Phenylpropanoyl Esters from Horseweed (*Conyza canadensis*) and Their Inhibitory Effects on Catecholamine Secretion

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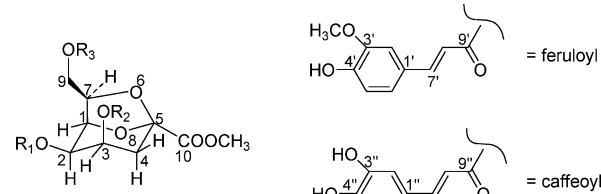
Three unique phenylpropanoyl 2,7-anhydro-3-deoxy-2-octulosonic acid derivatives were isolated from *Conyza canadensis* (horseweed). Their structures were defined as *rel*-(1*S*,2*R*,3*R*,5*S*,7*R*)-methyl 7-caffeoyloxymethyl-2-hydroxy-3-feruloyloxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate (**1**), *rel*-(1*S*,2*R*,3*R*,5*S*,7*R*)-methyl 7-feruloyloxymethyl-2-hydroxy-3-feruloyloxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate (**2**), and *rel*-(1*R*,2*R*,3*R*,5*S*,7*R*)-methyl 7-feruloyloxymethyl-2-feruloyloxy-3-hydroxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate (**3**). Compound **1** and a 5:3 mixture of compounds **2** and **3** were demonstrated to inhibit the catecholamine secretion induced by acetylcholine with IC_{50} values of 94.65 and 42.35 μM, respectively, and to inhibit the catecholamine secretion induced by veratridine and high [K^+] at a dose of 100 μM in cultured bovine adrenal medullary cells.

Horseweed, *Conyza canadensis* (L.) Cronq. (formerly *Erigeron canadensis* L., Compositae), is a winter or summer annual native to North America and now found in most parts of the world. Other common names include mare's-tail, Canadian horseweed, Canadian fleabane, colts-tail, and butterweed.[1,2] Of the 10 *Conyza* species found in China, horseweed is the most widespread and found throughout the country. The whole plants have been used medicinally in China to treat a wide range of diseases including edema, hematuria, hepatitis, and cholecystitis.[3] The tender stems and leaves are also used as swine fodder. Prevoius chemical studies have led to the isolation and identification of several secondary metabolite types including sphingolipids,[4,5] triterpenoids,[5,6] acetylenes,[6,7] phenolic acids,[5] and steroids.[4,6]

Catecholamines (CA) secreted from central and peripheral nervous catecholamine systems mainly contain epinephrine, norepinephrine, and dopamine and play a key role in regulating cardiovascular function, body temperature, and emotions. Adrenal medullary cells derived from the embryonic neural crest share many physiological and pharmacological properties with postganglionic sympathetic neurons, for example, secretion of CA.[8] Previous studies have shown that cultured bovine adrenal chromaffin cells contain a variety of ion channels that are involved in CA secretion, nicotinic acetylcholine receptor (nAChR)-associated cation channels, voltage-dependent Na^+ channels, and voltage-dependent Ca^{2+} channels. They can be activated by acetylcholine (ACh), veratridine (Ver), and high [K^+] (56 mM), respectively.[9] Because of the similarity to that of the sympathetic neurons or brain noradrenergic neurons, the bovine adrenal chromaffin cells have been widely used as a model system for studying the effects of multiple drugs on CA secretion.[10]

In the present study we describe the isolation and structure elucidation of three unique phenylpropanoyl esters with a 6,8-dioxabicyclo[3.2.1]octane skeleton from horseweed and their inhibitory effects on CA secretion induced by acetylcholine, veratridine, and high [K^+] in cultured bovine adrenal medullary cells.

