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Identification and quantification of γ-glutamyl conjugates of biogenic amines in the nervous system of the snail, *Helix aspersa*, by gas chromatography–negative-ion chemical ionisation mass spectrometry

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ABSTRACT

The γ-glutamyl conjugates of *p*-octapamine and dopamine were identified unambiguously for the first time and quantified in a single cerebral ganglion or pleural plus pedal ganglia of the snail, *Helix aspersa*, by gas chromatography–negative-ion chemical ionisation mass spectrometry. A new method was used for synthesis of γ-glutamylamine standards. The concentration of γ-glutamyltyramine was found to be low in the tissues, therefore it was used as an internal standard. The γ-glutamylamines were extracted with water and derivatised with pentafluoropropionic anhydride and trifluoroethanol. Under negative-ion chemical ionisation conditions, the trifluoroethyl and pentafluoropropionyl derivatives produced significant ions which were sufficiently abundant to be suitable for selective ion monitoring. The method had a limit of detection of *ca.* 80 pg of γ-glutamyl conjugate per tissue and calibration curves were linear over the range examined.

INTRODUCTION

Recently, the presence of γ-glutamyl conjugates of biogenic amines in gastropod tissues (*Helix aspersa* and *Helisoma trivolvis*) [1,2] has been reported. The compounds were analyzed using high-performance liquid chromatography with electrochemical detection (HPLC–ED). It was suggested that conjugation with glutamic acid is a major route for the catabolism of endogenous and exogenous amines in the tissues of *H. aspersa* and *H. trivolvis*. Similar metabolic routes have also been reported for *Aplysia* and *Limulus* [3,4].

Various sensitive but non-specific methods are available for the detection of γ-glutamyl conjugates, ranging from paper chromatography and electrophoresis [5,6] to thin-layer chromatography (TLC) and liquid chromatography [3,7] to the more elegant method of HPLC–ED [1,2,4]. To our knowledge, no report has been found on analysis involving the use of gas chromatography–mass spectrometry (GC–MS) in this respect. GC–MS has the advantage over other methods in that compounds may be identified with high specificity by high-resolution capillary GC combined with the monitoring of characteristic ions in the mass spectrum of a compound. Analytes also afford characteristic ratios of ion intensities, or their *m/z* values may be changed in a predictable manner, to provide additional proof of identity, by the preparation of a different derivative of the same chemical class.

In the present investigation, in order to detect, identify and quantify conjugates in the tissues of

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the snail, *H. aspersa*, a series of γ-glutamylamines were synthesized as standards. A new method based on one used to synthesize γ-glutamyl-DOPA [8] was used to chemically synthesize the γ-glutamylamines. This paper reports, for the first time, the full characterisation of three synthetic γ-glutamylamines. We also report a specific and sensitive analytical procedure based on GC–negative-ion chemical ionisation (NICI) MS for the quantitation of γ-glutamylamine conjugates in gastropod tissue.

EXPERIMENTAL

Materials and reagents

All solvents used in extraction and derivatisation were HPLC grade (Rathburn Chemicals, Peebleshire, UK). Chemicals were obtained from the following sources: 2,2,2-trifluoroethanol (TFE), heptafluorobutyric anhydride (HFBA) and pentafluoropropionic anhydride (PFPA) (Fluorochem, Derbyshire, UK); *p*-tyramine hydrochloride (p-TAHCl), dopamine hydrochloride (DAHCl), (±)-*p*-octopamine hydrochloride (p-OCTHCl), acetic anhydride, phthalic anhydride, L-glutamic acid and hydrazine hydrate (Aldrich, Dorset, UK); Dowex AG1-X8 100–200 mesh (Bio-Rad, Hertfordshire, UK).

