Acetophenone Glycosides from Thyme (*Thymus vulgaris* L.)

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Four acetophenone glycosides were isolated from the butanol-soluble fraction of thyme extracts. Their structures were determined by spectral methods (MS, NMR, and 2D-NMR). Among them, two new compounds, 4-hydroxyacetophenone 4-*O*-[5-*O*-(3,5-dimethoxy-4-hydroxybenzoyl)-β-D-apiofuranosyl]-(1→2)-β-D-glucopyranoside (**1**) and 4-hydroxyacetophenone 4-*O*-[5-*O*-(4-hydroxybenzoyl)-β-D-apiofuranosyl]-(1→2)-β-D-glucopyranoside (**2**), were determined. Compound **1** showed weak cytotoxicity, inhibiting DNA synthesis of human leukemia cells.

Keywords: *Thyme; acetophenone glycoside; Thymus vulgaris L.*

INTRODUCTION

Thymus vulgaris L., an herb commonly known as thyme, is a spice cultivated in central Europe, East Africa, India, and North America. It is also an herbal medicine used as hyperaemic, antibacterial, deodorizing agent in inflammation of the mouth and throat, a diuretic, urinary disinfectant, and vermifuge (Bisset, 1994). Thyme is an aromatic plant containing 1.0−2.5% essential oil, with thymol and carvacrol as main components. Flavonoids (Haraguchi et al., 1996; Samejima et al., 1995, Miura and Nakatani, 1989; Broucke et al., 1982) and biphenyl compounds (Nakatani et al., 1989) have also been found in the extract of thyme.

In early pharmacological works, the extracts of thyme were reported to show antioxidant activity (Economou et al., 1991), estrogen and progestin bioactivity (Zava et al., 1998), and antimutagenic activity (Vukovic-Gacic et al., 1993). There also have been a number of reports on the bioactivity of the essential oil fraction. The essential oil has shown antifungal activity (Wilson et al., 1997), antioxidant capacities in the prevention of age-related macular degeneration (Recsan et al., 1997), antioxidant activity (Dorman et al., 1995), antimicrobial activity (Farag et al., 1989), and inhibited growth and aflatoxin production in *Aspergillus parasiticus* (Tantaoui-Elaraki and Beraoud, 1994). Compounds from thyme have been examined for different bioactivity: some methylated flavones were isolated from thyme and tested as antioxidants (Miura and Nakatani, 1989); three new biphenyl compounds were identified as deodorant components (Nakatani et al., 1989); luteolin was identified to be a strong antimutagen against Trp-P-2, one of the dietary carcinogens formed during cooking (Samejima et al., 1995); two compounds, 3,4,3′,4′-tetrahydroxy-5,5′-diisopropyl-2,2′-dimethylbiphenyl and eriodicytol, were found to protect biological systems against various oxidative stresses (Haragachi et al., 1996); and *p*-cymene-2,3-diol, carvacrol, and thymol were found to show antioxidant activity (Schwarz et al., 1996).

In the course of searching for bioactive components from spices, we recently reexamined the chemical components of thyme. Here we report the structures of four acetophenone glycosides isolated from the *n*-butanol-soluble fraction and their weak antitumor activity.

MATERIALS AND METHODS

General Procedures. Chemicals. Silica gel (130−270 mesh), HP-20 ion-exchange resin (Sigma Chemical Co., St. Louis, MO), Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO), and Lichroprep RP-18 columns (Pharmacia Biotech Inc, Piscataway, NJ) were used for column chromatography. All solvents used for chromatographic isolation were analytical grade and purchased from Fisher Scientific (Springfield, NJ). [^{3}H]Thymidine (20 Ci/mmol) was purchased from DuPont Chemical Co., NEN Research Products (Boston, MA), and HL-60 cells and RPMI medium were purchased from the American Type Cell Cultures (Rockland, MD) and GIBCO BRL (Grand Island, NY), respectively.

Instrumental Analyses. ^{1}H NMR and ^{13}C NMR spectra were obtained on a VXR-200 instrument (Varian Inc., Melbourne, Australia). ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC were performed on a Varian U-500 instrument (Varian Inc., Melbourne, Australia). The desorption chemical ionization mass spectrum was measured on a JEOL SX-102 mass spectrometer (JEOL USA Inc., Peabody, MA). FAB mass spectra were recorded on a Finnigan MAT-90 instrument (Finnigan Inc., San Jose, CA). Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (250 μm thickness, 2−25μm particle size, Aldrich, Milwaukee, WI), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. Radioactivity was determined in a Beckman LS 1701 scintillation counter (Beckman Instruments Inc., Brea, CA).

