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γ-Glutamyl dipeptides in *Petiveria alliacea*

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Abstract

Three γ-glutamyl dipeptides have been isolated from *Petiveria alliacea* L. roots. These dipeptides include ($S_{C2}R_{C7}$)-γ-glutamyl-*S*-benzylcysteine together with two diastereomeric sulfoxides, namely ($S_{C2}R_{C7}R_S$)- and ($S_{C2}R_{C7}R_S$)-γ-glutamyl-*S*-benzylcysteine *S*-oxides (γ-glutamyl-petiveriins A and B, respectively). Their structures and absolute configurations have been determined by NMR, MALDI-HRMS, IR and CD spectroscopy, and confirmed by comparison with authentic compounds obtained by synthesis.

Keywords: *Petiveria alliacea*; Phytolaccaceae; Glutamyl dipeptide; Petiveriin; *S*-benzylcysteine sulfoxide; *S*-benzylcysteine

1. Introduction

Petiveria alliacea L. (Phytolaccaceae) is a perennial shrub indigenous to the Amazon Rainforest, although it grows abundantly in other areas, including tropical and Central America, the Caribbean and the southeastern United States. It is a popular folk medicine used to treat a wide variety of disorders. Preparations from this plant reportedly exhibit anticancer, antimicrobial and antiinflammatory effects, among many others.

We recently reported isolation of six novel sulfur-containing amino acids from *P. alliacea* roots, including (R_SR_C)/(S_SR_C)-*S*-benzylcysteine sulfoxides (petiveriins A and B) and (R_SR_C)/(S_SR_C)-*S*-(2-hydroxyethyl)cysteine sulfoxides (6-hydroxyethiins A and B) (Kubec and Musah, 2001; Kubec et al., 2002). We demonstrated that these cysteine derivatives are enzymatically cleaved upon tissue disruption in a fashion similar to that observed in *Allium* plants, yielding four thiosulfinates in total. We also identified the lachrymatory principle of the plant as the unique sulfine (*Z*)-thiobenzaldehyde *S*-oxide (Kubec et al., 2003). It is likely that the petiveriins serve as precursors to this sulfine.

Typically, *S*-substituted cysteines and their sulfoxides are accompanied by the corresponding γ-glutamyl dipeptides in the plant tissue. The biochemical role of these dipeptides is not completely clear, but it is believed that they serve as storage compounds of nitrogen and sulfur, among other functions. They also are intermediates in the biosynthesis of *S*-alk(en)ylcysteine sulfoxides (Kasai and Larsen, 1980; Fenwick and Hanley, 1985; Lancaster and Shaw, 1989, 1991).

This co-existence in many plants of *S*-substituted cysteine derivatives and their dipeptide forms encouraged us to search for the presence of such dipeptides in *P. alliacea*. The present paper describes our investigations on isolation and identification of three novel γ-glutamyl dipeptides from the roots of this plant.

2. Results and discussion

The roots of *P. alliacea* were extracted with boiling MeOH and the extract was subjected to ion-exchange chromatography. A dipeptide-containing fraction was

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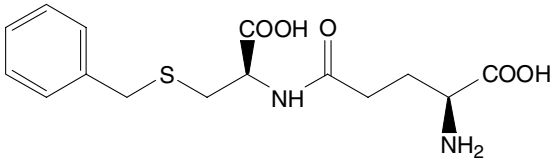
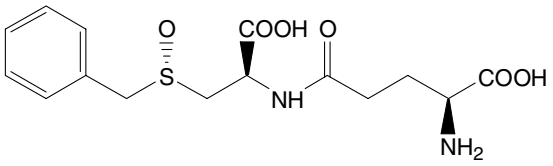
obtained by a combination of cation (Amberlite 200, H^+) and anion-exchange (Dowex 1 × 8, acetate) chromatography. HPLC/MS analysis of the dipeptide fraction revealed the presence of one component having [MH^+] of 341, together with two minor ones each exhibiting [MH^+] of 357. These three compounds were subsequently isolated by prep. HPLC as white and very hygroscopic solids.