The whole plants of horseweed were extracted using aqueous EtOH, and the extract was concentrated, suspended in H_2O, and partitioned with petroleum ether, $CHCl_3$, EtOAc, and *n*-BuOH. The BuOH extract was subjected to D101 macroporous resin column chromatography, and the 50% EtOH eluate (CCB50) showed an inhibitory effect on CA secretion at a dose of 50 μg/mL. Further fractionation led to the isolation of three unique phenylpropanoyl esters (**1**−**3**) with a 6,8-dioxabicyclo[3.2.1]octane skeleton (Figure 1).



1 R_1=H, R_2=feruloyl, R_3=caffeoyl
2 R_1=H, R_2=feruloyl, R_3=feruloyl
3 R_1=feruloyl, R_2=H, R_3=feruloyl

Figure 1. Structures of compounds **1**−**3**.

Compound **1** was obtained as a pale yellow, amorphous powder. The molecular formula was determined to be $C_{28}H_{28}O_{13}$ according to a quasi-molecular peak at *m*/*z* 571.1452 [M − H]$^-$ in the HRESIMS and its ^{1}H and ^{13}C NMR spectroscopic data. The ^{1}H NMR spectrum of **1** showed signals at δ 7.54 (1H, d, J = 16.0 Hz, H-7′), 6.54 (1H, d, J = 16.0 Hz, H-8′), 7.49 (1H, d, J = 16.0 Hz, H-7″), and 6.35 (1H, d, J = 16.0 Hz, H-8″), for two pairs of trans-coupled olefinic protons, signals at δ 7.32 (1H, d, J = 2.0 Hz, H-2′), 6.78 (1H, d, J = 8.5 Hz, H-5′), 7.10 (1H, dd, J = 8.5, 2.0 Hz, H-6′), 7.06 (1H, d, J = 2.0 Hz, H-2″), 6.77 (1H, d, J = 8.5 Hz, H-5″), and 7.02 (1H, dd, J = 8.5, 2.0 Hz, H-6″) for two 1,2,4-trisubstituted benzene rings, and signals at δ 3.83 (3H, s) for a methoxy group. These signals were assigned to caffeoyl and feruloyl groups (Table 1), respectively, on the basis of ^{1}H and ^{13}C NMR chemical shifts, splitting patterns, and coupling constants and respective 2D NMR correlations. The remaining ^{1}H and ^{13}C NMR signals (Table 1) suggested the presence of a methyl carboxylate (δ_H 3.72, s, 3H, and δ_C 167.3 and 52.6), an oxymethylene group (δ_H 5.35 and 4.36, each dd, 1H, and δ_C 62.8), and a 6,8-dioxabicyclo[3.2.1]octane with C-2 and C-3 oxygenated by a combined use of COSY, HMQC, and HMBC experiments.[11,12] HMBC correlations were used to place the feruloyl group at C-3 (H-3, δ

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Table 1. NMR Data (500 MHz, DMSO-d_6) of Compounds **1**−**3**[a]