Plant Material. Thyme (*T. vulgaris* L.) is harvested from California in 1997 when the plants are in bloom. The flowering tops are cut off, together with several inches of the tender, leafy stem. The remainder is shade-dried under ambient temperature to preserve the grayish-green color and used as spice.

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Extraction and Isolation Procedures. The dried thyme (4.5 kg) was extracted with 95% ethanol (10 L) at room temperature for 2 weeks. The extract was concentrated to dryness under reduced pressure; the residue was suspended in water (2 L) and partitioned successively with hexanes (3 × 2 L), ethyl acetate (3 × 2 L), and *n*-butanol (3 × 2 L). The *n*-butanol-soluble fraction (50 g) was subjected to column chromatography (CC) using 500 g of HP-20 ion-exchange resin, eluted with water−MeOH as eluent with increasing MeOH content (water, 3000 mL; water−MeOH, 3:7, 3000 mL; methanol, 3000 mL), and 3000 mL fractions were collected.

The fraction eluted by water−MeOH (3:7, 3000 mL) was dried and subjected to column chromatography (CC) on 1000 g of silica gel, eluted with ethyl acetate−methanol−water as eluent with increasing methanol and water content (12:1:1, 11:1:1, 10:1:1, 8:1:1, 6:1:1, 2:1:0.5, each 3000 mL), and 1000 mL fractions were collected. A total of 18 fractions (1−18) were collected. Fraction 2 was subjected to a Sephadex LH-20 column (eluted with methanol) to get two fractions; then the first fraction was subjected to silica gel chromatography using ethyl acetate−methanol−water (14:1:1) to get five subfractions. A 2 mg sample of compound **2** was obtained from subfraction 2 using RP-18 chromatography and eluted with methanol−water (3:7). Further chromatography of subfraction 3 on a Lichroprep RP-18 column using methanol−water (3:7) and a silica gel column using methylene chloride−methanol−water (7:1:0.1) yielded 25 mg of compound **3** and 17 mg of compound **1**.

Fractions 4−6 were combined together and rechromatographed on a Lichroprep RP-18 column using methanol−water (1:3, 1000 mL; 2:3, 1000 mL) and methanol (500 mL) to obtain 13 subfractions. Seven milligrams of compound **4** was obtained from subfraction 1 using silica gel column chromatography eluted with chloroform−methanol−water (6:1:0.1).

Antitumor Activity Test with Human Leukemia Cell HL-60. The HL-60 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin−streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C. HL-60 cells were then harvested by centrifugation for 10 min at 1000*g*. The pellets were suspended in RPMI medium without fetal calf serum and penicillin−streptomycin to a concentration of 5×10^5 cells/mL. To a series of 13 × 100 mm test tubes containing 1 mL of cell suspension wad added 2 μL of DMSO (as control) or various concentration of tested compounds. For the measurement of DNA synthesis, 3 μL of [^{3}H]thymidine (50 μCi/μmol) was added to every tube. The tubes were incubated at 37 °C for 2 h. The reaction was then terminated by adding of 1 mL of ice cold phosphate buffer saline (PBS) and centrifuged for 10 min at 1000*g*. The supernatant was discarded and cells were lysed with 1 mL of ice cold deionized water and 1 mL of 10% trichloroacetic acid (TCA) solution. The macromolecular substances were collected and washed three times with 3 mL of 5% TCA and two times with 3 mL of acetone. The dry filters were placed in scintillation vials with 5 mL of Scient Varse fluid (Fisher Scientific, Springfield, NJ) and radioactivity was determined in an Beckman LS 1701 scintillation counter.

RESULTS AND DISCUSSION

The *n*-butanol fraction of a 95% ethanol extract of thyme was chromatographed over Sephadex LH-20, silica gel, and C_{18} reversed-phase silica gel to yield compounds **1**−**4** (Figure 1).