The ^{1}H NMR spectrum of the first compound indicated the presence of the glutamyl (δ 2.14, 2.45 and 3.76 ppm, 5H) and cysteinyl (ABX system; δ 2.83, 2.98 and 4.34 ppm, 3H) moieties along with an unsubstituted benzyl group (δ 7.33–7.42 ppm, 5H). The ^{13}C NMR spectrum revealed the presence of 13 magnetically non-equivalent carbon atoms, with four of them being aromatic (δ 129.0, 129.3, 129.4 and 130.7 ppm) and three of them being carbonyl/carboxyl carbons (δ 173.9, 174.5 and 175.0 ppm). The IR spectrum contained a broad band at 3500–2850 cm^{-1} and MALDI-HRMS showed [MH^+] of 341.1169 ($C_{15}H_{20}N_2O_5S$ req. 341.1166). Based on the spectral data, it was concluded that the compound was a glutamyl dipeptide of *S*-benzylcysteine. Because both α- and γ-glutamyl dipeptides generally have very similar NMR spectra, the method of Kasai and Sakamura (1973) was used to determine the type of CO–NH linkage in the isolated dipeptide. The method is based on the sensitivity of the glutamyl α-proton to pH changes, leading to significant ^{1}H NMR signal shift differences. Addition of DC1 caused a downfield shift ($\Delta\delta = +0.31$ ppm) and addition of NaOD an upfield shift ($\Delta\delta = -0.58$ ppm) of the proton signal, indicating that the isolated compound was γ-glutamyl-*S*-benzylcysteine (**1**). The correct structural assignment was further confirmed by comparison with authentic ($S_{C2}R_{C7}$)-γ-glutamyl-*S*-benzylcysteine obtained by synthesis. Because the synthetic compound had identical spectral characteristics in all respects with the isolated dipeptide, we could unambiguously determine the absolute configuration of **1** to be $S_{C2}R_{C7}$ (Fig. 1).

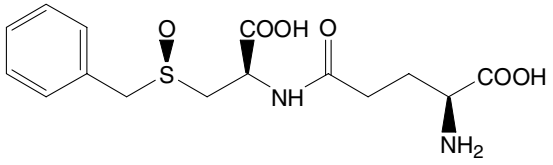
The ^{1}H and ^{13}C NMR spectra (incl. 2D NMR) of the two minor compounds were very similar. The proton spectra showed the presence of the glutamyl moiety, an unsubstituted benzyl group (δ 7.39–7.49 ppm, 5H) and two pairs of heterosteric methylene protons. The ^{13}C NMR spectra revealed the presence of 13 magnetically non-equivalent carbon atoms, with four being aromatic and three being carbonyl/carboxyl carbons. As with **1**, addition of DC1 and NaOD caused a significant downfield ($\Delta\delta = +0.19$ ppm) and upfield ($\Delta\delta = -0.55$ ppm) shift, respectively. Along with a broad band between 3500 and 2850 cm^{-1}, both IR spectra contained a strong absorption band near 1015 cm^{-1}, indicating the presence of a sulfoxide group. The MALDI-HRMS data showed *m*/*z* [$MH]^+$ of 357.1115 and 357.1116, respectively, corresponding to a molecular formula of $C_{15}H_{20}N_2O_6S$ for both compounds (req. 357.1115). In accordance with the above spectral data, the two minor



1, ($S_{C2}R_{C7}$)-γ-Glutamyl-*S*-benzylcysteine



2a, ($S_{C2}R_{C7}S_S$)-γ-Glutamyl-*S*-benzylcysteine sulfoxide (γ-L-glutamyl-petiveriin B)



2b, ($S_{C2}R_{C7}R_S$)-γ-Glutamyl-*S*-benzylcysteine sulfoxide (γ-L-glutamyl-petiveriin A)

Fig. 1. The structures of the γ-glutamyl dipeptides isolated from *Petiveria alliacea* roots.

dipeptides were identified as diastereomers of γ-glutamyl-*S*-benzylcysteine sulfoxide (**2a**/**2b**). This conclusion was further verified by comparison with authentic compounds obtained by synthesis.

The absolute configurations about the sulfur atoms in **2a** and **2b** were determined by CD and ^{1}H NMR spectroscopy together with enzymatic hydrolysis. The CD spectra of the dipeptides were almost mirror images, both exhibiting a maximum around 224 nm. The CD spectrum of the first eluting isomer (**2a**) showed a negative sign of the Cotton effect ($\Delta\varepsilon_{max} = -2.7$; 225 nm), whereas the second one (**2b**) gave a CD spectrum with a positive sign of the Cotton effect ($\Delta\varepsilon_{max} = +3.0$; 224 nm). As we showed in a previous contribution (Kubec and Musah, 2001), the negative sign of the Cotton effect correlates with the *S* configuration about the sulfur atom. Employing this rule, we determined the absolute configuration of **2a** and **2b** as $S_{C2}R_{C7}S_S$ and $S_{C2}R_{C7}R_S$, respectively (Fig. 1).

