Characterization of Sinapyl Derivatives in Pineapple (*Ananas comosus* [L.] Merill) Juice

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Three previously unidentified phenolic compounds were found in pineapple (*Ananas comosus* [L.] Merill) juice in substantial concentrations and were isolated by semipreparative reverse phase HPLC. The structures were elucidated from UV spectra, acid hydrolysis, and subsequent amino acid analysis, mass spectrometry, and two-dimensional NMR spectroscopy. The compounds are identified as *S*-sinapyl-L-cysteine, *N*-L-γ-glutamyl-*S*-sinapyl-L-cysteine, and *S*-sinapylglutathione.

Keywords: *Pineapple juice; Ananas comosus [L.] Merill; phenolics; sinapyl derivatives; glutathione*

INTRODUCTION

Although pineapple (*Ananas comosus* [L.] Merill) juice is a major commercial product in international commerce, its phenolics profile has not been well characterized, and only a few papers have been published on the phenolic composition of pineapple fruit (Macheix et al., 1990), juice (Fernández de Simón et al., 1992), and shell fiber (Larrauri et al., 1997). We found, however, in a preliminary investigation of pineapple juice phenolics by reverse phase HPLC/DAD, that the retention time and UV spectra of the major peaks did not match the phenolic compounds previously reported to be present. Three late-eluting peaks had identical UV spectra with a maximum absorption at 285 nm, suggesting the possibility of their being derivatives of the same basic phenolic compound. This publication reports the structures of these three new phenolic compounds, which were determined by mass spectrometric and heteronuclear two-dimensional NMR techniques combined with acid hydrolysis and subsequent amino acid analysis.

MATERIALS AND METHODS

Source. Authentic pineapple juice concentrate samples were provided by the National Food Processors Association (Washington, DC) and Dole Food Co., Inc. (Westlake Village, CA), and stored at −20 °C until analysis.

Extraction. Pineapple juice concentrate (400 g) was diluted 4-fold with deionized water and ultracentrifuged at 23000*g* for 20 min. The supernatant was collected and filtered through Whatman No. 1 filter paper with Celite diatomaceous earth filtration aid (Celite Corp., Lompoc, CA). The filtrate (100 mL) was passed through a MeOH-activated C_{18} cartridge (5 g resin) from Alltech Associates (Deerfield, IL), followed by a 100 mL 0.01% aqueous HCl wash, and then eluted with 50 mL of MeOH. The MeOH eluate was combined and kept at −10 °C overnight and then passed through a 0.45 μm Millipore HA membrane to remove the white precipitate that had formed, presumably polysaccharides or proteins. The filtrate was rotary evaporated to dryness at 35 °C, then dissolved in 10 mL of 0.01% aqueous HCl/MeOH (70: 30), and filtered through a 0.45 μm Millipore HV membrane prior to semipreparative HPLC isolation.

Semipreparative HPLC. Two Dynamax SD-300 pumps were used with a semipreparative Microsorb C_{18} column (25 cm × 21.4 mm, 5 μm) from Rainin Instrument Co. (Woburn, MA). The end of the column was connected to a flow divider, which split 1 mL/min flow to an HP 1040A diode array detector (DAD) with detection at 280 nm and the rest of the flow (19 mL/min) to an outlet for manual peak collection. The following gradient employing solvent A (methanol) and solvent B (0.07 M KPO_4 buffer, pH 2.4) was used: 7 min from 35 to 45% solvent A, then 3 min from 45 to 48% solvent A, and holding for 5 min. The peak purity was verified by analytical HPLC. Each peak collected from the preparative HPLC was rotary evaporated at 35 °C for 10 min to remove MeOH and then extracted with an Alltech C_{18} cartridge (5 g). The MeOH eluate from the cartridge was rotary evaporated to dryness and stored at −15 °C until analysis. Approximately 5 mg of compound **1**, 50 mg of compound **2**, and 50 mg of compound **3** were isolated.

