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γ-GLUTAMYLPEPTIDES FROM *PHILADELPHUS CORONARIUS*

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Key Word Index—*Philadelphus coronarius*; Saxifragaceae; γ-glutamylpeptides; unsaturated amino acids.

Abstract—Two new γ-glutamylpeptides (γ-L-glutamyl-L-2-amino-3-methylenepentanoic acid and γ-L-glutamyl-2-amino-3-methylene-4-pentenoic acid) and one new unsaturated amino acid, 2-amino-3-methylene-4-pentenoic acid have been isolated from leaves of *Philadelphus coronarius*. Structures have been determined by chemical and physical methods.

INTRODUCTION

In the course of our investigations of the free amino acids and peptides of higher plants, we have identified in the leaves of *Philadelphus coronarius* several new uncommon compounds which give yellow or purple colours with ninhydrin. These compounds are described in the present paper.

RESULTS AND DISCUSSION

2D-PC and high voltage electrophoresis surveys revealed that leaves of *P. coronarius* contain eight to nine unusual amino acids or glutamylpeptides. We have now identified four which occur in relatively large concentrations (**1**–**4**). **1** and **2** give purple colouration with ninhydrin. At pH 3.6, **1** and **2** are more acidic that aspartic acid; **2** is more acidic that **1**. **3** and **4** are revealed on ninhydrin-treated chromatograms as spots coloured yellow, brown and then purple after heating for 20 min at 100°. This is an indication that these compounds are unsaturated. They are neutral at pH 3.6. On PC, only proline reacts with isatin and none bleached iodoplatinate reagent. Another neutral compound, **5**, is coloured yellow–orange with ninhydrin but it has not been isolated.

Leaves were then extracted with 75% (v:v) ethanol–water; the extract was treated with Amberlite CG 120, pyridine form and the amino acids were eluted with 1 N pyridine. The eluate was concentrated, dissolved in water and applied to a column of Dowex 1 × 8, acetate form, washed with water. Elution with water gives neutral amino acids. Acidic amino acids and peptides were separated with 2 N HOAc. **1** was eluted first and crystallized, **2** was eluted together with some **1** but was pure in the last fractions. **2** was lyophilized. Further fractionations of neutral amino acids were carried out on Dowex 1 × 2, Cl^-; separation on a cellulose column and by PC gave a few milligrams of **3** and **4**. Elementary analysis indicated for **1** the formula $C_{11}H_{18}O_5N_2$, with one double bond, and for **2** the formula $C_{11}H_{16}O_5N_2$, with two double bonds or one triple bond. Unsaturation was also confirmed by the instability of **1** and **2** to treatment with acidic permanganate and bromine.

The IR spectra show absorption bands characteristic of dicarboxylic amino acids or γ-glutamylpeptides. They also support the presence of a CH_2=CHR or CH_2=CRR′ group at 909 cm^{-1} for **1** and 915 cm^{-1} for **2**. Levenberg [1] reported that the $=CH_2$ group in β-methylenenorvaline was characterized by absorption peaks at 909 and 1690 cm^{-1}. **1** and **2** are completely hydrolysed by heating with 2 N HCl for 3 hr at 100°; this lability to dilute mineral acid is typical of γ-glutamyl-amino acids. Treatment of **1** results in the production of L-glutamic acid and an unusual compound, **3**, having yellow–brown, similar ninhyrdin chromophores as unsaturated amino acids. This product moves on PC, with different solvents, like 2-amino-5-methyl-4-hexenoic acid or 2-amino-3-methylenepentanoic acid (β-methylenenorvaline). An aliquot was taken for analysis with a LKB 4400 amino acids analyser and revealed an approximate 1:1 ratio of glutamic acid and β-methylenenorvaline. Treatment of **2** also results in the production of glutamic acid and of a similar new compound inseparable from **4**. **2** is very unstable and it was impossible to isolate **4**. Hydrogenation using Adams Platinum dioxide catalyst converts **1** and **2** to a unique spot, purple with ninhydrin, and located on 2D-PC at the same place as γ-glutamylleucine or γ-glutamylisoleucine. Microhydrogenation of **1** results in the absorption of one mole of hydrogen; **2** absorbed 1.5–2 moles of hydrogen. Hydrolysis of hydrogenated **1** and **2** results, for both, in the production of glutamic acid and a 1:1 mixture of isoleucine and allo-isoleucine. This was confirmed by profile elution of amino acids from the LKB 4400 analyser.

The structure of **1** was confirmed by isolation of products of acid hydrolysis of a large quantity of peptide and by comparisons of IR, ^1H NMR and ^{13}C NMR spectra and optical rotation measurements with those of authentic materials. **1** is therefore γ-L-glutamyl-L(+)-2-amino-3-methylenepentanoic acid. The γ-glutamyl structure of both peptides was confirmed by ion-exchange chromatography and NMR studies at different pH [2]. IR and NMR spectra allowed us to assign the structure of **3** as β-methylenenorvaline. The formula of **2** is more difficult to establish. The above results strongly suggest that the neutral fraction of hydrogenated **2** has an isoleucine skeleton and that the unsaturated amino acid

has a terminal acetylenic linkage or two double bonds (allene or conjugated diene). When **2** is oxidized with acidic potassium permanganate, no β-methylaspartic acid or other amino acid was identified; the acetylenic linkage seems then impossible.

