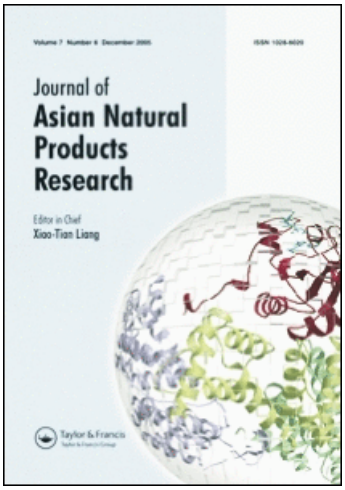

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Cytotoxic activity of flavonoids from the flowers of *Chrysanthemum morifolium* on human colon cancer Colon205 cells

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A new *p*-hydroxyphenylacetyl flavonoid, diosmetin 7-(6″-*O*-*p*-hydroxyphenylacetyl)-*O*-β-D-glucopyranoside (**1**), was isolated from the flowers of *Chrysanthemum morifolium* Ramat. 'huaiju' cv. nov. (Compositae), together with five known flavonoids, luteolin (**2**), diosmetin (**3**), diosmetin 7-*O*-β-D-glucopyranoside (**4**), diosmin (**5**), and scolimoside (**6**), and four known caffeoylquinic acid derivatives, macranthoin F (**7**), 3,5-dicaffeoylquinic acid (**8**), 1,3-dicaffeoyl-*epi*-quinic acid (**9**), and chlorogenic acid (**10**). The structure of **1** was elucidated by UV, IR, ESI-TOF-MS, 1D, and 2D NMR spectroscopic methods. Cytotoxic activity of compounds **1**–**5** against human colon cancer cell Colon205 was investigated using MTT assays. Compounds **2** and **3** showed significant cytotoxicities against Colon205, with their IC_{50} values being 96.9 and 82.9 μM, respectively. However, compounds **1**, **4**, and **5** showed little cytotoxic activity.

Keywords: *Chrysanthemum morifolium* Ramat.; flavonoids; cytotoxicity; Colon205

1. Introduction

Flos Chrysanthemi (the flowers of *Chrysanthemum morifolium* Ramat., Compositae) has been traditionally used to treat *wind-heat*-type common cold, headache, dizziness, and dim sight [1,2]. Some potentially useful natural compounds, including flavonoids, triterpenoids, and caffeoylquinic acid derivatives, have been isolated from *Flos Chrysanthemi* in the previous studies [3–8]. They exhibited wide pharmacological effects, such as the inhibitory activities of HIV-1 integrase and aldose reductase and antioxidant, anti-inflammatory, anti-mutagenic, and anti-allergic activities [3,8–13]. Recently, the inhibitory effect of luteolin on the development of cancer cells was investigated *in vitro* and *in vivo*. Its antitumor mechanism was associated with the protection against carcinogenic stimuli, inhibition of tumor cell proliferation, induction of cell cycle arrest, and induction of apoptosis via intrinsic and extrinsic signaling pathways [14]. Diosmetin also showed significant cytotoxicity on some human tumor cells, such as colon carcinoma cell (HT-29, Caco-2) and breast carcinoma cell (MCF-7) [15,16].

The crude materials of *Flos Chrysanthemum* are rather complicated due to different habitats. Traditionally, *Flos Chrysanthemum* was strictly divided into two sorts according to their different usages. Those for medicinal use included

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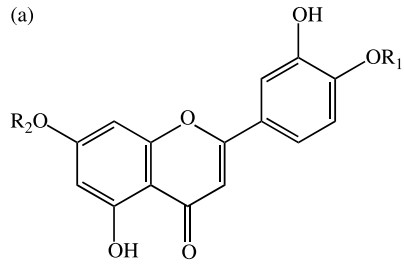
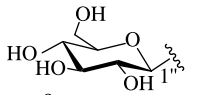
C. morifolium cv. Huai-ju, Bo-ju, Qi-ju, Ji-ju, and Chu-ju, while *C. morifolium* cv. Hang bai-ju, Hang huang-ju, and Gong-ju were generally used for tea [17]. Although Huai-ju, botanically from the flowers of *C. morifolium* Ramat. 'huaiju' cv. nov., is a typical representative for medicinal uses, only the chemical studies on its essential oils have been reported [18].