Analytical HPLC. A Supelco LC-18 column (25 cm × 4.6 mm, 5 μm) was used with an HP 1040A DAD set at 280 nm. The flow rate was 1.0 mL/min, and injection was 50 μL. The following gradient employing methanol (solvent A), acetonitrile (solvent B), and solvent C (0.07 M KPO_4 buffer, pH 2.4) was used: 10 min from 10% A/0% B to 22% A/0% B, then 25 min from 22% A/0% B to 22% A/25% B, the remainder being C.

Acid Hydrolysis. About 0.5 mg each of compounds **2** and **3** was acid hydrolyzed in a Teflon-lined screw-cap test tube with 5 mL of 2 N HCl at 100 °C for 30 min in the dark (10 min N_2 flushing before hydrolysis) and then cooled in an ice bath and extracted with a C_{18} Sep-Pak (360 mg resin) from Waters Associates (Milford, MA). The MeOH eluate of the Sep-Pak was rotary evaporated to dryness, dissolved in 0.01% aqueous HCl, and subjected to HPLC analysis using the same conditions as stated above.

Amino Acid Analysis. Two hundred microliters of the MeOH eluate from the isolation step above was transferred to a 1.5 mL Teflon-lined screw-cap reaction tube, flushed dry with N_2, dissolved in 120 μL of 6 N HCl, N_2 flushed for another minute, and then capped. The hydrolysis was performed in a heating block at 95 °C for 3 h. The hydrolysate was subjected to amino acid analysis by PITC derivatization as described by Hagen et al. (1993), and the identities of hydrolyzed amino acids were confirmed by spiking the sample with the standard PITC derivatives prepared.

Mass Spectrometry. Electrospray ionization MS (ESI-MS) was performed on a Perkin-Elmer Sciex API III+ triple-quadrupole ionspray mass spectrometer (Ontario, Canada) by pneumatically assisted electrospray. The operating conditions were standard. Tandem MS/MS spectra were recorded on the same

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Table 1. ^{1}H NMR Data for Compounds 1−3 [Parts per Million in $D_2O/CD_3OD/CF_3COOD$ (600 MHz)][a]

H	1	2	3
2, 6	6.44 s	6.35 s	6.40 s
7	6.13 d (15.6)	6.04 d (15.6)	6.08 d (15.6)
8	5.78 dt (15.6, 7.8)	5.69 dt (15.6, 7.8)	5.73 dt (15.6, 7.8)
9	3.07 d (7.8)	2.94 d (7.8)	3.00 d (7.8)
1″, 2″	3.54 s	3.47 s	3.51 s
2′		3.58 s	
4′	3.95 dd (7.8, 4.2)	4.20 t (6.6)	4.26 dd (8.4, 4.8)
6′		2.18 t (7.2)	2.21 t (7.2)
7′		1.86 dt (7.2, 7.2), 1.80 dt (7.2, 7.2)	1.90 dt (7.2, 7.2), 1.84 dt (7.2, 7.2)
8′		3.69 t (6.6)	3.74 t (6.6)
10′	2.86 dd (15.0, 4.2), 2.71 dd (15.0, 7.8)	2.60 dd (13.8, 4.8), 2.44 dd (13.8, 8.4)	2.69 dd (13.8, 4.8), 2.52 dd (13.8, 8.4)

[a] Coupling constants (J in Hz) given in parentheses. Chemical shifts assigned by comparison to chemical shift pattern of compounds **1**−**3** and glutathione standard and also from COSY, TOCSY, HMQC, and HMBC data.