	1		2		3	
no.	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)
1	76.3	4.55 dd (4.5, 4.0)	76.2	4.57 dd (4.5, 4.0)	74.2	4.76 dd (4.5, 4.0)
2	65.6	4.13 dd (5.0, 5.5)	65.5	4.15 ddd (5.0, 5.0, 5.0)	69.5	5.06 dd (5.0, 5.0)
2-OH		5.67 br s		5.67 d (5.0)		
3	65.9	5.37 br dd (5.5, 5.5)	65.8	5.39 br dd (5.5, 5.5)	61.2	4.28 ddd (5.0, 5.0, 5.0)
3-OH						5.36 d (4.5)
4	37.6	2.11 br d (15.0)	37.5	2.13 br d (15.0)	40.0	2.20 br d (14.5)
		2.37 dd (15.0, 5.5)		2.39 dd (15.0, 5.5)		2.30 dd (14.5, 5.0)
5	102.7		102.6		103.3	
7	79.0	4.42 ddd (9.5, 2.5, 3.5)	79.0	4.44 ddd (9.5, 2.5, 3.5)	78.5	4.37 ddd (8.0, 4.0, 4.0)
9	62.8	4.36 dd (2.5, 12.5)	62.8	4.41 dd (12.0, 2.5)	62.8	4.84 dd (11.5, 4.0)
		4.36 dd (2.5, 12.5)		4.41 dd (12.0, 2.5)		4.84 dd (11.5, 4.0)
10	167.3		167.3		167.3	
CH_3	52.6	3.72 s	52.5	3.74 s	52.5	3.75 s
1′	125.6		125.5		125.4	
2′	110.6	7.32 d (2.0)	110.1	7.36 d (1.5)	110.1	7.28 d (1.5)
3′	148.0		147.9		147.9	
4′	149.4		149.4		149.4	
5′	115.5	6.78 d (8.5)	115.5	6.81 d (8.5)	115.4	6.77 d (8.5)
6′	123.4	7.10 dd (8.5, 2.0)	123.2	7.13 dd (8.5, 2.0)	122.8	7.13 dd (8.5, 2.0)
7′	144.8	7.54 d (16.0)	145.2	7.57 d (16.0)	145.6	7.63 d (16.0)
8′	114.9	6.54 d (16.0)	114.9	6.53 d (16.0)	114.0	6.40 d (16.0)
9′	165.9		165.8		165.2	
OCH_3	55.6	3.83 s	55.6	3.83 s	55.6	3.82 s[b]
1″	125.5		125.5		125.4	
2″	115.0	7.06 d (2.0)	110.7	7.31 d (2.0)	111.5	7.28 d (1.5)
3″	145.6		147.9		147.9	
4″	148.5		149.4		149.4	
5″	115.8	6.77 d (8.5)	115.5	6.79 d (8.5)	115.6	6.80 d (8.5)
6″	121.4	7.02 dd (8.5, 2.0)	123.2	7.11 dd (8.5, 2.0)	123.1	7.06 dd (8.5, 2.0)
7″	145.4	7.49 d (16.0)	144.7	7.55 d (16.0)	145.2	7.53 d (16.0)
8″	113.8	6.35 d (16.0)	114.3	6.59 d (16.0)	114.1	6.51 d (16.0)
9″	166.7		166.7		166.5	
OCH_3			55.6	3.83 s	55.6	3.80 s[b]

[a] The assignments are based upon COSY, HMQC, HMBC, and NOESY experiments. [b] The assignments are interchangable.

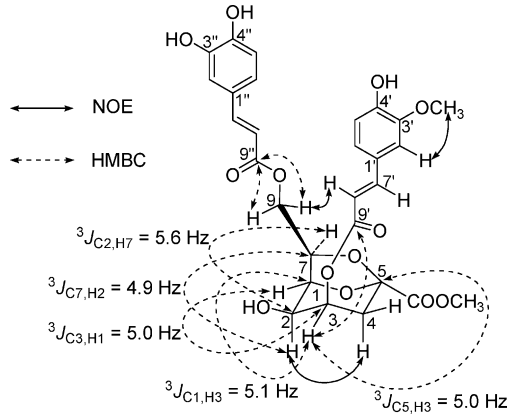

Figure 2. Selected NOESY correlations and $^3J_{C,H}$ values of compound **1**.

5.37, and C-9′, δ 165.9), and the caffeoyl group at C-9 (H-9, δ 5.35 and 4.36, and C-9″, δ 166.7) through ester linkages (Figure 2). Three-bond ($^3J_{CH}$) ^{13}C−^{1}H spin-couplings were used to determine the relative configurations at C-1, C-2, C-3, and C-7. The large couplings between C-7 and H-2 ($^3J_{CH}$ = 4.9 Hz), C-1 and H-3 ($^3J_{CH}$ = 5.1 Hz), C-5 and H-3 ($^3J_{CH}$ = 5.0 Hz), C-3 and H-1 ($^3J_{CH}$ = 5.0 Hz), and C-2 and H-7 ($^3J_{CH}$ = 5.6 Hz) suggested that C-7 and H-2, C-1 and H-3, C-5 and H-3, C-3 and H-1, and C-2 and H-7 were antiperiplanar or nearly so (Figure 2).[11,13−15] NOEs between H-2 (δ 4.13) and H-4a (δ 2.37) and between H-9a (δ 5.35) and H-8′ (δ 6.54) confirmed the above conclusion. Thus, the structure of compound **1** was established as *rel*-(1*S*,2*R*,3*R*,5*S*,7*R*)-methyl 7-caffeoyloxymethyl-2-hydroxy-3-feruloyloxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate (Figure 1). The absolute configuration of **1** was not determined.