The present study describes the isolation and structural elucidation of a new *p*-hydroxyphenylacetyl flavonoid (**1**), together with nine known compounds including five polyhydroxylated flavonoids (**2**–**6**) and four caffeoylquinic acid derivatives (**7**–**10**) (Figure 1). Moreover, cytotoxic activities of compounds **1**–**5** against human colon cancer cell Colon205 were investigated using MTT assays.

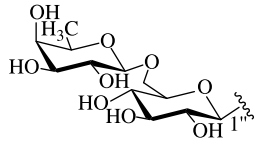
2. Results and discussion

The 95% EtOH extract of the flowers of *C. morifolium* Ramat. 'huaiju' cv. nov. was prepared by the reflux method. The petroleum ether- and water-soluble fractions were obtained by partitioning the crude extract. The water fraction was subjected to DA101 column chromatography, eluting with an EtOH–H_2O gradient to give H_2O-, 30% EtOH-, 60% EtOH-, and 95% EtOH-eluted fractions. A new *p*-hydroxyphenylacetyl flavonoid, diosmetin 7-(6″-*O*-*p*-hydroxyphenylacetyl)-*O*-β-D-



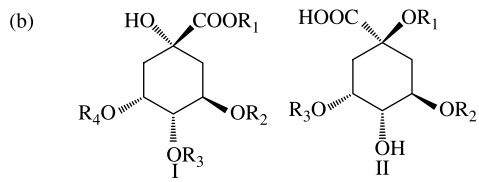


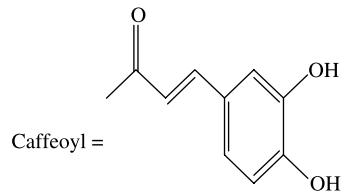
β-D-glucopyranosyl



α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl

Compounds	R_1	R_2
diosmetin 7-(6"-*O*-*p*-hydroxyphenylacetyl) -*O*-β-D-glucopyranoside (**1**)	CH_3	6"-*O*-*p*-hydroxyphenylacetyl-glc
luteolin (**2**)	H	H
diosmetin (**3**)	CH_3	H
diosmetin 7-*O*-β-D-glucopyranoside (**4**)	CH_3	-glc
diosmin (**5**)	CH_3	-glc-rham
scolimoside (**6**)	OH	-glc-rham





Compounds		R_1	R_2	R_3	R_4
macranthoin F (**7**)	I	H	caffeoyl	H	caffeoyl
3,5-dicaffeoylquinic acid (**8**)	I	CH_3	caffeoyl	caffeoyl	H
1,3-dicaffeoyl-*epi*-quinic acid (**9**)	II	caffeoyl	caffeoyl	H	H
chlorogenic acid (**10**)	I	H	H	H	caffeoyl

Figure 1. Structures of compounds **1**–**10**.

glucopyranoside (**1**), was isolated from the 60% EtOH-eluted fraction, together with five known polyhydroxylated flavonoids, luteolin (**2**), diosmetin (**3**), diosmetin 7-*O*-β-D-glucopyranoside (**4**), diosmin (**5**), and scolimoside (**6**). Moreover, four caffeoylquinic acid derivatives, macranthoin F (**7**), 3,5-dicaffeoylquinic acid (**8**), 1,3-dicaffeoyl-*epi*-quinic acid (**9**), and chlorogenic acid (**10**), were obtained from the 30% EtOH-eluted fraction. The structure of compound **1** was elucidated using UV, IR, ESI-TOF-MS, 1D, and 2D NMR techniques. Compounds **2**–**9** were identified by comparing their NMR and MS spectral data with reported values [5,8,19–24].