Table 2. ^{13}C NMR Spectral Data for Compound 2 [Parts per Million in $D_2O/CD_3OD/CF_3COOD$ (150 MHz)][a]

C	ppm	C	ppm
1	128.7	1″, 2″	56.3
2	104.0	1′	172.6
3	147.7	2′	41.0
4	134.3	3′	172.7
5	147.7	4′	53.2
6	104.0	5′	174.0
7	132.6	6′	31.0
8	123.6	7′	25.5
9	34.0	8′	52.2
		9′	171.2
		10′	31.9

[a] 1′ and 3′ are overlapping.

instrument with Ar/N_2 (9:1) as target gas. The collision energy was 15 or 25 V. Operating conditions were standard. The FAB-MS sample was prepared in a matrix of 0.1 N toluenesulfonic acid in 1:1 glycerol/3-nitrobenzyl alcohol, and the positive spectra were recorded on a Kratos M550TC instrument (Manchester, U.K.) at a scan speed of 10 s/decade using a resolution of 1100, with the gun producing a 7 kV beam of xenon atoms.

NMR. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded at 25 °C on a Bruker DRX 600 NMR spectrometer (Billierica, MA). A mixed solvent was used ($D_2O/CD_3OD/CF_3COOD$, 60:30:10 by volume), and the residual D_2O resonance was used as internal chemical shift reference. ^{1}H−^{1}H COSY and TOCSY and ^{1}H−^{13}C HMQC and HMBC experiments were performed using standard pulse sequence. Spectral widths of 10 and 200 ppm were used in the ^{1}H and ^{13}C dimensions, respectively.

***S*-Sinapyl-L-cysteine (compound 1)** was a slightly yellow solid: ESI-MS, m/z 314.0 (MH^+), 192.8 (MH^+ − cysteine), 160.8; MS/MS of daughter ion (m/z 192.8, collision energy 25 V), m/z (rel int) 192.8 (20), 161.2 (100), 143.2 (15), 133.2 (100), 115.0 (60), 105.2 (100), 79.0 (15), 55.0 (8); UV spectrum, see Figure 6; λ_{max} 285 nm (mobile phase); ^{1}H NMR, see Table 1.

***S*-Sinapylglutathione (compound 2)** was a slightly yellow solid: ESI-MS, m/z 500.0 (MH^+), 308.0 (glutathione·H^+), 192.8 (MH^+ − glutathione); UV spectrum, same as compound **1**; ^{1}H and ^{13}C NMR, see Tables 1 and 2; ^{1}H−^{13}C HMQC, see Table 3.

***N*-L-γ-Glutamyl-*S*-sinapyl-L-cysteine (compound 3)** was a slightly yellow solid: ESI-MS, m/z 442.8 (MH^+), 250.8 (Glu-Cys·H^+), 192.8 (MH^+ − Glu-Cys); FAB-MS, m/z 443.1473 (calcd for $C_{19}H_{27}O_8N_2S_1$, 443.1458; deviation, −3.4 ppm); UV spectrum, same as compounds **1** and **2**; ^{1}H NMR, see Table 1.

Sinapyl alcohol standard (Aldrich, Milwaukee, WI): ESI-MS, m/z 193.0 (MH^+ − H_2O); MS/MS (collision energy 15 V), m/z (rel int) 192.8 (16), 161.2 (80), 143.2 (5), 133.2 (83), 115.0 (45), 105.2 (100), 79.0 (10), 55.0 (5); UV spectrum, same as other sinapyl derivatives (mobile phase).

Table 3. Cross-Peaks in HMBC Spectra of Compound 2

H	C 3-bond	C 2-bond
2, 6	6, 2, 4, 7	3, 5
7	2, 6, 9	1
8	1	9
9	7, 10′	8
1″, 2″	3, 5	
2′	3′	1′ (?)
4′	5′	3′, 10′
6′	8′	5′, 7′
7′	5′, 9′	6′, 8′
8′	6′	7′, 9′
10′	9, 3′	4′