Gas chromatography–mass spectrometry

GC–MS in the NICI mode was carried out using Hewlett-Packard 5988A gas chromatograph–mass spectrometer interfaced with an HP RTE-6/VM data system. The following MS conditions were used: the instrument was tuned in the NICI mode to the ions at *m/z* 452, 595 and 633 from the perfluorotributylamine (PFTBA) calibrant, source temperature was 140°C, electron energy 200 eV and methane reagent gas was introduced to give a source pressure ~1.2 mbar. The gas chromatograph was fitted with a Restek Rtx 20 fused-silica capillary column (15 m × 0.25 mm I.D., Belmont Instruments, Glasgow, UK). The stationary phase of the column was cross-linked

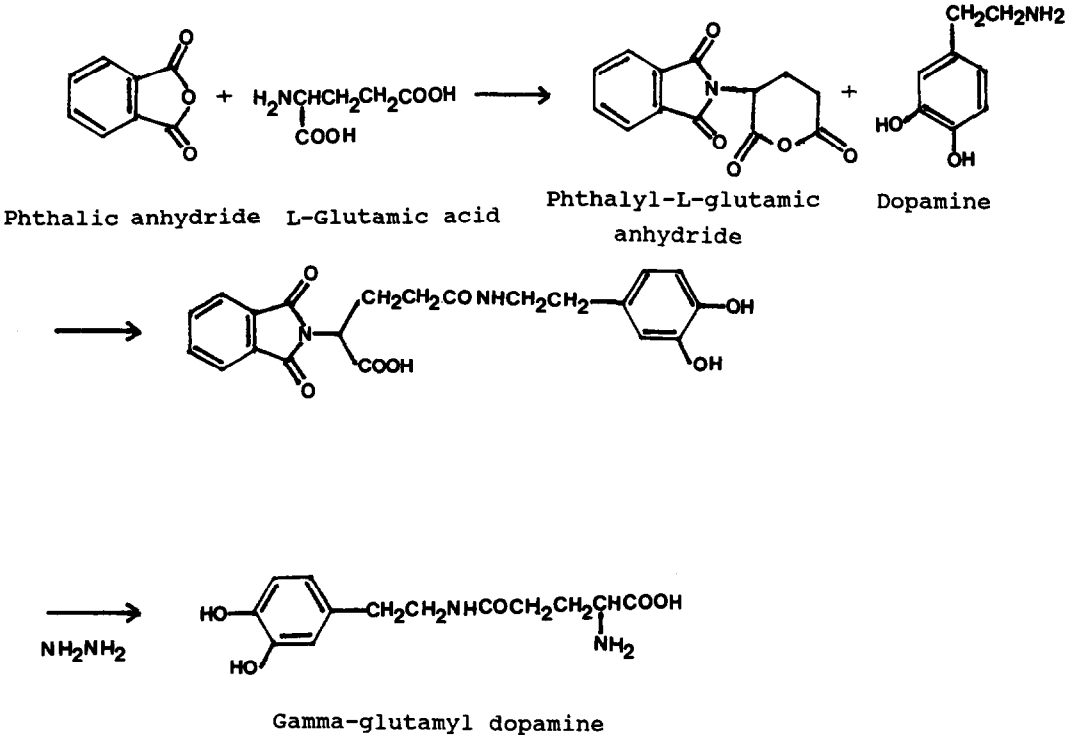

Fig. 1. Synthesis of γ-glutamyldopamine.

80% dimethyl–20% diphenylpolysiloxane (film thickness 0.25 μm). Helium carrier gas was used with a head pressure of 0.5 bar for the 15.0-m column.

The GC conditions were as follows: injector temperature 250°C, transfer line temperature 280°C, the oven temperature was maintained at 100°C for 1 min, then programmed at 10°C/min to 300°C. Injections were made using a Grob splitless injection system.

Chemical synthesis of standards

γ-Glutamyldopamine (γ-glu-DA). The procedure was a modified version of that described by Wilk *et al.* [8] (Fig. 1). L-Glutamic acid (14.7 g, 0.1 mol) and phthalic anhydride (14.8 g, 0.1 mol) were ground separately to a fine powder, mixed and heated to 135–145°C for 20 min. The clear melt was then cooled to 100°C and acetic anhydride (17.5 ml) was added. The mixture was heated for 40 min at 100°C and then xylene (52.5 ml) was added. The mixture was cooled to 0°C, and allowed to stand at this temperature overnight when phthaloyl-L-glutamic anhydride (13.3 g, 50%, m.p. 198–200°C) was obtained.