Compounds **2** and **3** were identified as a 5:3 mixture. For the major component, the well-resolved ^{1}H and ^{13}C NMR spectra suggested the presence of two feruloyl groups (Table 1). The remaining resonances were similar to those for compound **1**. After the assignments of all proton and carbon signals (Table 1) were accomplished by a combination of COSY, HMQC, HMBC, and NOESY experiments, the correlations between H-3 (δ 5.39) and C-9′ (δ 165.8) and between H-9 (δ 5.33, 4.41) and C-9″ (δ 166.7) in the HMBC spectrum showed the locations of the two feruloyl groups at C-3 and C-9. Consequently, compound **2** was identified as *rel*-(1*S*,2*R*,3*R*,5*S*,7*R*)-methyl 7-feruloyloxymethyl-2-hydroxy-3-feruloyloxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate.

The ^{1}H and ^{13}C NMR spectra of compound **3**, the minor component in the mixture, showed resonances for two feruloyl groups (Table 1) and the 6,8-dioxabicyclo[3.2.1]octane skeleton. Analysis of ^{1}H and ^{13}C NMR chemical shifts, splitting patterns, and coupling constants, and COSY, HMQC, HMBC, and NOESY spectra resulted in the conclusion that one of the two feruloyl groups was attached to C-2 by the HMBC correlations between H-2 (δ 5.06) and C-9′ (δ 165.2), and the other was at C-9 like in compound **2**. Hence, compound **3** was elucidated as *rel*-(1*R*,2*R*,3*R*,5*S*,7*R*)-methyl 7-feruloyloxymethyl-2-feruloyloxy-3-hydroxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate.

Octulosonic acid derivatives are rarely observed in natural products research. Ten 2,7-anhydro-3-deoxy-2-octulosonic acid derivatives have been reported from *Erigeron breviscapus* with configuration undetermined, eight of which were tentatively characterized by UV and MS spectra.[12,16] In addition, three 2,7-anhydro-2-octulosonic acid derivatives have been reported from *E. breviscapus* via UV and MS data,[16] and two from *Smallanthus sonchifolius* identified on the basis of NMR data.[11]

Compound **1** and the 5:3 mixture of **2** and **3** were examined for their effects on CA secretion since CCB50 at a dose of 50 μg/mL showed an inhibitory effect on CA secretion induced by ACh (300

