Pergamon

PII: S0031-9422(98)00025-9

Phytochemistry, Vol. 48, No. 4, pp. 643–645, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

γ-GLUTAMYL-*S*-ETHENYLCYSTEINE: A DIPEPTIDE FROM *VICIA NARBONENSIS*

DIRK ENNEKING,† IAN M. DELAERE and MAX E. TATE*

Department of Plant Science, Waite Agricultural Research Institute, The University of Adelaide, Glen Osmond 5064, South Australia; † Co-operative Research Centre for Legumes in Mediterranean Agriculture, The University of Western Australia, Nedlands, 6009, Western Australia

(*Received in revised form* 8 *December* 1997)

Key Word Index—*Vicia narbonensis*; Vicieae; Narbon bean; γ-glutamyl-*S*-ethenylcysteine; photolabile; chemotaxonomy; non-protein amino acid; off-flavour precursor; grain legume.

Abstract—γ-Glutamyl-*S*-ethenylcysteine has been isolated and crystallised from aqueous ethanolic extracts of the seeds of Narbon bean, (*Vicia narbonensis*). The γ-glutamyl peptide is present at 0.7% in the Australian Temperate Crops Collection cultivar ATC 60105. Depending upon genotype and environment, seeds with up to 3% peptide have been observed. This photolabile peptide is the precursor responsible for the repulsive odour of germinating Narbon beans. It is a useful chemotaxonomic character for Vicieae, as well as a significant storage form of seed sulfur. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In general, legumes are usually deficient in sulfur-containing amino acids [1]. The Narbon bean, (*Vicia narbonensis*) is an exception, with an extractable total sulfur content of ca 0.1–0.2% (dry wt) of the seed. It is, used in some societies for human consumption, is a minor Mediterranean grain legume and forage crop, with good adaptation to southern Australian dryland farming systems [2].

The sulfur containing γ-glutamyl peptide described in this paper was initially observed in extracts of Narbon bean during an investigation of antinutritional factors in *Vicia* species using a porcine feed intake bioassay (Enneking, D., personal communication). It appeared as a strong UV (254 nm) absorbing ninhydrin-positive spot on paper electrophoretograms of ethanolic extracts. It shows the electrophoretic characteristics of the chemotaxonomic character previously reported by Bell and Tirimanna as VA_3 in *V. narbonensis* [3]. Structural assignment was based upon its elemental analysis, electrophoretic-charge properties and spectroscopic analysis. These data are consistent with a γ-glutamyl derivative of S-ethenylcysteine. The absolute molecular structural configuration of the two chiral centres and confirmation of the γ-glutamyl substituent sequence are the subject of an X-ray crystallographic study. S-Ethenyl cysteine, the non-protein amino acid moiety of the peptide, again of uncertain chirality, has been previously reported as a component of *Olax phyllanthi* [4].

RESULTS AND DISCUSSION

Seeds were extracted with 30% ethanol and examined by paper electrophoresis to reveal a previously uncharacterised cationic (pH 1.76), ninhydrin-positive, Ehrlich-positive, UV absorbing spot, with an electrophoretic mobility which was intermediate between γ-glutamyl-β-cyanoalanine [5] and glutamic acid. Irradiation of the air-dried electrophoretogram with UV light (254 nm) yielded a strong sulphurous odour from the UV absorbing band, which was similar to that from the germinating seed. A similar repulsive odour is observed when *O. phyllanthi* leaves are crushed.

The unknown non-protein amino acid was isolated and its structure established in the following way. Paper electrophoresis consistently showed a ninhydrin-positive spot with relative mobilities towards Orange G (RM O.G = −0.43, pH 1.7; RM O.G = +0.74, pH 5.0) indicating its suitability for isolation by anion-exchange chromatography. The relative electrophoretic mobility/pH behaviour is similar to that of γ-glutamyl-β-cyanoalanine [5] and is consistent with the presence of a γ-glutamyl-α-amino acid dipeptide, exhibiting two carboxyls with two overlapping pK_a values near 2.1 and 3.7. The alternative α-glutamyl-α-amino acid dipeptide would be expected to have two overlapping pK_a values near 3.0

* Author to whom correspondence should be addressed.