The correct assignment of the absolute configurations was further confirmed by ^{1}H NMR spectroscopy. Although the ^{13}C NMR spectra of **2a** and **2b** were nearly identical, the ^{1}H spectra displayed significantly different signal patterns for the S(O)C*H*$_2$CH(NH$_2$) methylene protons. As discussed previously (Kubec and Musah, 2001), this typical feature of all *S*-substituted cysteine sulfoxides and their glutamyl dipeptides allows convenient determination of the absolute configuration

about the sulfur. Whereas the methylene proton signals in **2a** appeared as two distinctive doublets of doublets (δ 3.11 and 3.49 ppm; $J_{AX} = 7.8$ Hz and $J_{BX} = 6.0$ Hz), those in **2b** were represented by a poorly resolved multiplet (δ 3.28 ppm). These splitting patterns showed that the sulfoxide oxygen and the amino group of **2a** are on the same face of the molecule (i.e., $S_{C2}R_{C7}S_S$).

Furthermore, the absolute configurations were determined by enzymatic hydrolysis of **2a** and **2b** by γ-glutamyltranspeptidase (EC 2.3.2.2). Indeed, **2a** was cleaved into glutamic acid and (R_CS_S)-*S*-benzylcysteine sulfoxide (petiveriin B). Analogously, **2b** yielded glutamic acid along with (R_CR_S)-*S*-benzylcysteine sulfoxide (petiveriin A). Petiveriins A and B thus show a reverse elution order on a C-18 column to that of their γ-glutamyl dipeptides (**2b** and **2a**, respectively) (Kubec and Musah, 2001).

We also searched for the presence of the analogous dipeptide forms of the minor cysteine derivatives present in *P. alliacea*, i.e., γ-glutamyl-*S*-(2-hydroxyethyl)cysteine and its sulfoxides. We were able to confirm that synthetically obtained γ-glutamyl-*S*-(2-hydroxyethyl)cysteine and its stereoisomeric sulfoxides yield the expected [MH^+] of 294 and 310, respectively, on HPLC/MS analysis. However, no compounds showing the corresponding [MH^+] of 294 or 310 were detected in the dipeptide-containing fraction by HPLC/MS. Given the fact that the content of γ-glutamyl dipeptides in plants is known to vary significantly throughout their growth cycle, being very low or even non-detectable at times (Kasai and Larsen, 1980; Lancaster and Shaw, 1991; Matsuura et al., 1996), it is reasonable to expect a similar seasonal variation in the γ-glutamyl dipeptide content in *P. alliacea*. Thus, we believe that our current observation is a consequence of the *temporary* absence or very low content of these compounds as a result of the particular growth stage of the sample we analyzed.

In *Allium* and *Brassica* plants, γ-glutamyl dipeptides have been shown to be biochemical precursors of the corresponding *S*-alk(en)ylcysteines and their sulfoxides (Kasai and Larsen, 1980; Fenwick and Hanley, 1985; Lancaster and Shaw, 1989, 1991). During sprouting, the dipeptide forms are cleaved by γ-glutamyltranspeptidases to yield *S*-alk(en)ylcysteines and *S*-alk(en)ylcysteine sulfoxides. Our observation of **1** and **2a**/**2b** suggests that analogous biochemical pathways are operative in the biosynthesis of *S*-benzylcysteine sulfoxides (petiveriins A/B) in *P. alliacea*. However, the origin of the benzyl moiety in petiveriins A/B remains unknown.

3. Experimental

3.1. Plant material

Fresh roots of *P. alliacea* were collected in Hillaby, St. Andrew (Barbados) by Mr. Jeff Chandler (Department of Horticulture, University of the West Indies at Cave Hill, Barbados) in April 2004. They had been stored at 4 °C for 3 weeks prior to being analyzed. A voucher specimen is deposited at the National Herbarium of Barbados, University of the West Indies, Cave Hill Campus, St. Michael, Barbados (Accession No. JC37).

3.2. General experimental procedures

HPLC separations were performed on a Dynamax SD-200 binary pump system, employing a Varian PDA 330 detector and a C-18 reverse phase column (Rainin Microsorb-MV 100 Å, 250 × 4.6 mm, 5 μm). Alternatively, a preparative C-18 reverse phase column (Rainin Dynamax-100 Å, 250 × 21.4 mm, 8 μm) was used. 10 mM of NH_4OAc buffer (solv. A, pH 5.6) and MeCN (solv. B) were used as the mobile phase, with a flow rate of 0.9 mL min^{-1} (18 mL min^{-1} with the prep. column). The gradient was as follows: A/B 97/3 (0 min), 90/10 (in 15 min), 65/35 (in 25 min) and 97/3 (in 30 min). Other instrumentation used was identical to that described previously (Kubec and Musah, 2001).