Compound **1** was isolated as a yellowish amorphous powder. The molecular formula was determined to be $C_{30}H_{28}O_{13}$ from the molecular ion peak at *m/z* 595.1469 in the ESI-TOF-MS. The UV absorption maxima at 226.5, 252.5, and 346.4 nm revealed a flavonoid skeleton. A positive reaction with the $AlCl_3$ reagent suggests that it is a hydroxyl-substituted flavonoid. The IR spectra showed absorption bands for a hydroxyl group (3408 cm^{-1}), a carbonyl group (1650 and 1722 cm^{-1}), and an aromatic ring (1607, 1497, 1443 cm^{-1}). The ^1H NMR spectrum of **1** (Table 1) revealed six aromatic proton signals due to the aglycone moiety, a

Table 1. ^1H and ^{13}C NMR spectral data for compound **1**.

Position	δ_C[a], mult.	δ_H[b] (*J* in Hz)	HMBC[c]
2	165.1		
3	103.9	6.85 (s)	2, 4, 10, 1′
4	182.0		
5	161.5		
6	100.5	6.37 (d, $J = 2.1$)	5, 7, 8, 10
7	163.2		
8	94.1	6.81 (d, $J = 2.1$)	6, 7, 9, 10
9	157.5		
10	105.9		
1′	122.9		
2′	113.4	7.84 (d, $J = 2.1$)	3′, 4′, 6′, 2
3′	151.7		
4′	148.8		
5′	112.3	6.99 (d, $J = 8.7$)	3′, 4′, 2, 6′
6′	118.0	7.45 (dd, $J = 8.7, 2.1$)	2, 2′
1″	100.8	4.94 (d, $J = 7.1$)	7
2″	73.3	3.21 (m)	1″, 3″, 4″
3″	76.0	3.28 (m)	1″, 2″, 4″
4″	70.2	3.08 (m)	3″, 5″
5″	75.0	3.68 (t)	3″, 4″, 6″
6″-α	63.9	3.79 (m)	5″
6″-β		4.66 (d, $J = 11.2$)	8‴
1‴	121.1		
2‴	128.7	7.95 (brd, $J = 8.7$)	4‴, 3‴
3‴	116.2	6.95 (brd, $J = 8.7$)	
4‴	164.1		
5‴	116.2	6.95 (brd, $J = 8.7$)	
6‴	128.7	7.95 (brd, $J = 8.7$)	
7‴	45.3	3.05 (m)	
8‴	169.1		

Notes: [a,b] Measured separately at 150 and 600 MHz, respectively.
[c] HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon. Some chemical shift assignments were done on the basis of the HMQC and HMBC techniques.

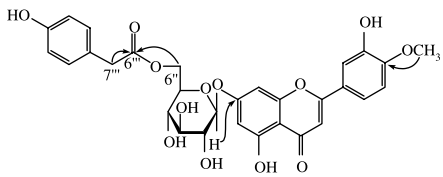


Figure 2. Key HMBC correlations of **1**.