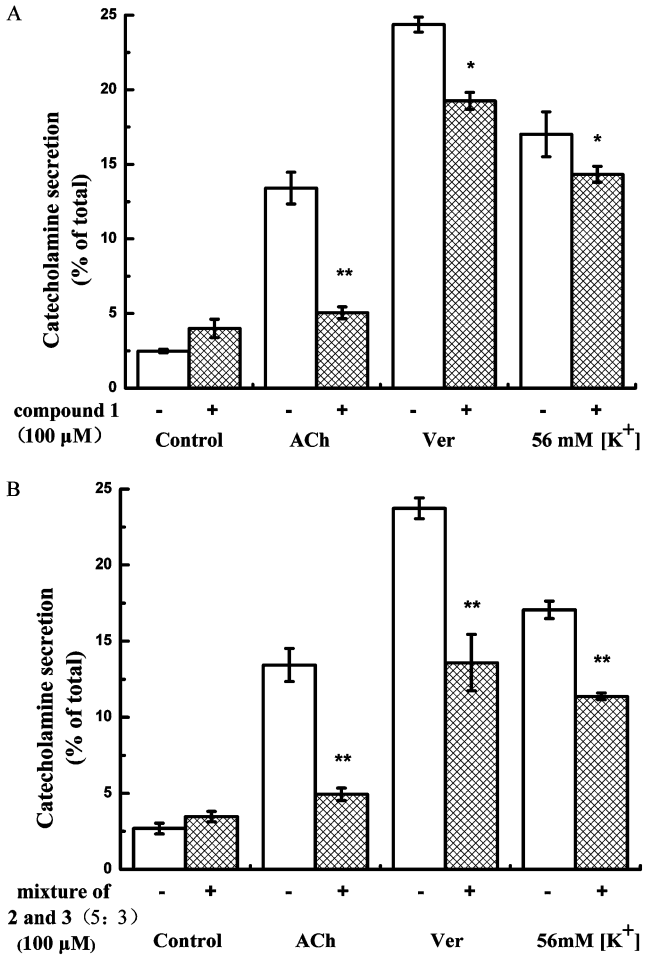

Figure 3. Effects of compound **1** (100 μM) (A) and a mixture of **2** and **3** (100 μM) (B) on CA secretion induced by Ach (300 μM), Ver (100 μM), and 56 mM [K^+] in cultured bovine adrenal medullary cells. Data are means ±SD from three separate experiments carried out in triplicate. *$P < 0.05$ and **$P < 0.01$, compared with each secretagogue alone.

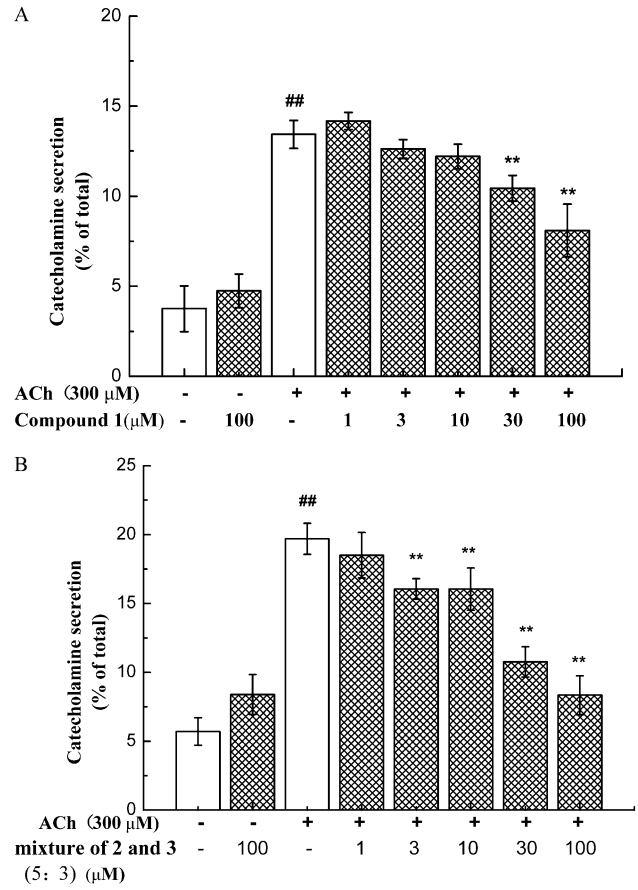

Figure 4. Effects of compound **1** (1, 3, 10, 30, 100 μM) (A) and the mixture of **2** and **3** (1, 3, 10, 30, 100 μM) (B) on CA secretion induced by ACh (300 μM) in cultured bovine adrenal medullary cells. Data are means ± SD from three separate experiments carried out in triplicate. ##$P < 0.01$, compared with control, **$P < 0.01$, compared with ACh.