Dopamine hydrochloride (1.9 g, 0.01 mol) was dissolved in a solution of Na_2CO_3 (50 ml, 0.5 *M*) under nitrogen. The flask was cooled in an ice bath to 0–5°C and a solution of phthaloyl-L-glutamic anhydride (5.2 g, 0.02 mol) in dry dioxane (30 ml) was added dropwise with stirring. The mixture was stirred for an additional 20 min and then acidified to approximately pH 1.0 by the addition of 6 *M* HCl. The mixture was extracted with several portions of ethyl acetate and the pooled extracts were dried with anhydrous solid sodium sulphate overnight. Ethyl acetate was removed from the extract by rotary evaporation and the residue was dissolved in methanol (50 ml). Hydrazine hydrate (99%, 3 ml) was added to the methanolic solution and the mixture was allowed to stand for two days at 26°C. Methanol was then removed by rotary evaporation and the residue was suspended in water (50 ml). The suspension was acidified to pH 3.0 by the dropwise addition of 1 *M* HCl. The precipitated white solid (phthaloylhydrazide) was removed by filtration and the pH of the filtrate was adjusted back to a value of 5.0 by adding dropwise a solution of Na_2CO_3 (0.5 *M*).

The solution was then applied to the top of a Dowex (AG1-X8, 100–200 mesh, acetate form) column (2 × 48 cm). The column was washed with 100 ml of 0.01 *M* acetic acid and then eluted with a linear gradient established between 2 l of 0.01 *M* acetic acid and 2 l of 2 *M* acetic acid. Fractions of 50 ml were collected.

The presence in the eluate of ninhydrin-positive material was determined by a spot test on Whatman No. 1 filter paper. The product of the reaction emerged from the column very rapidly when approximately 100 ml of the eluent had passed through it. The fractions (100–150 ml) containing γ-glutamyldopamine were a single ninhydrin-positive spot (R_F 0.38) obtained on the TLC on silica gel in a solvent system consisting of 1-butanol–acetic acid–water (4:1:1). A single peak was also observed on GC of the PFP–TFE ester derivative of this conjugate. This evidence suggested that the product was quite pure. The acetic acid was completely removed by rotary evaporation under reduced pressure. A yellow solid was obtained (1.2 g, yield 42.7%, m.p. 192–194°C). Found: C, 53.61%; H, 6.36%; N, 9.69%; calculated for $C_{13}H_{18}N_2O_5$; C, 55.31%, H, 6.43%; N, 9.93%. The infrared spectrum (KBr) showed amide carbonyl stretching vibration at 1650 cm^{-1}. The ^1H NMR spectrum (400 MHz, D_2O) exhibited the following signals: 2.07 (2H, M, CH_2CH_2CH), 2.35 (2H, m, $COCH_2CH_2$), 2.65 (2H, t, CH_2CH_2NH, $J_{12} = 6.84$ Hz), 3.36 (2H, t, CH_2CH_2NH, $J_{21} = 6.84$ Hz), 3.71 (1H, t, CH–COOH, $J_{54} = 6.12$ Hz), 6.66 (1H, dd, Ar–H_B, $J_{BA} = 2.0$ Hz, $J_{BC} = 8.1$ Hz), 6.78 (1H, d, Ar–H_A, $J_{AB} = 2.0$ Hz), 6.85 (1H, d, Ar–H_C, $J_{CB} = 8.1$ Hz). There were no NH and OH peaks at 4.80 ppm because they exchanged with the solvent (D_2O). The ^{13}C spectrum with proton decoupling and ^{13}C jmod spectrum indicated the following signals: 27.65 (1C, CH_2CH_2CH), 32.73 (1C, $COCH_2CH_2$), 34.79 (1C, CH_2CH_2NH), 41.71 (1C, CH_2CH_2NH), 55.22 (1C, CH–COOH), 117.26 (1C, $=CH_A$), 117.69 (1C, $=CH_C$), 122.21 (1C, $=CH_B$), 133.07 (1C, =C–CH), 143.35 (1C, $HOC_{4'}=$), 144.897 (1C, $HOC_{3'}=$), 174.92 (1C, CONH), 175.36 (1C, COO).