singlet at δ 6.85 due to H-3, a pair of doublet at δ 6.37 (1H, d, $J = 2.1$ Hz, H-6) and 6.81 (1H, d, $J = 2.1$ Hz, H-8) indicating A-ring with two substituents, an ABX aromatic proton system appeared at δ 7.84 (1H, d, $J = 2.1$ Hz, H-2′), 7.45 (1H, dd, $J = 2.1$, 8.7 Hz, H-6′), and 7.00 (1H, d, $J = 8.7$ Hz, H-5′) suggesting two substituents attached to the B-ring, and a singlet at δ 3.82 due to methoxyl protons. The location of the methoxyl at C-4′ was deduced from the HMBC correlation (Figure 2) between its protons and C-4′. The resonances for carbons and protons of the aglycone had a close resemblance to those of diosmetin [20], thus the aglycone of **1** was confirmed as diosmetin. The signals of an anomeric proton at δ 4.94 (1H, d, $J = 7.1$ Hz) and five protons (δ 4.66–3.08), together with the ^{13}C NMR spectral data, indicated the presence of a β-D-glucosyl moiety. The location of the sugar moiety at C-7 was deduced from the HMBC spectrum (Figure 2). The HMBC correlation was observed between H-1″ (δ 4.94) and C-7 (δ_C 163.2). The resonances for the carbons and protons of the aglycone and sugar moiety, except for those of C-6″, C-5″, as well as the related protons (Table 1), had a close resemblance to those of diosmetin 7-*O*-β-D-glucopyranoside (**4**), and they were assigned according to the literature values of the ^{1}H and ^{13}C NMR spectral data [5] for **4** as well as its own HSQC spectrum. The downfield shift of C-6″ (δ_C 63.9, + 3.7 ppm) and the upfield shift of C-5″ (δ_C 75.0, − 2.2 ppm) relative to the corresponding signals of compound **4** revealed the acylation of C-6″, which is also supported by the corresponding signals of taxifolin 3′-(6″-*O*-phenylacetyl)-*O*-β-D-glucopyranoside [25]. The resonances for the carbons and protons of the substituent in the ^{1}H and ^{13}C NMR as well as HSQC spectra included an AA′BB′ aromatic proton system appearing at δ 6.95 (2H, d, $J = 8.7$ Hz, H-3‴, 5‴; δ_C 116.2) and 7.95 (2H, d, $J = 8.7$ Hz, H-2‴, 6‴; δ_C 128.7), a methine (δ 3.05, 2H, m; δ_C 45.3), and a carbonyl (δ_C 169.1) as evidenced by the IR absorption bands at 1722 cm^{-1}. Compared with the ^{13}C NMR spectral data of taxifolin 3′-*O*-β-(6″-*O*-phenylacetyl)-glucopyranoside, the chemical shift of C-4‴ (δ_C 164.1) shifted downfield by 36.2 ppm, while that of C-3‴, 5‴ (δ_C 116.2), and C-1‴ (δ_C 121.1) shifted upfield by 13.2 and 12.4 ppm, respectively. The above NMR spectral data indicated the hydroxylation of C-4‴. The literature values for rengyolester [26] also support the presence of the 4‴-hydroxyl group. The *para*-substituted moiety, —CH_2—CO—, was deduced from the HMBC spectrum (Figure 2). Thus, the substituent was confirmed as *p*-hydroxyphenylacetyl. After mild alkaline hydrolysis of **1** with 0.05 N NH_4OH in 50% MeOH, a mixture of two aromatic compounds was recovered from the ethyl acetate extract of the hydrolysate. They were separated and identified as diosmetin 7-*O*-β-D-glucopyranoside and *p*-hydroxyphenylacetic acid. The above evidence suggested that compound **1** is a derivative of diosmetin 7-*O*-β-D-glucopyranoside, with *p*-hydroxyphenylacetyl as a substituent attached to the sugar moiety. The HMBC correlations between H-6″ (δ 3.79, m and δ 4.66, d, $J = 11.2$ Hz) and the carbonyl carbon suggested a linkage between the acyl moiety and C-6″ of glucose. Hence, compound **1** was assigned as diosmetin 7-(6″-*O*-*p*-hydroxyphenylacetyl)-*O*-β-D-glucopyranoside, a new flavonoid glycoside, which is the first reported acylated flavonoid glycoside with an acyl group of *p*-hydroxyphenylacetic acid.

The cytotoxic activity of compounds **1**–**5** on Colon205, which is a kind of non-metastatic human colon cancer cell lines, was assayed for the first time. Compounds **2** and **3** showed significant cytotoxicities against Colon205 cells, with the IC_{50} values being 96.9 and 82.9 μM, respectively. Our results suggest that luteolin and diosmetin may be of therapeutic potential for the treatment of colon cancer. The glycosidic flavonoids **1**, **4**, and **5** showed little cytotoxicity ($IC_{50} > 200$ μM).