μM), Ver (100 μM), and 56 mM [K^+] (% inhibition ratios 48.1, 10.8, 11.0, respectively) in bovine adrenal medullary cells. Two isolates inhibited CA secretion induced by acetylcholine with IC_{50} values of 94.65 and 42.35 μM, respectively, and CA secretion induced by veratridine and high [K^+] at a dose of 100 μM in cultured bovine adrenal medullary cells, as shown in Figures 3 and 4. It is known that the increase of intracellular calcium concentration ($[Ca^{2+}]_i$) is a prerequisite for CA secretion,[9] and we further investigated the effects of two isolates on the increase of $[Ca^{2+}]_i$ (Figure 5). The doses that produced 50% inhibition of the increase of $[Ca^{2+}]_i$ were 59.81 and 67.29 μM for two isolates, respectively. The results were consistent with CA secretion. This is the first report of bioactivity of octulosonic acid derivatives isolated from medicinal herbs.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Rudolph Research Analytical Autopol II automatic polarimeter. IR spectra were obtained on a Bruker Tensor 27 spectrometer. ^1H, ^{13}C NMR and 2D NMR spectra were obtained on a Bruker AVANCE DRX-500 instrument using DMSO-d_6 as a solvent. HRESIMS data were acquired in the negative ion mode on a Varian 7.0T FT-ICR mass spectrometer. Column chromatography was carried out on silica gel (200−300 mesh, Qingdao Haiyang Chemical Co., Ltd.), Sephadex LH-20 (Amersham Pharmacia Biotech AB), and polyamide (60−100 mesh). TLC was carried out using precoated plates with GF_{254} silica gel (Qingdao Haiyang Chemical Co., Ltd.). An Agilent 1100 system and an ODS analytical column (5 μm, 4.6 mm × 250 mm) were employed to detect the CA in cell medium by using an isocratic solvent system consisting of 10% MeOH, 1% MeCN, and 89% phosphate buffer (containing 13.6 g of KH_2PO_4, 2.5 g of sodium-octanesulfoacid, and 0.036 g of EDTA, pH adjusted to 3.3 with H_3PO_4) at a flow rate of 1 mL/min and a temperature of 35 °C. All solvents used were of analytical grade (Tianjin Jiangtian Chemical Technology Co., Ltd.). The composition of oxygenated Krebs-Ringer phosphate (KRP) buffer is as follows: 154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, and 10 mM glucose, adjusted pH to 7.4. Eagle's minimum essential medium (MEM) and newborn calf serum were purchased from Gibco (USA), collagenase was from Nitta Zerachin (Japan), and cytosine arabinoside, ACh, and Ver were from Sigma (St. Louis, MO). All the indicated drugs were dissolved in 100% DMSO and then diluted in a reaction medium before use at a final DMSO concentration not exceeding 0.5%, unless otherwise specified.

Plant Material. The whole plants of *C. canadensis* were collected from Ruicheng County, Shanxi Province, P. R. China, in August 2006, and authenticated by one of the authors (Y.S.). A voucher specimen (200608061) was deposited in the School of Pharmaceutical Science and Technology, Tianjin University, P. R. China.

Extraction and Isolation. The air-dried plant material of *C. canadensis* (15 kg) was extracted with 95% EtOH at room temperature and refluxed once with 95% EtOH and twice with 60% EtOH. The filtrate was combined and concentrated under reduced pressure to give a dark solid residue (2087 g). The residue was suspended in H_2O (8 L) and sequentially extracted with petroleum ether (60−90 °C) (39 L), $CHCl_3$ (39 L), EtOAc (55 L), and *n*-BuOH (58 L) to give the respective extracts. A portion of the BuOH extract (274 g) was subjected to D101 macroporous resin column chromatography eluted with H_2O (16 L)