From ^1H and ^{13}C NMR spectra, results are consistent with the structure:

$$\underset{1}{COOH}-\underset{2}{CH(NH_2)}-\underset{3}{CH_2}-\underset{4}{CH_2}-\underset{5}{CO}-NH-\underset{6}{CH}(\underset{7}{COOH})-\underset{8}{C}(=\underset{9}{CH_2})-\underset{10}{CH}=\underset{11}{CH_2}$$

^1H NMR spectra were determined in D_2O and in D_2O + TFA (pH < 1) containing 2,2,3,3-tetradeuterio-3-trimethylsilylpropionate as an internal standard. It showed a triplet at δ 3.84 (C-2), and multiplets at 1.95–2.30 (C-3) and 2.35–2.65 (C-4). In D_2O, the proton at C-6 is completely masked by the DOH peak but it appears as a shoulder at δ 4.92 in D_2O + TFA. The four olefinic protons (C-9, C-11) showed a multiplet at δ 5.00–5.45 and the proton at C-10 showed a quadruplet at δ 6.37. These values are in excellent agreement with those found by Suhr [3] for a similar structure. The absence, near δ 1.5, of a singlet for a methyl group is also observed. By a combination of chemical and physical methods, compounds were shown to be: **3**, L(+)-2-amino-3-methylenepentanoic acid; **2**, γ-L-glutamyl-2-amino-3-methylene-4-pentenoic acid and **4**, 2-amino-3-methylene-4-pentenoic acid.

More than twenty unsaturated aliphatic α-amino acids have been discovered in the plant kingdom. Many have been isolated from fungi; in particular, β-methylenenorvaline has been reported in *Lactarius helvus* [4]. Very few unsaturated amino acids have been found in the form of the γ-glutamylpeptides. Examples are γ-glutamyl-2-amino-4-methyl-4-hexenoic acid [5], γ-glutamyl-β-(methylenecyclopropyl)alanine [6] and γ-glutamyl-α-(methylenecyclopropyl)glycine [7].

EXPERIMENTAL

Material. Fresh leaves of *P. coronarius* were collected in June in Gembloux. A voucher specimen is deposited in the department of Chimie organique et Biologique.

General. IR were measured in KBr discs. ^{13}C NMR were recorded on a Brucker 15.08 MHz and the chemical shifts are given as δ values (ppm) with TMS as standard.

Chromatography and electrophoresis. 2D-PC were carried out using *n*-BuOH–HFo–H_2O (15:3:2, solvent 1), PhOH–H_2O in the presence of NH_3 vapour (8:2, solvent 2) and PhOH saturated with buffer pH 4.2 (citric acid–$Na_2HPO_4 \cdot 2H_2O$, 0.08 M, solvent 3). R_{Al} values in solvent 1 are: 1.2 (1), 1.1 (2), 1.5 (3), 1.5 (4), 1.5 (5); in solvent 3: 1.7 (1), 1.4 (2), 1.9 (3), 1.5 (4), 1.1 (5). High voltage electrophoresis was carried out at pH 3.6, 70 V/cm, 90 min.

Isolation of **1** *and* **2**. Leaves of *P. coronarius* (4400 g) were extracted with 85% EtOH and filtered. The filtrate was passed through columns of Amberlite CG 120, pyridine form. The resin was washed with EtOH and H_2O, amino acids were then eluted with 1 N pyridine. The eluate was concd (12 g) and the residue was diluted in H_2O. It was then applied to a column of Dowex 1 × 8 (4 × 60 cm). Elution with H_2O gives neutral amino acids and elution with 0.5–2 N HOAc gives acidic compounds, **1** (110 mg) and **2** (150 mg). **1** (Found: C, 50.80; H, 6.93; N, 10.60. $C_{11}H_{18}N_2O_5$ requires C, 51.16; H, 6.98; N, 10.89%.) $[\alpha]_D^{20} = +87.2°$ (*c* 2.05; H_2O), $+108°$ (*c* 2.00, 1 N HCl). **2** (Found: C, 50.90; H, 6.05; N, 10.40. $C_{11}H_{16}N_2O_5$ requires C, 51.56; H, 6.25; N, 10.94%.) The neutral fraction containing **3** and **4** was applied to a column of Dowex 1 × 2, Cl^- (4 × 95 cm), and eluted with H_2O. **3** and **4** were then separated on a column of cellulose (7 × 40 cm), eluted with *n*-BuOH–HFo–H_2O (15:3:1). Purification was achieved by preparative chromatography. **3**: $[\alpha]_D^{20} + 119°$ (*c* 0.98, H_2O), $+167°$ (*c* 0.9, 1 N HCl). Glutamic acid from peptides: $[\alpha]_D^{20} + 20.1°$ (*c* 0.88, H_2O); commercial glutamic acid: $[\alpha]_D^{20} + 17.7°$ (*c* 2, H_2O).

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