RESULTS AND DISCUSSION

Figure 1 shows the analytical HPLC chromatogram of pineapple juice with peaks 1−3 being the compounds investigated. The identities of other peaks and their concentration range for 54 authentic pineapple juice concentrate samples will be reported in another publication. Figure 2 is the semipreparative HPLC chromatogram of pineapple juice after C_{18} cartridge fractionation, which permitted collection of peaks 1−3 without impurities as determined by analytical HPLC. Figure 3 shows the acid hydrolysis pattern of compounds **2** and **3**. The acid hydrolysis of compound **2** generated compounds **1** and **3** and an additional compound with the same UV spectrum. Acid hydrolysis of compound **3** generated only compound **1**. Compound **1** was no longer hydrolyzed by acid. The odd number in molecular weight differences between compounds **2** and **3** and also between compounds **3** and **1**, as determined by ESI-MS, suggested the differences were caused by amino acid residues—glutamic acid and glycine. The release of those same amino acids from compounds **2** and **3** with acid hydrolysis was confirmed by amino acid analysis. Compound **1** has an odd-number molecular weight, so it should contain an odd number of nitrogens, and it is also possibly an amino acid derivative of a basic phenolic compound. Glutathione (Glu-Cys-Gly) is commonly found in plants, so it is possible that compound **2** is glutathionyl derivative of a phenolic compound, such as glutathionyl caftaric acid, which has been identified in grape juice (Cheynier et al., 1986). This structural proposal is in agreement with the ESI-MS fragmentation pattern of the three compounds. The ESI-MS shows a fragment dissociation of 121 units from compound **1**, which is the molecular weight of cysteine, 307 units (glutathione) from compound **2**, and 250 units (Glu-Cys) from compound **3**.

The molecular weight of compound **3** as determined by FAB-MS is 443.1473, with several different formulas being possible. The formula $C_{19}H_{27}O_8N_2S_1$ (MH^+) was chosen on the basis of the evidence for one sulfur atom (isotopic ratio ^{32}S:^{34}S = 100:4.4) and the maximum UV absorption at 285 nm. The proton NMR spectra of compounds **1**−**3** exhibit the characteristic pattern of Cys, Glu-Cys-Gly, and Glu-Cys except that the two β methylene protons of Cys were split (see Table 1). However, this splitting has been previously reported for a conjugated dipeptide with a similar type of structure (see Figure 4; Wemmer et al., 1993). The proton NMR spectra of these three compounds also exhibit the same resonances with chemical shifts at 6.4, 6.0, 5.5, 3.5, and 3.0 ppm. The 6.4 ppm chemical shift is the characteristic resonance of aromatic protons, whereas 6.0 and 5.5 ppm shifts are traits of double-bond protons. The final