3. Experimental

3.1 General experimental procedures

Melting points were measured using a micro melting point apparatus and are uncorrected (Yanamoto Manufactory Co., Kyoto, Japan). Optical rotations were recorded on a Perkin-Elmer 241MC automatic polarimeter. UV spectra were obtained with a Shimadzu UV-2201 spectrophotometer, and IR spectra were obtained with a Bruker IFS-55 infrared spectrometer. 1D and 2D NMR spectra were recorded separately on a Bruker ARX-600 or ARX-300 spectrometer. ESI-TOF-MS were acquired on a Micro TOF Bruker Daltonics mass spectrometer with a resolution of 25,000 (10% Valley). Preparative HPLC was carried out using a Shimadzu's LC-8A solvent delivery pump and Shimadzu's SPD-10AVP detector. Authentic chlorogenic acid was purchased from Wako Chemicals Co. (Osaka, Japan). Column chromatography was carried out on silica gel (200–300 mesh; Qingdao Haiyang Chem. Group Co., Qingdao, China), polyamide gel (Zhejiang Taizhou Luqiaosijia Biochemistry Plastic Co., Taizhou, China), DA101 (Fushun Xintai Fine Chemical Co., Fushun, China), Sephadex LH-20 (GE Healthcare, Uppsala, Sweden), DIAION HP-20 (Mitsubishi Chemical Co., Tokyo, Japan), ODS (YMC Co., Ltd, Kyoto, Japan), MDS-5 reverse-phase packing (200–300 mesh; Beijing Medicine Technology Center, Beijing, China), Varian BOND ELUT® C18 column (GL Science, Tokyo, Japan), and Shim-pack PRC-ODS column (2.0 × 25 cm; Shimadzu Co., Ltd, Tokyo, Japan).

3.2 Plant material

Flowers of *C. morifolium* Ramat. 'huaiju' cv. nov. were collected in Wuzhi County, Henan Province, China. They were authenticated by Professor Dan Yuan, Shenyang Pharmaceutical University. A voucher specimen (No. 021204) has been maintained in the Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University.

3.3 Biological materials

Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China). RMPI 1640 medium was purchased from Gibco BRL (Grand Island, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemicals (St Louis, MO, USA). Compounds **1**–**5** with their purities being more than 97% by the HPLC method were dissolved initially in DMSO and then were diluted with RMPI 1640 medium for experiments. DMSO at the highest concentration possibly present under the experimental conditions used (0.1%) was not toxic to cells.

3.4 Cell cultures

Human colon cancer cell Colon205 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RMPI 1640 supplemented with 10% FBS, L-glutamine (2 mmol/l), penicillin (100 U/ml), and streptomycin (100 mg/ml), and maintained at 37°C with 5% CO_2 in a humidified atmosphere.

3.5 Cell viability

The cytotoxic effect of compounds **1**–**5** on Colon205 cell was measured by the MTT assay as described previously [27]. In brief, the cells were dispensed in 96-well flat bottom microtiter plates at a density of 5×10^3 cells per well. After 24 h incubation, they were treated with various concentrations of compounds **1**–**5** for the indicated time periods. After various treatments, the cells were incubated with MTT (0.25 mg/ml) for 3 h at 37°C. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.7). The level of MTT formazan was determined by measuring its absorbance at the wavelength of 490 nm with a SPECTRA (shell) Reader (Tecan, Grödig, Austria).