γ-Glutamyl-p-octopamine (γ-glu-OCT). The synthesis of γ-glu-OCT was carried out in a manner essentially the same as that described above. A white solid was obtained (1.0 g, yield 35.7%, m.p. 203–204°C). Found: C, 53.66%; H, 6.15%; N, 9.72%; calculated for $C_{13}H_{18}N_2O_5$: C, 55.36%; H, 6.42%; H, 9.93%. The infrared spectrum (KBr) showed a single carbonyl stretching vibration at 1640 cm^{-1} and the ^1H NMR spectrum (400 MHz, D_2O) indicated signals at: 2.09 (2H, M, CH_2CH_2CH), 2.40 (2H, M, $COCH_2CH_2$), 3.50 (2H, M, $CHCH_2NH$), 3.72 (1H, t, CH_2CH–NH_2, $J_{54} = 5.96$ Hz), 6.94 (2H, d, Ar–($H_{2'}H_{6'}$), $J_{2'6'} = 8.36$ Hz), 7.31 (2H, d, Ar–($H_{3'}H_{5'}$), $J_{3'5'} = 8.40$ Hz). There were no NH, OH peaks at 4.78 ppm because they exchanged with the solvent (D_2O), and CHOH peaks was overlapped by solvent. The ^{13}C spectrum with proton decoupling and ^{13}C jmod spectrum indicated the following signals: 27.79 (1C, CH_2CH_2CH), 32.92 (1C, $COCH_2CH_2$), 47.11 (1C, $CHCH_2NH$), 55.47 (1C, CH–COOH), 72.87 (1C, $HOCHCH_2$), 116.75 (2C, $C_{3'}=C_{5'}$), 129.28 (2C, $C_{2'}=C_{6'}$), 134.28 (1C, $=C$–CHOH), 156.66 (1C, $=C$–OH), 175.22 (1C, CONH), 176.01 (1C, COOH).

γ-Glutamyl-p-tyramine (γ-glu-TA). The synthesis of γ-glu-TA was carried out in a manner essentially the same as γ-glu-OCT described above to yield a white solid (1.2 g, yield 45.11%, m.p. 219–220°C [9]). Found: C, 58.52%; H, 6.80%; N, 10.52%; calculated for $C_{13}H_{18}N_2O_4$; C, 58.63%; H, 6.81%; N, 10.52%. The ^1H NMR spectrum (400 MHz, CF_3COOD) showed the following signals: 2.54 (2H, M, CH_2CH_2CH), 2.92 (4H, m, $COCH_2CH_2$, CH_2CH_2NH), 3.67 (2H, t, CH_2CH_2NH, $J_{21} = 6.56$ Hz), 4.49 (1H, s, CHCOOH), 7.00 (2H, d, Ar–(H_2–C=C– and –C=C–$H_{6'}$), $J_{2'3' \text{ and } 6'5'} = 8.2$ Hz), 7.23 (2H, d, Ar–($H_{3'}$–C=C– and C=C–$H_{5'}$), $J_{3'2' \text{ and } 5'6'} = 8.16$ Hz). There were no NH and OH peaks because of exchange with the solvent (CF_3COOD). The ^{13}C spectrum with proton decoupling and ^{13}C jmod spectrum indicated the following signals: 27.51 (1C, CH_2CH_2CH), 34.42 (1C, $COCH_2CH_2$), 36.09 (1C, CH_2CH_2NH), 44.70 (1C, CH_2CH_2NH), 56.32 (1C, CHCOOH), 118.26 (2C, $C_{3'}=C_{5'}$), 132.55 (2C, $C_{2'}=C_{6'}$), 133.93 (1C, $=C$–CH_2), 154.88 (1C, HO–C=), 174.81 (1C, CONH), 177.98 (1C, COOH).

Animals and tissue preparation

H. aspersa (body weight including shell 5–9 g) were supplied by Blades Biological (Ebenbridge, UK). Snails were fed on fresh lettuce and water. They were put into bottles containing 0.1% pentobarbitone sodium solution [10]. After 6–7 h snails were fully anaesthetised and were then dissected to remove the cerebral and pleural plus pedal ganglia. Tissues were stored frozen at −20°C until analysis (usually less than ten days).

Extraction and derivatisation

A standard solution (20 μl, containing 20 ng of γ-glu-TA) was added to water (0.5 ml) containing a single cerebral ganglion (or pleural plus pedal ganglia) in a ground-glass homogeniser. The tissue was homogenised and then acetonitrile (0.5 ml) was added. The homogenate was centrifuged for 30 min at 2500 *g* (4500 rpm). The supernatant was transferred to a screw-capped vial (1 ml), and the solvent was removed under a stream of nitrogen. Dried tissue extract or standard was reacted (100°C, 30 min) with TFE (10 μl) and PFPA (100 μl) in a screw-capped vial. The excess reagents were evaporated with a stream of nitrogen and the residue redissolved in ethyl acetate (40 μl). For the formation of TFE–HFB derivatives, the extract or standard was heated with TFE (10 μl) and HFBA (100 μl) for 1 h at 100°C, and the excess reagents were removed under a stream of nitrogen. The residue was dissolved in ethyl acetate (40 μl). An aliquot of this solution (1 μl) was injected into the GC–MS system.