3.3. Extraction and purification

Several portions of fresh *P. alliacea* roots (755 g in total) were carefully cleaned and homogenized in MeOH (3 L) using a blender. The slurries obtained were refluxed for 10 min and filtered. The individual extracts were combined, concentrated to ca. 400 mL by vacuum evaporation (40 °C) and extracted with hexane (2 × 400 mL) to remove lipidic compounds. The polar fraction was adjusted to 600 mL by addition of 3% HCl. The precipitate that appeared on acidification was filtered off and the filtrate was passed through a cation-exchange column (20 × 2.1 cm, Amberlite 200, H^+ form, 20–50 mesh). After washing the column with 3% HCl (300 mL) and H_2O (300 mL), the dipeptide fraction was eluted with 500 mL of 1 M NH_4OH. The yellowish eluent obtained was concentrated to ca. 150 mL, the pH was adjusted to 7.0 by addition of 10% HOAc, and the resulting solution was applied to an anion-exchange column (15 × 2.1 cm, Dowex 1 × 8, acetate form, 200–400 mesh). The column was washed with 500 mL of H_2O (discarded) and the dipeptide fraction was eluted with 10% HOAc (500 mL). The eluate was evaporated to dryness and the yellowish residue redissolved in 15 mL of 10 mM NH_4OAc buffer (pH 5.6) and subjected to prep. HPLC. The fractions eluting at 16.8, 8.1 and 8.6 min were repeatedly collected, concentrated to ca. 3 mL, and again subjected to prep. HPLC using H_2O instead of NH_4OAC (to remove the buffer salt). The eluted fractions were evaporated to dryness to yield 19, 3 and 4 mg of **1**, **2a** and **2b**, respectively, as white hygroscopic solids.

3.4. Reference compounds

(*R*)-*S*-Benzylcysteine and both diastereomers of (R_C)-*S*-benzylcysteine sulfoxide (petiveriins A and B) were obtained as described previously (Kubec and Musah, 2001). ($S_{C2}R_{C7}$)-γ-Glutamyl-*S*-benzylcysteine (**1**) was synthesized according to the procedure of Orlowski and Meister (1971). The diastereomers of ($S_{C2}R_{C7}$)-γ-glutamyl-*S*-benzylcysteine sulfoxide (**2a** and **2b**) were obtained by oxidation of **1** with H_2O_2 and subsequent separation by prep. C-18 HPLC. *N*-Phthaloyl-L-glutamic anhydride was prepared from *N*-phthaloyl-L-glutamic acid (Aldrich) and acetic anhydride (King and Kidd, 1949). γ-Glutamyltranspeptidase (GTP) (EC 2.3.2.2) from equine kidney (11 U/mg) was purchased from Aldrich.

3.4.1. ($S_{C2}R_{C7}$)-γ-Glutamyl-S-benzylcysteine (*1*)

A white hygroscopic solid. M.p. 152–155 °C; $[\alpha]_D^{22}$: −14.7° (H_2O; *c* 0.03); CD $\Delta\varepsilon_{max}$ (H_2O; *c* 0.03; 22 °C): +0.51 (220 nm); IR (KBr) ν_{max} cm^{-1}: 3500–2850 (*s*, *br*), 1736 (*m*), 1226 (*m*); ^{1}H NMR (300 MHz; D_2O): δ 2.14 (2H, *m*, CH_2CH_2CH), 2.45 (2H, *m*, CH_2CH_2CH), 2.83 (1H, *dd*, *J* = 8.3, 13.9 Hz, SCH_2CH-*a*), 2.98 (1H, *dd*, *J* = 4.5, 13.9 Hz, SCH_2CH-*b*), 3.76 (1H, *m*, Glu-C*H*), 3.81 (2H, *s*, CH_2 SCH_2), 4.34 (1H, *dd*, *J* = 4.5, 8.0 Hz, Cys-C*H*), 7.33–7.42 (5H, *m*, H_{arom}); ^{13}C NMR (75 MHz; D_2O): δ 26.8 (CH_2CH_2CH), 31.7 (CH_2CH_2CH), 33.6 (SCH_2CH), 35.8 (CH_2SCH_2), 54.5 (Cys-*C*H), 54.6 (Glu-*C*H), 127.6 ($C_{arom,para}$), 129.0 ($C_{arom,meta}$), 129.1 ($C_{arom,ortho}$), 138.7 ($C_{arom,q}$), 174.3 (Glu-*C*OOH), 174.5 ($NHCOCH_2$), 177.1 (Cys-*C*OOH). MALDI-HRMS [MH^+] 341.1169 ($C_{15}H_{20}N_2O_5S$ req. 341.1166).