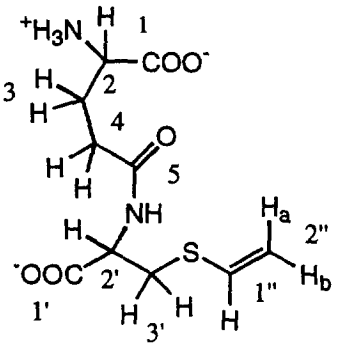
and 4.7 [6]. Examination of the column eluate by paper electrophoresis showed the presence of three ninhydrin-positive components, glutamic acid and two photolabile, UV absorbing and Ehrlich-positive spots. The major fraction contained the γ-glutamyl peptide and a minor component (RM O.G = −0.54, pH 5.0), whose electrophoretic and UV spectrum were consistent with the non-protein amino acid, *S*-ethenylcysteine [4]. The γ-glutamyl peptide was recrystallised from water-iso-propanol.

Micellar electrokinetic chromatography (MEKC) as its FMOC derivative, confirmed the homogeneity of the crystalline γ-glutamyl peptide with RR_{tGGP} = 8.13min. Quantitative MEKC analysis of seeds indicated the γ-glutamyl peptide concentration to be 0.7% in *V. narbonensis* accession ATC 60105. The peptide with 11.6% *S* accounts for ca 30% of the total *S* in the seed. The total *S* content due to the peptide has been observed to vary with genotype and environment with up to 0.3% (dry wt) in some instances.

Acid hydrolysis, followed by paper electrophoresis established the presence of glutamic acid and cysteine as the only detectable ninhydrin positive species. The FAB mass spectra were consistent with a M_r of 276 and the molecular formula of $C_{10}H_{16}N_2O_5S$ was determined by elemental analysis. The UV spectrum showed a distinct peak at λ_{max} 222 nm (log ε 3.74) and a shoulder at λ 241 nm (log ε 3.62) characteristic of a thio-vinyl group [7] and consistent with the reported *S*-ethenyl cysteine λ_{max} 223 nm (log ε 3.72) [8]. The diffuse reflectance IR absorbance spectrum showed peaks at ν_{max}^{KCl} cm^{-1} 1475 *s*, 2033 *w* (thio-vinyl) and vinyl double bond (ν_{max}^{KCl} cm^{-1} 2923 *w*).

The ^{13}C NMR at pD 6.0 showed three carbonyl groups (176.0, 176.2 and 178.7) and the presence of two ethylenic carbons (δ 114.9 and 133.4). The ^{1}H NMR, 2D COSY and 2D HETCOR spectra showed a high degree of multiplicity associated with five connected protons on three carbons, a resolved ABX-spin system, consistent with an alanyl residue, and three partially resolved ethylenic protons shifted up-field. Resolution of the coupling constants associated with the ethylenic protons using a temperature experiment (333°K) showed signals assignable to a thio-vinyl group (δ 5.73, 1H, *br d*, *J* = 16.8 Hz, H-2″a; 5.73, 1H, *br d*, *J* = 10.0 Hz, H-2″b; 6.87, 1H, *dd*, *J* = 16.8, 10.0 Hz, H-1″). These data are consistent with the presence of a 3-thio-substituted alanyl moiety [4]. Further decoupling experiments were used to assign coupling constants to C-2–C-4. These assignments were supported by NMR simulation [9] and were closely related to the values reported for the γ-glutamyl residue in glutathione [10]. Proton assignments have been made relative to HOD, consequently, absolute chemical shift values can be expected to show some minor variation due to changes in temperature and pD.

All these data are consistent with the major ninhydrin positive species (VA_3) reported by Bell and Tirimanna [3] in *V. narbonensis* being γ-glutamyl-*S*-ethenyl cysteine (**1**). An interesting aspect of γ-glutamyl-*S*-ethenyl cysteine is that it is photochemically labile to UV light and resembles γ-glutamyl-*S*-prop-1-enyl cysteine, the homologue from the seeds of chives (*Allium schoenoprasum*) [11]. The homologue behaves similarly on paper electrophoresis and evolves a characteristic strong sulphurous chive odour when the electrophoretograms are irradiated with UV light.