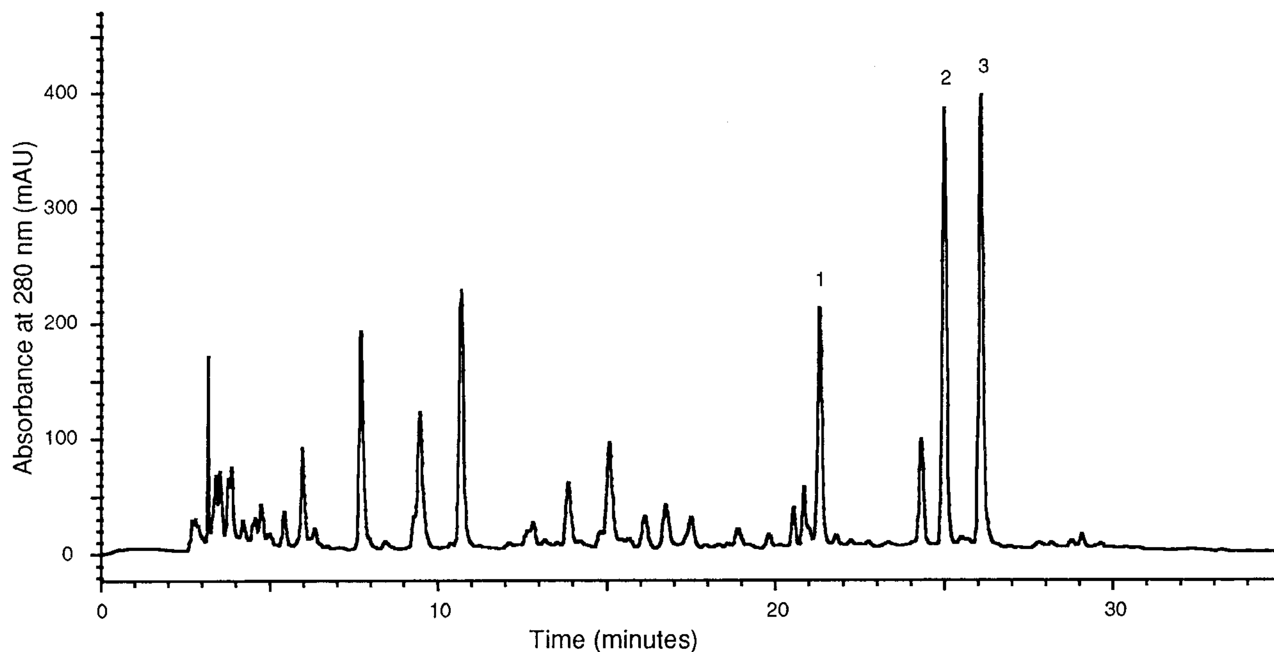

Figure 1. Analytical C_{18} reverse phase HPLC of pineapple juice. Peak identities: (1) *S*-sinapyl-L-cysteine; (2) *S*-sinapylglutathione; (3) *N*-L-γ-glutamyl-*S*-sinapyl-L-cysteine.

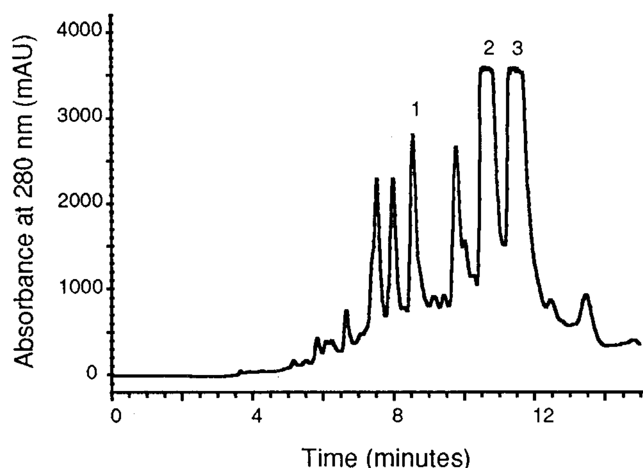

Figure 2. Semipreparative C_{18} reverse phase HPLC of pineapple juice (after C_{18} cartridge fractionation). Peak identities: (1) *S*-sinapyl-L-cysteine; (2) *S*-sinapylglutathione; (3) *N*-L-γ-glutamyl-*S*-sinapyl-L-cysteine.

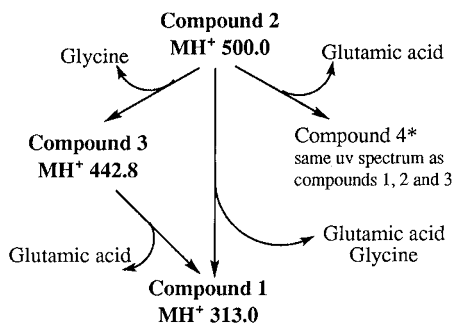

Figure 3. Acid hydrolysis pattern of compounds **2** and **3**.

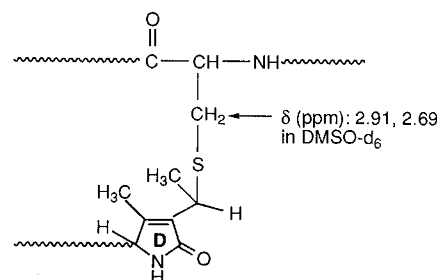

Figure 4. Partial structure of doubly linked 15,16-dihydrobiliverdin [redrawn from Wemmer et al. (1993)].