RESULTS AND DISCUSSION

To the best of our knowledge NMR data fully characterising the γ-glutamyl conjugates of biogenic amines has not been reported before. Using the synthetic procedure described in this paper, we obtained pure samples of three conjugates in good yield; our attempts to use a previously described method of synthesis [3] resulted in a prod-

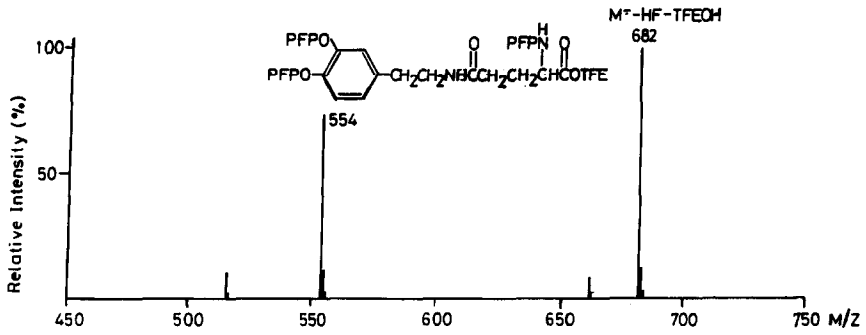

Fig. 2. NICI mass spectrum of the PFP–TFE derivative of γ-glutamyldopamine (PFP = C_2F_5CO, TFE = CF_3CH_2).

uct mixture that was very difficult to purify. Initially γ-glutamyl-3,4-dihydroxybenzylamine was prepared for use as an internal standard but its recovery on extraction from the biological tissues was very variable and also it gave an extra chromatographic peak which interfered with the quantitation of γ-glu-OCT. The concentration of γ-glu-TA in snail nervous tissue was found to be very low and therefore our synthetic sample of γ-glu-TA was used as an internal standard for the quantitation of γ-glu-DA and γ-glu-OCT. The PFP–TFE derivatives [11] of the γ-glutamyl conjugates were prepared for the GC–MS analysis; these derivatives were previously used to analyze amino acids in invertebrate nervous tissue [12]. Fig. 2 shows the major ions under NICI conditions obtained from the PFP–TFE derivative of γ-glu-DA. The HFB–TFE derivatives of the glutamyl conjugates were also prepared; the major ions under NICI conditions are shown in Fig. 3 for the HFB–TFE derivative of γ-glu-DA. The two derivatives gave very similar mass spectra, with ions differing by 150 a.m.u., but had slightly different chromatographic retention times. HFB–TFE derivatives were used to confirm the identity of the γ-glutamyl conjugates in invertebrate nervous tissue. Table I summarises the chromatographic data for the derivatives of γ-glutamyl conjugates and gives the principal ions in their mass spectra. The base peak in the case of the PFP–TFE derivative results from the loss of TFEOH + HF from the molecular ion, and in the case of the HFB–TFE derivative from the loss of TFEOH + HFBH; however, both derivatives contain two major ions arising from identical types of fragmentation. γ-Glu-OCT showed more tendency to produce reagent-specific ions in the low mass range. It was not possible to see a molecular ion for the derivatives under NICI, electron impact or positive-ion chemical ionisation conditions.