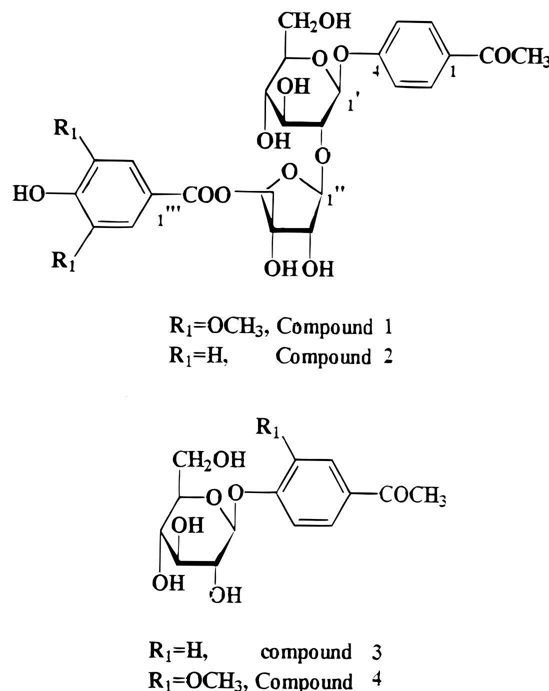

Figure 1. Chemical structures of compounds **1**−**4**.

Compound **1** was isolated as a white powder and exhibited prominent quasi-molecular ion peaks in the positive FAB mass spectrum at *m*/*z*: 611 $[M + 1]^+$ and 633 $[M + Na]^+$, together with ^{13}C NMR, suggesting a $C_{28}H_{34}O_{15}$ molecular formula for compound **1**. The ^{13}C NMR showed a total of 28 carbon peaks, including 13 methine, 9 quaternary, 3 methylene, and 3 methyl groups. Among them, signals at δ 199.2 (s), 162.4 (s), 132.2 (s), 131.5 (d, 2C), 116.6 (d, 2C), and 26.3 (q) demonstrated that compound **1** was an acetophenone glycoside (Ushiyama and Furuya, 1989), while signals at δ 167.5 (s), 148.7 (s, 2C), 142.1 (s), 120.9 (s), 108.3 (d, 2C), and 56.9 (q, 2C) suggested a 3,5-dimethoxy-4-hydroxybenzoyl moiety in **1** (Joe et al., 1996). This was supported by the ^{1}H NMR spectrum, which showed the signals for a 1,4-bisubsituted phenyl group [7.69 (2H, d, J = 9.0 Hz), 6.93 (2H, d, J = 9.0 Hz)] and a signal at 7.14 (s, 2H) for another phenyl group. The ^{13}C NMR also showed the signals for two sugars with anomeric sugar carbons at δ 99.6 and 110.2 ppm, while in the ^{1}H NMR spectrum, signals for anomeric sugar protons were at δ 5.05 (1H, d, J = 7.8 Hz) and 5.51 (1H, J = 0.7 Hz) suggesting the sugars were possibly β-D-glucose and β-D-apiose. ^{1}H−^{1}H COSY, HMBC, and HMQC spectra allowed full assignment of the ^{1}H and ^{13}C NMR data of sugar moiety. The data matched those previously reported for β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside (Takeda et al., 1997; Wang et al., 1998). Thus, the presence of two sugar moieties and two aromatic rings was deduced.

The linkage of the sugar moiety and two aromatic rings was deduced from the HMBC spectrum, in which the carbonyl group (δ 167.5) showed correlation with H-5″(apiose proton) at δ 4.18 and 4.33, while the 4-position carbon of acetophenone moiety showed correlation with a proton at 5.05 ppm assigned to H-1′ of the glucose moiety. So compound **1** was elucidated as 4-hydroxyacetophenone 4-*O*-[5-*O*-(3,5-dimethoxy-4-hydroxybenzoyl)-β-D-apiofuranosyl]-(1→2)-β-D-glucopyranoside. This structure was further confirmed by the NOESY spectrum with correlation cross-peaks between H-1″ of the apiose moiety and H-2′ of the glucose moiety and of H-1′ of the glucose moiety with H-3 and H-5 of the acetophenone moiety.

Compound **2** was isolated as a white powder, and the molecular formula $C_{26}H_{30}O_{13}$ was deduced from the ^{13}C NMR spectrum and FAB-MS showing $[M + 1]^+$ at 551 and $[M + Na]^+$ at 573. The ^{13}C NMR spectrum displayed typical signals for *p*-hydroxyacetophenone at δ 199.6 (s), 162.9 (s), 132.7 (s), 131.9 (d, 2C), 117.0 (d, 2C), and 26.7