1

EXPERIMENTAL

General

^{1}H NMR and ^{13}C NMR were recorded in D_2O solns at 300 MHz (HOD int. std) and 75 MHz (*t*-BuOH ext. std; $-CH_3$: 31.6 ppm), respectively. Micellar electrokinetic chromatography analysis of FMOC-derivatised EtOH extracts of seed tissue and purified compound were performed with a capillary electrophoresis system fitted with a UV-DAD detector (272 ± 10 nm), equipped with a untreated fused silica capillary (0.57 m × 50 μm-i.d.) using borate-SDS-based buffers (20 mM/19.5 mM, pH 9.52), voltage 23.7 kV (+ve polarity) and a column temp. of 30° [12]. IR analysis were performed on a double beam dispersive infrared spectrometer, fitted with a diffuse reflectance attachment. FAB-MS analysis was carried out on a triple stage quadrupole instrument using Xe as bombardment gas; establishment voltage was 7–10 keV and ion current 0.5 mA. Liquid matrices: acidified glycerol (5% HOAc, +ve ion mode) and triethanolamine (−ve ion mode). Sample concentration was 1 μg μl^{-1}.

Extraction and isolation. Seeds (1 kg) were ground and extracted in 1:10 *w/v* 30% EtOH for 24 h at room temp. and filtered. The following is a typical preparative extraction procedure. The filtrate was applied to a column of Amberlite IR 400 [OAc^- form, anion-exchange, 500 ml bed-vol], and eluted with a 0.1–1.0 M ammonium acetate gradient. Frs were examined by paper electrophoresis (1 M HCO_2H-0.75 M HoAc, pH 1.76, voltage gradient 80 V cm^{-1}; citrate

buffer, pH 5, voltage gradient 48 V cm^{-1}) using UV and ninhydrin in acetone for detection. Frs were pooled and rotary evaporated to dryness. The dried residue was extracted with warm MeOH and filtered through a sintered glass funnel to remove ammonium glutamate. The partially purified mixt. (0.96 g) was dissolved in 1:10 *w/v* H_2O with 68 mg activated charcoal and left at room temp. for 15 min and filtered. The yellow filtrate was chilled to 4°, left overnight and decanted leaving a yellow oily residue which was discarded. Stepwise additions of *iso*-PrOH (30, 20 and 20 ml) to incipient turbidity, followed by overnight refrigeration at 4° produced a yellow oil which was discarded. The decanted supernatant was induced to crystallise by scratching and further refrigeration resulting in a crop (150 mg) of a mixt. of twinned and long linear crystals suitable for X-ray crystallographic analysis. Refrigeration and additional *iso*-PrOH provided white amorphous material (350 mg) containing no appreciable ninhydrin-positive components other than γ-glutamyl-*S*-ethenylcysteine providing a total yield of 500 mg (52%) from the crude product.

Acid hydrolysis. γ-Glutamyl-*S*-ethenylcysteine. (0.5 mg/ml^{-1}), sealed tube (6 M HCl at 110° for 4 h). Paper electrophoresis (pH 1.7) showed glutamic acid and cysteine only.

