A New γ-Glutamyltripeptide in *Juncus* Species

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Some years ago an acidic peptide was found in this laboratory in the ethanol extract of *Juncus conglomeratus* (Fig. 1). On a two-dimensional paper chromatogram the acid gave a violet spot on spraying with ninhydrin. After strong hydrolysis (6 N HCl, 20 h, 108°C), as well as after a mild one (0.5 N HCl, 10 h, 100° C), the spot was not detected on the chromatogram any longer, and hence it was apparently an easily decomposed peptide. The peptide was isolated in fairly pure form and was found to contain an easily split off glutamic acid molecule [1]. This peptide has now been isolated in completely pure form and its structure has been established.

For the isolation of the peptide 5 kg of *Juncus conglomeratus* was extracted in 70 % ethanol. The ethanol extract was run through an Amberlite IR-120 column. Amino acids were eluted by 1 N NH_4OH. When ammonia had been distilled off *in vacuo*, the residue was dissolved into 100 ml of water. The insoluble substance was filtered off and the filtrate was run through an Amberlite IR-45 column in acetate form. Neutral and basic amino acids were removed by water and the acidic ones by 0.5 N acetic acid. The unknown peptide (I) remained in the fraction with acidic amino acids, being accordingly acidic. After acetic acid had been distilled off there also was a substance (II) in the fraction of acidic amino acids which at first turned yellow on spraying with ninhydrin and then violet. This substance had apparently been formed through the partial decomposition of the original peptide. This peptide (I) was separated from the acidic amino acids on a cellulose powder column using butanol-acetic acid-water (630—100—270) as solvent. 160 fractions of 10 ml were taken and fractions 130—158 contained only the peptide without any free amino acids. These fractions were combined and the solvent was distilled off. The residue obtained was brown, however.

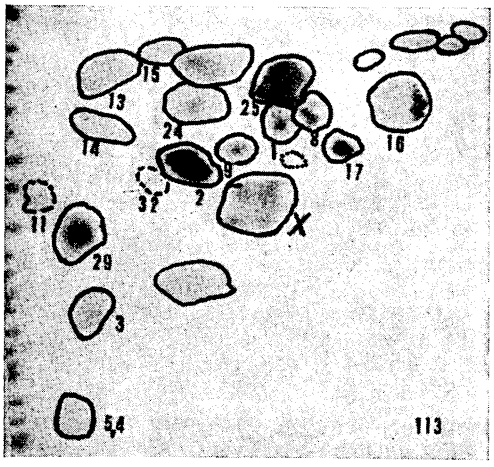

Fig. 1. Paper chromatogram of free amino acids (and some lowmolecular peptides) in *Juncus conglomeratus*. Solvents in text. × = unknown acidic peptide, 1 = gly, 2 = ala, 3 = val, 9 = threo, 16 = asp, 17 = glu, 29 = γ-amino-butyric acid.

As another method Dowex 1 resin (200—400 mesh, in acetate form) was used for the separation. About 100 mg of amino acids, eluted by 1 N NH_4OH from the Amberlite IR-120 column, were dissolved into 2 ml of 0.5 N acetic acid, and the solution was run through the Dowex 1 column (0.9 × 30 cm). The elution was performed by 0.5 N acetic acid and 1 ml fractions were taken. Also by this method the unknown peptide could be isolated, but the residue obtained after acetic acid had been distilled off was still brown.

After this both preparations were combined and once again run through the cellulose powder column, using water-saturated butanol as solvent. Now the brown impurity emerged from the column before the peptide, and the peptide-containing fractions were uncoloured. When the solvent was distilled off, the residue was white. The yield of peptide I was 160 mg.

When fractionating the original peptide from the cellulose powder column, the substance II was also collected. Since also this fraction was slightly coloured it was once again run through the cellulose powder column with water-saturated butanol. The yield obtained was 45 mg.

When hydrolysing the original peptide with 0.5 N HCl for 20 h at 100°C, glutamic acid was formed, as well as the substance

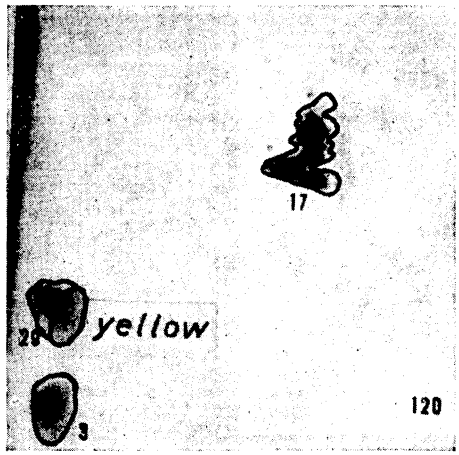

Fig. 2. Paper chromatogram of the unknown peptide after hydrolysis with 0.5 N HCl for 20 h at 100°C. 17 = glu, yellow = dipeptide formed on hydrolysis, 29 = added γ-amino-butyric acid, 3 = added val.

II which turned yellow on spraying with ninhydrin, and later on violet (Fig. 2). On hydrolysis of this substance in strong acid (6 N HCl, 20 h, 108°C) glutamic acid and valine were formed, and hence this substance also is a peptide. The same amino acids were also formed when hydrolysing the original peptide in strong acid.