Fig. 4 shows the selected ion monitoring (SIM) traces for PFP–TFE derivatives of γ-glu-DA and γ-glu-OCT extracted from snail cerebral ganglion

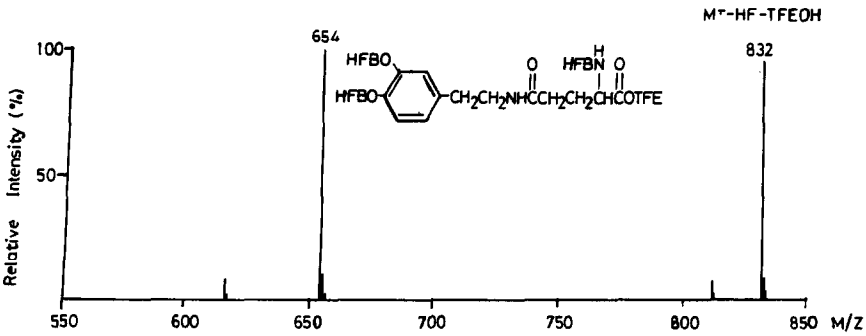

Fig. 3. NICI mass spectrum of the HFB–TFE derivative of γ-glutamyldopamine (HFB = C_3F_7CO).

TABLE I

KOVATS' INDICES AND INTENSITIES OF RELEVANT IONS OF PFP–TFE AND HFB–TFE DERIVATIVES OF γ,GLU-TA, γ-GLU-OCT AND γ-GLU-DA

Amine	Kovats' index	M^-	Other major ions (*m/z*)
PFP–TFE derivatives			
γ-Glu-TA	2230	640 (0%)	520 (100%)
			500 (33%)
			392 (80%)
γ-Glu-OCT	2165	802 (0%)	682 (100%)
			702 (90%)
			554 (20%)
			163 (10%)
γ-Glu-DA	2190	802 (0%)	682 (100%)
			554 (60%)
HFB–TFE derivatives			
γ-Glu-TA	2260	740 (0%)	620 (100%)
			442 (98%)
γ-Glu-OCT	2200	952 (0%)	832 (100%)
			852 (80%)
			654 (43.5%)
			194 (58%)
γ-Glu-DA	2220	952 (0%)	832 (70%)
			654 (100%)

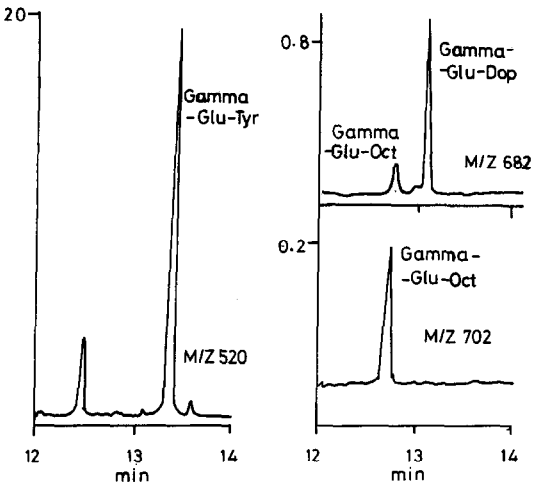

Fig. 4. SIM trace showing γ-glutamyldopamine and γ-glutamyl-octopamine which were extracted from a cerebral ganglion of *H. aspersa* and converted to PFP–TFE derivatives. A 20-ng amount of γ-glutamyltyramine was added as an internal standard prior to extraction.

and also the trace for the added 20 ng of γ-glu-TA internal standard. Calibration curves were constructed over the range 200 pg–40 ng and had correlation coefficients >0.99. On a day-to-day basis instrumental variations were corrected by running a standard mixture with each batch of samples. Blank samples containing 20 ng of γ-glu-TA put through the extraction and derivatisation procedure were also run with each batch of samples. In addition one tissue in each batch was run without addition of γ-glu-TA to ensure that there was no danger of elevated levels of endogenous γ-glu-TA interfering in the analysis. The limit of detection for the PFP–TFE derivatives was *ca.* 80 pg per sample. Repeated analysis run of the same sample gave between-run coefficients a variation with standard deviations of ±7.6% ($n = 5$) for the PFP–TFE derivative of γ-glu-DA and ±5.0% ($n = 5$) for the PFP–TFE derivative of γ-glu-OCT. The average concentrations and ranges of γ-glu-DA and γ-glu-OCT determined in cerebral ganglia and pleural plus pedal ganglia for *H. aspersa* are shown in Tables II and III.