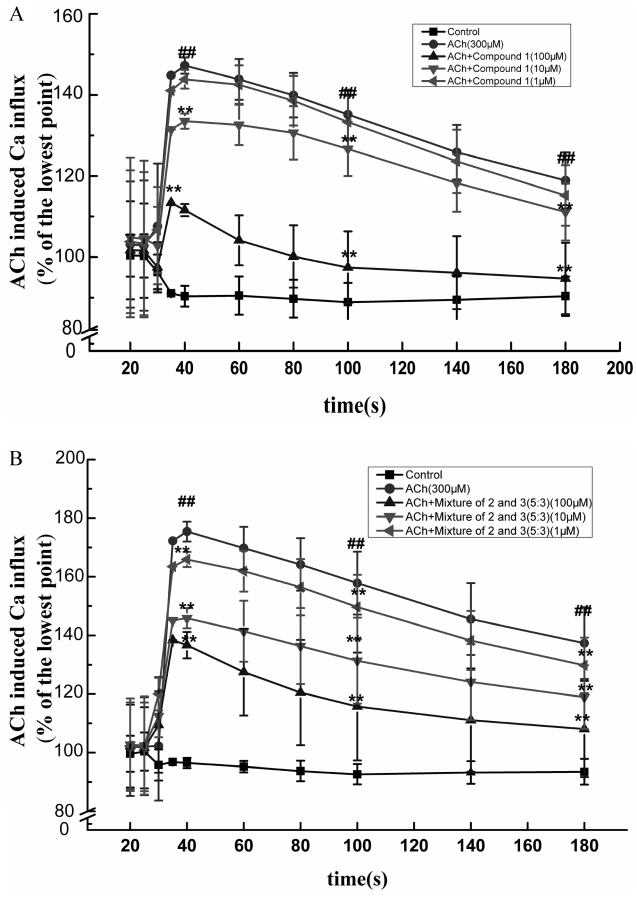

Figure 5. Effects of compound **1** (1, 10, 100 μM) (A) and a mixture of **2** and **3** (1, 10, 100 μM) (B) on Ca^{2+} influx induced by ACh (300 μM) in cultured bovine adrenal medullary cells. Data are means ±SD from three separate experiments carried out in triplicate. $^{\#\#}P < 0.01$, compared with control, $^{**}P < 0.01$, compared with ACh at the corresponding time.

and 30% (12 L), 50% (14 L), 70% (12 L), and 95% EtOH. The 50% eluate (CCB50, 40 g) was chromatographed over a silica gel column (Φ 6.5 cm; 100−200 mesh, 1000 g) with CH_2Cl_2−MeOH, 92:8 (8500 mL) and 87:13 (8000 mL), and finally CH_2Cl_2−MeOH, 70:30, saturated with H_2O (7500 mL) to give 41 fractions (Fr1−Fr41). Fr10−11 and Fr12−13 were separately chromatographed over a Sephadex LH-20 column (Φ 2.3 cm; 50 g) with MeOH. Subfractions 5−11 of Fr10−11 and subfractions 8−9 of Fr12−13 were combined and exposed to a polyamide column (Φ 3.3 cm; 60−100 mesh, 50 g) with a gradient elution of $CHCl_3$−MeOH (93:7 2250 mL, 85:15 650 mL) to give 13 fractions. Fractions 2 and 3 afforded compound **1** (150 mg) and a mixture (50 mg) of compounds **2** and **3** by a Sephadex LH-20 column (Φ 2.3 cm; 50 g) eluted with MeOH and a further silica gel column (Φ 1.5 cm; 200−300 mesh, 17 g) eluted with $CHCl_3$−MeOH (98:2.6 480 mL). An additional sample of compound **1** (200 mg) was also purified from Fr14−15 by polyamide (Φ 3.3 cm; 60−100 mesh, 120 g; $CHCl_3$−MeOH, 93:7, 2500 mL) and Sephadex LH-20 (MeOH) column (Φ 2.3 cm; 50 g) chromatography.