The amounts of amino acids formed on hydrolysis were determined quantitatively using the copper salt method [2]. It was then found that on hydrolysis in dilute acid one molecule of glutamic acid was split off from the original peptide, and from the substance II one molecule glutamic acid and one molecule valine on hydrolysis in strong acid. The latter hydrolysis was not quite complete.

Tripeptide (I) (mild hydrolysis)	49	μg	glutamic	acid,	calc.	value*	50 μg
	45	»	»	»	»	»	50 »
Dipeptide (II) (strong hydrolysis)	25	»	»	»	»	»	39 »
	23	»	valine		»	»	31 »

The *molecular weight* of the original peptide, determined according to the method of Childs [3], was 341 (calc. value for a tripeptide containing two glutamic acid molecules and one valine molecule is 375). Glycyl-L-tyrosine, mol. wt. 238, was used as reference substance. The value obtained can be considered satisfactory when the low molecular weight of the known substance is taken into consideration.

The *nitrogen content* of the original peptide was determined by Kjeldahl according to Beet [4]; found 10.9 % N, calc. value for a tripeptide containing two glutamic acid molecules and one valine molecule ($C_{15}H_{25}O_8N_3$) 11.2 %.

The nitrogen content of the dipeptide formed on hydrolysis of the tripeptide in dilute acid was 10.81 %, calc. value for a dipeptide containing glutamic acid and valine ($C_{10}H_{18}O_5N_2$) 11.38 %.

R_F-values:	BuOH—AcOH—H_2O	Phenol
Tripeptide	0.31	0.49
Dipeptide	0.38	0.84

The position of glutamic acid and valine in the dipeptide was determined according to the method of Sanger [5]. It was found that the amino group in valine is free. Accordingly, the dipeptide is valyl-glutamic acid. Since the first glutamic acid molecule is split off from the tripeptide already on hydrolysis in dilute acid, it has to be linked to the dipeptide by its γ-carboxyl group, and the structure of the tripeptide is thus γ-glutamyl-valyl-glutamic acid HOOC · $CH(NH_2)$ · CH_2 · CH_2 · CO · NH · $CH(C_3H_7)$ · CO · NH · $CH(C_3H_5O_2)$ · COOH [6].

The tripeptide has been found by paper chromatography also in *Juncus effusus* and *Juncus filiformis*. There is about 100 mg/1 kg fresh plants of the tripeptide in *Juncus conglomeratus*.

Thanks are due to Miss Pirkko Kovakoski for her assistance in the isolation of the peptide and in the closer investigation of it in the first stage of the work in 1955.

* In the calculated values the original peptide has been supposed to be a tripeptide containing two glutamic acid molecules and one valine molecule.

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Mass Spectrometric Evidence Regarding the Structural Relations between Dextropimaric, *Iso*dextropimaric, and Cryptopimaric Acids *

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A study of the mass spectra of the methyl esters of rosin acids, performed with a high-mass, high-resolution mass spectrometer with heated inlet system [1], has provided new evidence regarding the structural relations between dextropimaric acid, *iso*dextropimaric acid, and cryptopimaric acid [2].

The mass spectrum of the methyl ester of cryptopimaric acid (Fig. 1a) is strikingly similar to that of the methyl ester of dextropimaric acid (Fig. 1b). The base peak of the spectra is at *m/e* 121, and strong peaks occur at *m/e* 316 (molecular peak), 180, 181, 241, 257, and 301. The methyl ester of *iso*dextropimaric acid gives a quite different mass spectrum (Fig. 1c) that has the base peak at *m/e* 241 and strong peaks at *m/e* 316 (molecular peak), 256, 257, 287, and 301.

We interpret these results as showing that dextropimaric acid and cryptopimaric acid can be structurally different only with regard to the orientation of the methyl and vinyl groups attached to C7, and that dextropimaric acid and *iso*dextropimaric acid must be different with regard to the geometry of the ring system. The opinion of Wenkert [3] that the two last mentioned acids differ in the configuration at C13 is thus supported by the present evidence: As the proposed stereochemical difference at C7 [4] has recently been confirmed [5] it follows that the acids are stereochemically different both at C7 and C13.

The above conclusions seem to tally with monolayer [6] and infrared absorption [7-9] data.

Experimental. The cryptopimaric acid used had $[\alpha]_D^{12} - 21.7°$ and m.p. 160—162°.** Because of the small amount of material, the optical rotation was not checked by us. Keimatsu *et al.*[2] give m.p. 159—161° and $[\alpha]_D^{17.5} - 18.99°$ (in ethanol). The sample of dextropimaric acid had m.p. 207—208°, $[\alpha]_D^{25} + 70° \pm 3°$ ** (ethanol, micro tube), and the *iso*dextropimaric acid m.p. 160°, $[\alpha]_D^{25} \pm 0.00°$ ** in ethanol. The acids were converted into methyl esters by means of fresh diazomethane in ether solution. The mass spectra were run on 0.5 mg samples with the inlet system kept at a temperature of 200°.

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* This work was reported at the meeting of the Swedish Biochemical Society at Uppsala, June 6—7, 1958.

** Determined by us.