coelute under our normal conditions (pH 7.0), but are resolved at pH 5.0) Re-examination of the hydrolysis mixture from A (above) by HPLC(OPT) showed 1.00 mol β-Phe formed per mol of **1**. Elution of the AG-1 × 4 column (above) with 0.5 N HOAc (10 ml) gave Glu (41 mg, 90%) after recrystallization from EtOH–H_2O.

Ninhydrin oxidation [2]. A soln of the dipeptide (1 ml), and 25 mg of ninhydrin were sealed in a 6 ml 'Hypovial', evacuated and placed in a boiling H_2O bath for 10 min. After cooling to room temp. and pressurizing to 1 atm, an aliquot of the gas phase was analysed for CO_2 by GLC (Porapak Q, 42°, He, TC detector). Calibration was achieved by substituting 1 ml of 10 mM Glu for the dipeptide soln. The yield of CO_2 was 1.17 mol/mol of dipeptide (duplicate samples).

Configuration of Glu. A sample of the Glu from hydrolysis of **1** was incubated (25°) with L-glutamic decarboxylase (type V, Sigma) at pH 4.7 (acetate), and periodically analysed by HPLC(OPT). After *ca* 90 min, less than 2% of the original GLU remained, and a peak corresponding to γ-ABA appeared [5].

Configuration of β-Phe. The isolated β-Phe exhibited circular dichroism identical to that of authentic D(−)-β-Phe and opposite that of the L(+)-isomer. The D- and L-isomers used were obtained from the racemic acid (Aldrich Chemical Co.) according to the lit. [6].

Documentation of plant materials. Azolla caroliniana Willdenow; Azollaceae or Salviniaceae; voucher specimens and living material deposited with curator of ferns, New York Botanical Garden. Taxonomy of new world species in revision by Fowler, Portsmouth Polytechnic, U.K. State of taxonomic understanding in genus *Azolla* has been reviewed [7].

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