***rel*-(1*S*,2*R*,3*R*,5*S*,7*R*)-Methyl 7-caffeoyloxymethyl-2-hydroxy-3-feruloyloxy-6,8-dioxabicyclo[3.2.1]octane-5-carboxylate (1):** pale yellow, amorphous powder; $[\alpha]^{30}_D$ +126.8 (*c* 0.97, MeOH); IR (KBr) ν_{max} 3423, 1690, 1631, 1514, 1273, 1156 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 571.1452 (calcd for $[M - H]^-$ $C_{28}H_{27}O_{13}$, 571.1457).

Mixture of Compounds 2 and 3: yellow, amorphous powder; ^1H and ^{13}C NMR data see Table 1; HRESIMS *m/z* 585.1611 (calcd for $[M - H]^-$ $C_{29}H_{29}O_{13}$, 585.1614).

Isolation and Primary Culture of Bovine Adrenal Medullary Cells. Adrenal glands were obtained from a slaughterhouse in Tianjin. Bovine chromaffin cells were isolated following reported methods.[17] Cells were planted into 24-well plates at a density of 50 000 cells/well and cultured in modified Eagle medium (MEM) supplemented with 10% newborn calf serum, 10 μM cytosine arabinoside, 50 U/mL penicillin, and 50 g/mL streptomycin under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. All the cells were used for experiments between 2 and 7 days of culture.

CA Secretion from Cultured Bovine Adrenal Medullary Cells and Detection. Chromaffin cells were washed three times before experiments. After preincubation with compound **1** and the mixture of compounds **2** and **3** at different concentrations for 10 min, cells were incubated with or without various secretagogues at 37 °C for another 10 min in the presence or absence of compound **1** and the mixture of compounds **2** and **3** of the corresponding concentrations. The incubation medium was transferred immediately to a test tube containing perchloric acid (PCA) (final concentration 0.4 M). After being adsorbed by $Al(OH)_3$, CA (norepinephrine and epinephrine) secreted into the medium was directly measured by HPLC coupled with an electrochemical detector with a potential of +0.7 mV. The injection volume was 20 μL.[17,18]

Measurements of Intracellular Concentration of Ca^{2+}. Ca^{2+} mobilization was studied using a Flexstation III fluorometric imaging plate reader (Molecular Devices, USA). Cells were cultured in black-walled clear-base 96-well plates (Greiner, USA) at a density of 50 000 cells per well in culture media and grew for 24−36 h in a CO_2 incubator at 37 °C. In Flex experiments, the cells were incubated with the Calcium 4 reagent (Molecular Devices, Sunnyvale, CA) for 60 min in a CO_2 incubator at 37 °C. The Calcium 4 reagent-loaded cells were placed into the Flexstation III to monitor cell fluorescence (λ_{ex} = 485 nm, λ_{em} = 525 nm). Compound **1** and the mixture of compounds **2** and **3** were dissolved in assay buffer (final DMSO concentration kept below 1%) in 96-well plates and added to the loaded cells by the automated pipettor in the Flexstation III. Intracellular Ca^{2+} mobilization responses were measured as relative fluorescence units (RFU) and expressed as the percentage of RFU of the lowest points.

Statistical Analysis. All experiments were performed in triplicate, and each experiment was repeated at least three times. Data are presented as means ±SD. The significance of differences between means was evaluated using *t* test or one-way ANOVA. When a significant *F* value was found by ANOVA, Dennett's tests for multiple comparisons were used to identify differences among the groups. Values were considered statistically significant for $P < 0.05$. Statistical analyses were performed using SPSS 11.5 software.

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Supporting Information Available: ^1H, ^{13}C, HMQC, COSY, HMBC, and NOESY NMR spectra, HRMS, and IR spectra for **1** and ^1H, ^{13}C, HMQC, COSY, HMBC, NOESY NMR spectra and HRMS spectra for **2** and **3** are available free of charge via the Internet at http://pubs.acs.org.

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