Table 1. ^{13}C NMR Data of Isolated Compounds 1−4

	1 (CD_3OD)	**2** (CD_3OD)	**3** (CD_3OD)	**4** (DMSO-d_6)
1	132.2 (s)	132.7 (s)	132.9 (s)	131.1 (s)
2	131.5 (d)	131.9 (d)	131.9 (d)	111.2 (d)
3	116.6 (d)	117.0 (d)	117.5 (d)	150.9 (s)
4	162.4 (s)	162.9 (s)	163.4 (s)	148.9 (s)
5	116.6 (d)	117.0 (d)	117.5 (d)	114.4 (d)
6	131.5 (d)	131.9 (d)	131.9 (d)	123.0 (d)
7	199.2 (s)	199.6 (s)	199.7 (s)	196.7 (s)
8	26.3 (q)	26.7 (q)	26.8 (q)	26.7 (q)
1′	99.6 (d)	100.1 (d)	101.8 (d)	99.7 (d)
2′	77.6 (d)	78.1 (d)	75.1 (d)	73.3 (d)
3′	78.8 (d)	79.0 (d)	78.2 (d)	77.4 (d)
4′	71.3 (d)	71.6 (d)	71.5 (d)	69.8 (d)
5′	78.2 (d)	78.5 (d)	78.6 (d)	77.1 (d)
6′	62.4 (t)	62.7 (t)	62.7 (t)	60.8 (t)
1″	110.2 (d)	110.5 (d)		
2″	78.7 (d)	78.9 (d)		
3″	79.2 (s)	79.5 (s)		
4″	75.2 (t)	75.6 (t)		
5″	68.2 (t)	68.2 (t)		
1‴	120.9 (s)	122.1 (s)		
2‴	108.3 (d)	133.1 (d)		
3‴	148.7 (s)	116.4 (d)		
4‴	142.1 (s)	163.8 (s)		
5‴	148.7 (s)	116.4 (d)		
6‴	108.3 (d)	133.1 (d)		
7‴	167.5 (s)	167.9 (s)		
OCH_3	56.9 (q)			55.9 (q)

(q), while in the ^{1}H NMR spectrum, the signals for it were at δ 7.01 (2H, d, $J = 9.0$ Hz) and 7.71 (2H, d, $J = 9.0$ Hz). The ^{1}H NMR spectra ^{13}C NMR spectra also showed the exact signals for β-D-apiofuranosyl-(1→2)-β-D-glucopyranoside, as in compound **1**. But the signals for the 3,5-dimethoxy-4-hydroxybenzoyl moiety disappeared; instead, the signal for a 4-hydroxybenzoyl moiety appeared in both ^{1}H NMR (at δ 6.72, 2H, d, $J = 9.0$ Hz and 7.75, 2H, d, $J = 9.0$ Hz) and ^{13}C NMR [at δ 167.9 (s), 163.8 (s), 133.1 (d, 2C), 122.1 (s), 116.4 (d, 2C)] spectra (Okuyama et al., 1998). So compound **2** was elucidated as 4-hydroxyacetophenone 4-*O*-[5-*O*-(4-hydroxybenzoyl)-β-D-apiofrunosyl]-(1→2)-β-D-glucopyranoside.

Compounds **3** and **4** were identified as picein and androsin, respectively, by comparison of spectral data with literature. In the antitumor test, the initial rate of incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)-insoluble material was utilized to estimate the rate of DNA synthesis. Compound **1** showed weak cytotoxicity (IC_{50}, 40 μM) and was in a dose dependent manner.

4-Hydroxyacetophenone 4-*O*-[5-*O*-(3,5-dimethoxy-4-hydroxybenzoyl)-β-D-apiofuranosyl]-(1→2)-β-D-glucopyranoside (1): white powder; FAB-MS *m/z* 611 [M + 1]$^+$, 633 [M + Na]$^{\bullet +}$; ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) see Tables 1 and 2.

4-Hydroxyacetophenone 4-*O*-[5-*O*-(4-hydroxybenzoyl)-β-D-apiofuranosyl]-(1→2)-β-D-glucopyranoside (2): white powder; FAB-MS *m/z* 551 [M + 1]$^+$, 573 [M + Na]$^+$; ^{1}H NMR (200 MHz, CD_3OD) 7.75 (2H, d, $J = 9.0$ Hz, H-2‴and H-6‴), 7.71 (2H, d, $J = 9.0$ Hz, H-2 and H-6), 7.01 (2H, d, $J = 9.0$ Hz, H-3 and H-5), 6.72 (2H, d, $J = 9.0$ Hz, H-3‴ and H-5‴), 5.52 (1H, br s, H-1″), 5.09 (1H, d, $J = 7.6$ Hz, H-1′), 4.23−4.33 (3H, m), 3.97 (1H, br s, H-2″), 3.29−3.94 (m), 2.46 (3H, s, H-8); ^{13}C NMR see Table 1.