3.4.2. ($S_{C2}R_{C7}S_S$)-γ-Glutamyl-S-benzylcysteine sulfoxide (*2a*, γ-L-glutamyl-petiveriin B)

A white hygroscopic solid. M.p. 138–140 °C; $[\alpha]_D^{22}$: −26.2° (H_2O; *c* 0.06); CD $\Delta\varepsilon_{max}$ (H_2O; *c* 0.06; 22 °C): −2.7 (225 nm); IR (KBr) ν_{max} cm^{-1}: 3500–2850 (*s*, *br*), 1610 (*s*, *br*), 1015 (*s*); ^{1} H NMR (300 MHz; D_2O): δ 2.15 (2 H, *m*, CH_2CH_2CH), 2.48 (2H, *t*, *J* = 7.6 Hz, CH_2CH_2CH), 3.11 (1H, *dd*, *J* = 8.3, 13.6 Hz, $SOCH_2CH$-*a*), 3.49 (1H, *dd*, *J* = 5.5, 13.6 Hz, SCH_2CH-*b*), 3.80 (1H, *t*, *J* = 6.2 Hz, Glu-*C*H), 4.14 (1H, *d*, *J* = 13.2 Hz, CH_2SO-*a*), 4.35 (1H, *d*, *J* = 13.2 Hz, CH_2SO-*b*), 4.66 (1H, *dd*, *J* = 5.5, 8.3 Hz, Cys-C*H*), 7.39–7.49 (5H, *m*, H_{arom}); ^{13}C NMR (75 MHz; D_2O): ^{13}C NMR (75 MHz; D_2O; DSS): δ 26.3 (CH_2CH_2CH), 31.7 (CH_2CH_2CH), 50.2 (Cys-*C*H), 53.3 ($SOCH_2CH$), 54.3 (Glu-*C*H), 56.9 (CH_2SOCH_2), 129.0 ($C_{arom,para}$), 129.3 ($C_{arom,meta}$), 129.5 ($C_{arom,q}$), 130.7 ($C_{arom,ortho}$), 173.9 (Glu-*C*OOH), 174.3 ($NHCOCH_2$), 174.7 (Cys-*C*OOH). MALDI-HRMS [MH^+] 357.1115 ($C_{15}H_{20}N_2O_6S$ req. 357.1115).

3.4.3. ($S_{C2}R_{C7}R_S$)-γ-Glutamyl-S-benzylcysteine sulfoxide (*2b*, γ-L-glutamyl-petiveriin A)

A white hygroscopic solid. M.p. 126–129 °C; $[\alpha]_D^{22}$: +3.2° (H_2O; *c* 0.06); CD $\Delta\varepsilon_{max}$ (H_2O; *c* 0.06; 22 °C): +3.0 (224 nm); IR (KBr) ν_{max} cm^{-1}: 3500–2850 (*s*, *br*), 1610 (*s*, *br*), 1012 (*s*); ^{1}H NMR (300 MHz; D_2O): δ 2.15 (2H, *m*, CH_2CH_2CH), 2.51 (2H, *t*, *J* = 7.5 Hz, CH_2CH_2CH), 3.28 (2H, *m*, $SOCH_2CH$-*a*, *b*), 3.81 (1H, *t*, *J* = 6.2 Hz, Glu-C*H*). 4.17 (1H, *d*, *J* = 13.2 Hz. CH_2SO-*a*), 4.33 (1H, *d*, *J* = 13.2 Hz, CH_2SO-*b*), 4.64 (1H, *dd*, *J* = 5.0, 9.2 Hz, Cys-C*H*), 7.39–7.49 (5H, *m*, H_{arom}); ^{13}C NMR (75 MHz; D_2O): δ 26.4 (CH_2CH_2CH), 31.7 (CH_2CH_2CH), 49.6 (Cys-*C*H), 53.0 ($SOCH_2CH$), 54.3 (Glu-*C*H), 56.8 (CH_2SOCH_2), 129.0 ($C_{arom,para}$), 129.3 ($C_{arom,meta}$), 129.4 ($C_{arom,q}$), 130.7 ($C_{arom,ortho}$), 173.9 (Glu-*C*OOH), 174.5 ($NHCOCH_2$), 175.0 (Cys-*C*OOH). MALDI-HRMS [MH^+] 357.1116 ($C_{15}H_{20}N_2O_6S$ req. 357.1115).

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