γ-Glutamyl-*S*-ethenyl cysteine. ^1H NMR (300 MHz, D_2O, $T = 297$ K): δ 2.16 (2H, *m*, $J = 7.4$ Hz, H-3), 2.48 (1H, *t*, $J = 7.4$ Hz, H-4a), 2.48 (1H, *d*, $J = 8.6$ Hz, H-4b), 3.00 (1H, *dd*, $J = 14.0$ Hz, 8.3 Hz, H-3′a), 3.24 (1H, *dd*, $J = 14.0$, 4.3 Hz, H-3′b), 3.75 (1H, *dd*, $J = 6.7$, 5.6 Hz, H-2), 4.41 (1H, *dd*, $J = 8.3$, 4.3 Hz, H-2′), 5.28 (1H, *br d*, $J = 16.3$ Hz, H-2″a), 5.29 (1H, *br d*, $J = 10.2$ Hz, H-2″b), 6.43 (1H, *dd*, $J = 16.8$ Hz, 10.0 Hz, H-1″), the coupling constants of the ethylenic protons H-1″, H-2″a and H-2″b were not fully resolved at 297°K; ^1H NMR (300 MHz, D_2O, $T = 333$ K). δ 2.60 (2H, *m*, $J = 7.4$ Hz, H-3), 2.92 (1H, *t*, $J = 7.4$ Hz, H-4a), 2.92 (1H, *d*, $J = 8.6$ Hz, H-4b), 3.48 (1H, *dd*, $J = 14.0$, 8.3 Hz, H-3′a), 3.69 (1H, *dd*, $J = 14.0$, 4.3 Hz, H-3′b), 4.20 (1H, *dd*, $J = 6.7$, 5.6 Hz, H-2), 4.86 (1H, *dd*, $J = 8.3$, 4.3 Hz, H-2′), 5.73 (1H, *br d*, $J = 16.8$ Hz, H-2″a), 5.73 (1H, *br d*, $J = 10.0$ Hz, H-2″b), 6.87 (1H, *dd*, $J = 16.8$, 10.0 Hz, H-1″); ^{13}C NMR (75.47 MHz, D_2O): δ 28.4 (C-3), 33.7 (C-4), 35.6 (C-3′), 56.3 (C-2), 56.5 (C-2′), 114.9 (C-2″), 133.4 (C-1″), 176.0 (C-1), 176.2 (C-5), 178.7 (C-1′). FAB-MS, *m/z* $[M+H]^+$ 277, *m/z* $[M-H]^-$ 275. IR, ν_{max}^{KCl} cm^{-1} 775 *s* (NH_2), 1074 *s* (CH), 1121 *s* (NH_2), 1217 *s* (CH_2), 1250 *s* (CO) 1332 *s* (OH) 1434 *s* (CH_2), 1475 *s* (S—CH=CH_2), 1523 *s* (amide II), 1581 *s* (COO^-), 1645 *s* (amide I), 2033 *w* (S—CH=CH_2), 2923 *w* (CH=CH_2), 3327 *vs* (NH_2). Elemental analysis (Found: C, 43.4; H, 6.0; N, 10.3; S, 11.7. $C_{10}H_{16}N_2O_5S$ requires: C, 43.5; H, 5.8; N, 10.1; S, 11.6). $[\alpha]_D^{23}$ −5.0° (H_2O; *c* 2.0). UV, λ_{max}^{H2O} nm (log ε) 222 (3.74).

Acknowledgements—We gratefully acknowledge the assistance of Mr Phil Clements (Department of Chemistry, University of Adelaide) for acquisition of NMR spectra and Dr Yogi Hayasaka (Australian Wine Research Institute) for collection of FAB-MS spectra. Support for this work by the Grains Research Development Corporation is gratefully acknowledged.

REFERENCES

1. Arora, S. K., in *Tropical Legumes in Animal Nutrition*, ed. J. P. F. D'Mello and C. Devendra. CAB International, Wallingford, UK, 1995, 67.
2. Siddique, K. H. M., Loss, S. P. and Enneking, D., *Australian Journal of Experimental Agriculture*, 1996, **36**, 53.
3. Bell, E. A. and Tirimanna, A. S. L., *Biochemical Journal*, 1965, **97**, 53.
4. Thumfort, P. P., Pate, J. S., Rasins, E. and Ghisalberti, E. L., *Phytochemistry*, 1993, **34**, 657.
5. Delaere, I. M., Tate, M. E., Jones, G. P. and Tiekink, E. R. T., *Acta Crystallographica Section C.*, 1995, **C51**, 289.
6. Kasai, T. and Larsen, P. O., in *Progress in The Chemistry of Organic Natural Products*, ed. W. Herz, H. Grisebach, G. W. Kirby. Springer: Vienna, 1980, **39**, 173.
7. Price, C. C. and Zomlefer, J., *Journal of American Chemical Society.*, 1950, **72**, 14.
8. Däbritz, E. and Virtanen, A. I., *Chemische Berichte*, 1965, **98**, 781.
9. Hägele, G., Spiske, R., Hoffken, H. W., Lenzen, T., Weber, U. and Goudetsidis, S., *Phosphorus, Sulfur and Silicon.*, 1993, **77**, 262.
10. Podanyi, B. and Reid, R. S., *Journal of American Chemical Society.*, 1988, **110**, 3805.
11. Block, E., *Angewandte Chemie.*, 1992, **31**, 1135.
12. Albin, M., Weinberger, R., Sapp, E. and Moring, S., *Analytical Chemistry*, 1991, **63**, 417.