The synthesis of γ-glutamylamines described in the text has several advantages over previous procedures [3,6,9,13,14]. Tsuji *et al.* [6] synthesized γ-glutamylamines using carbobenzoxy-γ-glutamylhydrazide to react with $NaNO_2$ to form azide, which was in turn reacted with biogenic amines. Previous workers [3] synthesized γ-glutamylamines by the following method. The N-Boc-α-L-glutamate-*tert.*-butyl ester was reacted with free-base biogenic amine in the presence of dicyclohexylcarbodiimide and then the syn-

TABLE II

AVERAGE CONCENTRATIONS OF γ-GLU-DA AND γ-GLU-OCT EXTRACTED FROM A SINGLE CEREBRAL GANGLION OF SNAIL AS PFP–TFE DERIVATIVES

Amine	*n*	Concentration (mean ± S.D.) (ng/tissue)	Range (ng/tissue)
γ-Glu-DA	12	3.88 ± 2.88	0.68–8.86
γ-Glu-OCT (SIM *m/z* 682 + 702)	12	0.75 ± 0.26	0.43–1.26

TABLE III

AVERAGE CONCENTRATIONS OF γ-GLU-DA AND γ-GLU-OCT EXTRACTED FROM PLEURAL PLUS PEDAL GANGLIA OF SNAIL AS PFP–TFE DERIVATIVES

Amine	*n*	Concentration (mean ± S.D.) (ng/tissue)	Range (ng/tissue)
γ-Glu-DA	8	10.40 ± 4.64	4.11–15.87
γ-Glu-OCT (SIM *m/z* 682 + 702)	8	1.20 ± 0.34	0.85–1.84

thesized peptidoamine was deprotected by TFA. They found that complete deprotection was difficult to achieve.

The method used in the present experiment was based on that of Wilk *et al.* [8]. In the first-step reaction, the mixture was heated for 40 min (instead of 3 min as reported in their experiment) after acetic anhydride had been added. It was found that heating for a long period could help the cyclization reaction to form phthaloyl-L-glutamic anhydride. At the end of the reaction sequence, when the crude final product was passed through the ion-exchange column, the γ-glutamylamine emerged after *ca.* 100 ml of the eluent had passed through the column (as compared with 2 l in the synthesis of the DOPA-glutamyl conjugate [3]), this was presumably due to the product containing only one carboxyl group, compared to two in DOPA-glutamyl conjugate.

In the past, γ-glu-DA and γ-glutamyl-5-hydroxytryptamine (γ-glu-5HT) were detected (but not quantified) in the nervous tissue of *H. aspersa* (Gastropoda) by HPLC–ED [1]. γ-Glu-DA and γ-glu-5HT were detected and quantified by HPLC–ED in the nervous tissue of *H. trivolvis* [2]. In both of these previous studies the snails were preinjected with precursors or the γ-glutamyl biogenic amine conjugates before dissection. In our current investigation preincubation with precursors was not carried out.

There is now a large amount of evidence suggesting that the formation of amine conjugates serves to inactivate these amines [3,7]. These studies also suggested that γ-glutamylation of a series of biogenic amines, including histamine, *p*-octopamine, *p*-tyramine, tryptamine, dopamine, 5-hydroxytryptamine and phenylethylamine, may also serve to inactivate these biogenic amines. Other evidence has suggested that conjugates of biogenic amines can function as neurotransmitters, neuromodulators and neurohormones. In *Limulus*, γ-glu-OCT can modulate visual sensitivity whereas γ-glu-TA has little effect [4]. However, it has not yet been established whether or not OCT and γ-glutamyl conjugates are released from efferent fibres in response to electrical stimulation or endogenous activation. In the snail it was suggested that the conversion of amines to γ-glutamylamines occurs through the action of a γ-glutamylamine synthetase and this is thought to be a pathway for amine inactivation [2]. However, very few of the synthetic enzymes have been purified and characterized. An exception is found in the investigations reported previously [15], where some of the characteristics of the enzyme, γ-glutamylhistamine synthetase, were described. However, the properties of the enzyme were not fully explored and it was not possible to establish whether the formation of all the amine conjugates was catalyzed by a particular synthetase or whether each conjugate was synthesized by a separate enzyme.

GC–MS provides a highly specific and sensitive method for the detection, identification and quantification of γ-glutamyl conjugates. The method should be applicable to investigations of γ-glutamylamine synthetase activity in *H. aspersa* and other organisms. γ-Glutamyl formation appears to be the chief pathway for metabolism of biogenic amines in *H. aspersa* since N-acetates and amine oxidase metabolites are absent from its neurological tissues [16].

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