3.6 Extraction and isolation

The air-dried flowers (3.7 kg) of *C. morifolium* Ramat. 'huaiju' cv. nov. were extracted with EtOH (2× 30 L) using the reflux method. The condensed extract (710 g) was dissolved in water (1 L), and successively partitioned with petroleum ether (3× 1 L) to obtain petroleum ether fraction (203 g) and water fraction (506.6 g). The water fraction was subjected to macro porus resin DA101 column chromatography, eluting with an EtOH–H_2O gradient, to give H_2O-, 30% EtOH-, 60% EtOH-, and 95% EtOH-eluted fractions. The 30% EtOH-eluted fraction (78.6 g) was passed through polyamide gel column chromatography, eluting with an EtOH–H_2O gradient to yield five fractions (1–5). Fraction 2 (50 mg) eluted with EtOH–H_2O (10:90) was passed through a Sephadex LH-20 column chromatography (1.5 × 30 cm) to give compound **10** (29 mg). Fraction 3 (5.99 g) eluted with EtOH–H_2O (30:70) was further subjected to a Sephadex LH-20 column (2.0 × 60 cm) eluting with MeOH, and then further purified by vacuum liquid chromatography using silica gel (6.0 × 6.0 cm) eluting with the $CHCl_3$–MeOH gradient to yield five fractions (6–10). Fraction 8 (500 mg) eluted with $CHCl_3$–MeOH (10:1) was further separated with preparative HPLC eluting with MeOH–H_2O (40:60) at a flow rate of 9.0 ml/min to afford compound **7** (21 mg). Fraction 8 (590.5 mg) eluted with $CHCl_3$–MeOH (8:1) was further subjected to an ODS open column chromatography (3.0 × 60 cm) eluting with MeOH–H_2O (50:50) to give compound **9** (211.4 mg). Fraction 4 (5.22 g) was repeatedly purified with silica gel column chromatography, and, finally, with preparative HPLC using MeOH–H_2O–DMC (15:81:4) plus 0.5% formic acid as an eluent at a flow rate of 20 ml/min to afford compound **8** (37 mg). The 60% EtOH-eluted fraction (76.3 g) was passed through silica gel column chromatography eluting with the $CHCl_3$–MeOH gradient to yield 10 fractions (11–20). Fraction 12 (500 mg) eluted with $CHCl_3$–MeOH (15:1) was further separated with preparative HPLC eluting with MeOH–H_2O (60:40) at a flow rate of 20 ml/min to afford compound **3** (76.4 mg). Fraction 15 (100 mg) eluted with $CHCl_3$–MeOH (10:1) was repeatedly recrystallized with MeOH to give compound **2** (81.5 mg). Fraction 18 (1.07 g) eluted with $CHCl_3$–MeOH (8:1) was purified by an ODS open column chromatography eluting with MeOH–H_2O (50:50) to give compound **4** (18.9 mg). Fraction 19 (5.0 g) eluted with $CHCl_3$–MeOH (5:1) was subjected to a DIAION HP-20 column (4.0 × 90 cm) eluting with the MeOH–H_2O gradient, and then further separated with an open column chromatography packed with MDS-5 reverse-phase packing particles eluting with the MeOH–H_2O gradient to yield five major fractions (21–25). Fraction 21 (121.6 mg) eluted with MeOH–H_2O (50:50) was further purified with a Sephadex LH-20 column eluting with MeOH, and, finally, with a Varian BOND ELUT® C_{18} column

(1.0 × 4.0 cm) using MeOH–H_2O (30:70) as an eluent to give compound **1** (8.9 mg). Fraction 20 (2.06 g) eluted with $CHCl_3$–MeOH (3:1) was subjected to a Sephadex LH-20 column eluting with MeOH to yield compound **5** (8.0 mg) and compound **6** (6.0 mg).

*3.6.1 Diosmetin 7-(6″-O-p-hydroxyphenylacetyl)-O-β-D-glucopyranoside (**1**)*

A yellowish amorphous powder, mp 215–218°C. $[\alpha]_D^{24}$ −28 ($c = 0.2$, MeOH). UV (MeOH) λ_{max}: 226, 253, 346 nm. IR (KBr) ν_{max}: 3408, 2924, 1722, 1650, 1607, 1497, 1443, 1072 cm^{-1}. ^1H and ^{13}C NMR spectral data, see Table 1. ESI-TOF-MS (negative) *m/z*: 595.1469 $[M-H]^-$ (calcd for $C_{30}H_{27}O_{13}$, 595.1446).

3.7 Mild alkaline hydrolysis of 1

A solution of compound **1** (5 mg) in 0.05 N NH_4OH–50% MeOH was stirred at room temperature for 1 h. The reaction mixture was neutralized with Dowex HCR-W2 (H^+ form) and the resin was removed by filtration. The hydrolysate was extracted with EtOAc. After purification of the extract by a Varian BOND ELUT® C_{18} column (1.0 × 4.0 cm; GL Science) eluting with a MeOH–H_2O gradient (20% MeOH → 50% MeOH → MeOH). Two aromatic compounds, **1a** (2 mg) and **1b** (1 mg), were obtained. Compound **1a** was identified as diosmetin-7-*O*-β-D-glucopyranoside by co-chromatography (TLC silica gel) in $CHCl_3$–MeOH–H_2O (7:3:1) and EtOAc–MeOH–H_2O (4:1:1) with an authentic sample. Compound **1b** turned out to be *p*-hydroxyphenylacetic acid, ^1H NMR ($CDCl_3$): δ 4.08 (2H, s, H-7‴), 7.54 (2H, brd, $J = 8.7$ Hz, H-3‴, 5‴), and 7.74 (2H, brd, $J = 8.7$ Hz, H-2‴, 6‴).

Acknowledgements

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