Picein (3): amorphous powder; DCI-MS *m/z* 316 [M + NH_4]$^+$; ^{1}H NMR (200 MHz, CD_3OD) δ 7.98 (2H, d, $J = 8.0$ Hz, H-2 and H-6), 7.17 (2H, d, $J = 8.0$ Hz, H-3 and H-5), 5.04 (1H, d, $J = 7.2$ Hz, H-1′), 3.90 (1H, m, H-6′), 3.71 (1H, dd, $J = 12.0$; 5.6 Hz, H-6′), 3.31−3.52 (4H, m, H-2′; H-3′; H-4′ and H-5′), 2.56 (3H, s, H-8); ^{13}C NMR, see Table 1, are identical with the literature (Ushiyama and Furuya, 1989).

Table 2. ^{1}H NMR Data, HMBC, and NOESY Correlation of Compound 1 in CD_3OD

	^{1}H NMR	$HMBC_{C\to H}$	NOESY
1		H-3, 5, 8	
2	7.69 (1H, d, $J = 9.0$ Hz)	H-6	H-8, 3
3	6.93 (1H, d, $J = 9.0$ Hz)	H-2, 5	H-2, 1′
4		H-2, 3, 5, 6, 1′	
5	6.93 (1H, d, $J = 9.0$ Hz)	H-3, 6	H-6, 1′
6	7.69 (1H, d, $J = 9.0$ Hz)	H-2	H-8, 5
7		H-2, 6, 8	
8	2.43 (3H, s)		H-2, 6
1′	5.05 (1H, d, $J = 7.6$ Hz)	H-2′	H-5′, 3, 5, 3′
2′	3.70 (1H, dd, $J = 9.0$, 7.6 Hz)	H-3′	H-4′, 1″
3′	3.63 (1H, dd, $J = 9.0$, 9.0 Hz)	H-2′	H-1′, 5′
4′	3.39 (1H, d, $J = 9.0$ Hz)	H-3′, 5′, $6'_a$, $6'_b$	H-2′, $6'_a$, $6'_b$
5′	3.45 (1H, m)	H-4′	H-1′, 3′, $6'_a$
$6'_a$	3.86 (1H, dd, $J = 2.2$, 12.2 Hz)	H-4′	H-4′, 5′, $6'_b$
$6'_b$	3.67 (1H, dd, $J = 5.6$, 12.2 Hz)		H-4′, $6'_b$
1″	5.51 (1H, d, $J = 0.7$ Hz)	H-2′, 2″, $4''_a$	H-2′, 2″
2″	4.00 (1H,d, $J = 0.7$ Hz)		H-1″, $5''_a$, $5''_b$
3″		H-$4''_b$, $5''_a$, $5''_b$	
$4''_a$	3.90 (1H, d, $J = 9.5$ Hz)	H-$5''_a$, $5''_b$, 1″,	H-$4''_b$
$4''_b$	4.27 (1H, d, $J = 9.5$ Hz)		H-$4''_a$
$5''_a$	4.18 (1H, d, $J = 11.2$ Hz)	H-$4''_a$, $4''_b$, 2″	H-2″, $5''_b$
$5''_b$	4.33 (1H, d, $J = 11.2$ Hz)		H-2″, $5''_a$
1‴		H-2‴, 6‴	
2‴	7.14 (1H, s)	H-6‴	OCH_3
3‴		H-2‴ and OCH_3	
4‴		H-2‴, 6‴	
5‴		H-6‴ and OCH_3	
6‴	7.14 (1H, s)	H-2‴	OCH_3
7‴		H-2‴, $5''_a$, $5''_b$,6‴	
OCH_3			H-2‴, 6‴

Androsin (4): white powder; FAB-MS *m/z* 329 [M + 1]$^+$, 351 [M + Na]$^+$; ^{1}H NMR (200 MHz, DMSO-d_6) δ 7.58 (1H, dd, $J = 8.0$, 1.8 Hz, H-6), 7.48 (1H, d, $J = 1.8$ Hz, H-2), 7.19 (1H, d, $J = 8.0$ Hz, H-5), 5.07 (1H, d, $J = 7.2$ Hz, H-1′), 3.13−4.12 (m, H-glu), 3.84 (3H, OCH_3), 2.56 (3H, s, H-8); ^{13}C NMR, see Table 1, are identical with the literature (Junior, 1986; Stuppner et al., 1989).

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