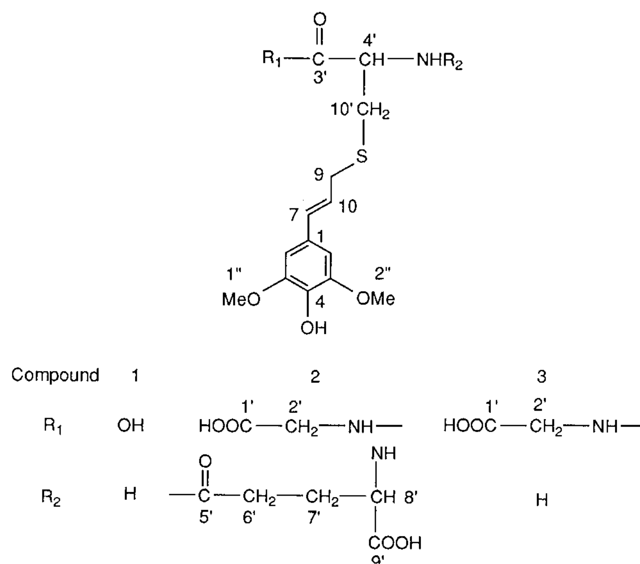

Figure 5. Structures of compounds **1**–**3**.

structure (Figure 5) was established by ^{13}C NMR (Table 2) and HMQC and HMBC (Table 3). The sinapyl core structure was further confirmed by the same UV spectrum (Figure 6) and fragmentation pattern as for sinapyl alcohol standard. It was found that the two protons at the C-2 and C-6 positions could undergo deuterium exchange under very acidic conditions when kept at room temperature for over 2 days. This exchange was followed by ^{1}H NMR, resulting in the almost complete loss of proton chemical shifts at 6.4 ppm. The deuterium exchange was further confirmed by a 2 mass unit increase in the fragment ion corresponding to the

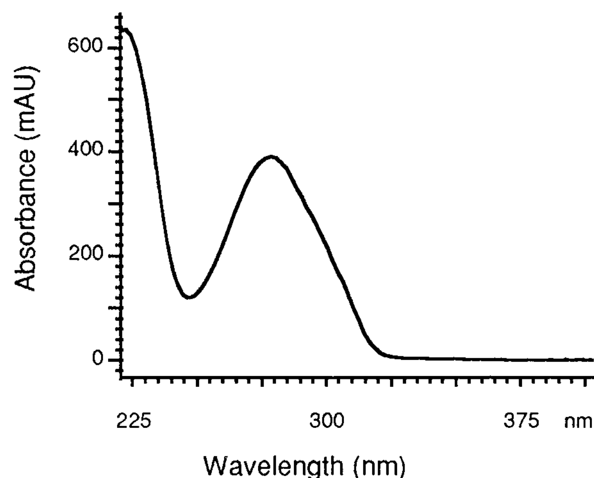

Figure 6. UV spectrum of compound **1**. Compounds **2** and **3** and sinapyl alcohol have the same UV spectrum as compound **1**.

sinapyl moiety [*m*/*z* (without deuterium exchange) 193] in the ESI-MS. This deuterium exchange can be used to establish the fragmentation pathway during MS/MS by comparing the fragmentation pattern of the compound with that of the deuterium-exchanged compound.

Glutathione is a common reducing compound in biological systems. It is involved in biosynthesis and metabolism and also plays a role in plant protection. For the glutathionyl derivative of caftaric acid identified in grape juice (Cheynier et al., 1986), glutathione was directly attached to the aromatic ring as a reducing agent after the oxidation of caftaric acid. The mechanism for the formation of the glutathionyl derivative of sinapyl alcohol in pineapple juice is not known. Furthermore, it is not clear if compounds **1** and **3** are formed independently or if they are the hydrolytic products of compound **1**. To our knowledge, this is the first report of the presence of glutathionyl derivatives of sinapyl alcohol.

ABBREVIATIONS USED

COSY, correlation spectroscopy; TCOSY, total correlation spectroscopy; HMQC, heteronuclear multiple bond quantum correlation; HMBC, heteronuclear multiple bond correlation; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry.

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