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•Research article•

Three new coumarins and a new coumarin glycoside from *Micromelum integerrimum*

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[ABSTRACT] Three new coumarins, integmarins A–C (**1**–**3**), and a new coumarin glycoside, integmaside A (**4**) were isolated from the leaves and stems of *Micromelum integerrimum*. Their structures were elucidated on the basis of 1D and 2D NMR and MS data, and their absolute configurations were assigned according to the ECD data of the in situ formed transition metal complexes and comparison of experimental and calculated ECD data. Compounds **1** and **2** are two rare coumarins with butyl and propyl moieties at the C-6 position; compound **3** is a novel coumarin with a highly oxidized prenyl group, and compound **4** is a rare bisdihydrofuranocoumarin glycoside.

[KEY WORDS] Rutaceae; *Micromelum integerrimum*; Coumarins; Coumarin glycoside; ECD
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Introduction

The genus *Micromelum* belongs to the Rutaceae family, including nearly ten species around the world. Many of *Micromelum* species have been used for the treatment of various diseases in folk [1, 2]. *Micromelum integerrimum* (Buch.-Ham.) Roem. is a small tree widely distributed in South China, Vietnam, Thailand, and India, and often used as a herb remedy for influenza, cough, ostalgia, gastralgia, swelling and pain [3]. Previous chemical investigations revealed that some chemical components, such as coumarins, flavonoids, and alkaloids with potent anti-inflammatory, antibacterial, and anticancer effects had been isolated from *M. integerrimum* [3–5]. In an ongoing search for new natural products from *M. integerrimum*, the 95% aqueous EtOH extract of its leaves and stems was investigated, obtaining four new coumarin derivatives, including three new coumarins, integmarins A–C (**1**–**3**), and a new coumarin glycoside, integmaside A (**4**) (Fig. 1). Compounds **1** and **2** are two rare coumarins with butyl and propyl moieties at the C-6 position; compound **3** is a novel coumarin with a highly oxidized prenyl group, and compound **4** is a rare bisdihydrofuranocoumarin glycoside. We report here the isolation and structure elucidation of these four compounds.

Results and Discussion

Integmarin A (**1**) was obtained as a pale yellow oil, $[\alpha]_D^{25}$ –84 (*c* 0.1, MeOH). The molecular formula was determined as $C_{14}H_{14}O_5$ on the basis of HR-ESIMS (*m/z* 263.0917 $[M + H]^+$, Calcd. for $C_{14}H_{15}O_5$, 263.0919) and ^{13}C NMR data. The UV spectrum showed maximum absorptions at 221 nm and 327 nm, suggesting a coumarin nucleus [6, 7]. The ^1H NMR data (Table 1) revealed a set of typical characteristic coumarin signals [δ_H 6.25 (1H, d, J = 9.5 Hz, H-3), 7.92 (1H, d, J = 9.5 Hz, H-4)], two aromatic proton singlets at δ_H 7.59 and 6.99 (each 1H, s, H-5 and H-8), and a methoxy group [δ_H 3.98 (3H, s)]. Moreover, a methyl [δ_H 0.95 (3H, t, J = 7.3 Hz)], a methylene [δ_H 2.41 (1H, dq, J = 18.1, 7.3 Hz), 2.56, (1H, dq, J = 18.1, 7.3 Hz)], an oxidized methine [δ_H 5.40 (1H, s)] protons were also observed. The ^{13}C NMR (Table 1) exhibited 14 carbon signals, including nine carbons of a coumarin nucleus, a methoxy, a carbonyl, and three aliphatic carbons. The methoxy group was deduced to be located at C-7 based on the HMBC correlation of the methoxy protons with C-7 (δ_C 161.1) (Fig. 2). The HMBC correlations from the oxidized methine proton to C-5/6/7/2′ (δ_C 210.1), from the

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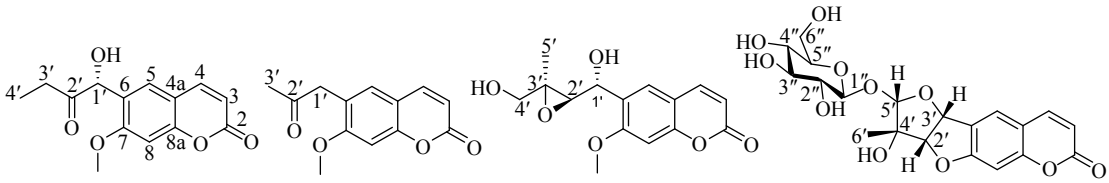
These authors have no conflict of interest to declare.



1 **2** **3** **4**

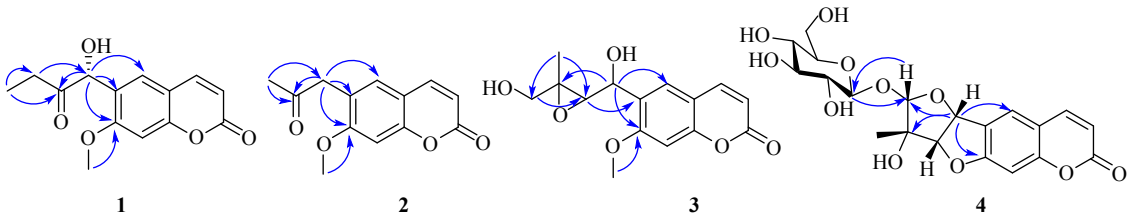
Fig. 1 Structures of compounds 1–4

Table 1 NMR data for 1–4 (δ in ppm, *J* in Hz, ^{1}H: 500 MHz for 1 and 2 and 400 MHz for 3 and 4, ^{13}C: 125 MHz for 1 and 2 and 100 MHz for 3 and 4)

Position	**1** (measured in acetone-d_6)		**2** (measured in acetone-d_6)		**3** (measured in acetone-d_6)		**4** (measured in methanol-d_4)	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2		160.9		161.1		161.1		162.9
3	6.25, d (9.5)	114.1	6.22, d (9.5)	113.8	6.23, d (9.5)	113.9	6.25, d (9.4)	113.4
4	7.92, d (9.5)	144.6	7.87, d (9.5)	144.6	7.94, d (9.5)	144.9	7.90, d (9.4)	145.9
4a		113.3		112.9		113.2		115.0
5	7.59, s	129.2	7.41, s	131.1	7.76, s	128.1	7.66, s	127.2
6		126.5		122.6		129.4		125.3
7		161.1		161.8		161.8		165.1
8	6.99, s	100.2	6.94, s	99.7	6.94, s	99.9	6.84, s	99.3
8a		156.8		156.3		156.5		158.4
1′	5.40, s	74.6	3.76, s	44.6	4.99, d (7.7)	66.4		
2′		210.1		205.1	3.06, d (7.7)	67.1	5.09, d (6.6)	94.5
3′	2.41, dq (18.1, 7.3) 2.56, dq (18.1, 7.3)	31.6	2.14, s	29.6		62.4	5.92, d (6.6)	80.7
4′	0.95, t (7.3)	7.9			3.81, s	64.8		81.3
5′					1.36, s	20.5	4.95, s	108.9
6′							1.29, s	19.8
1″							4.51, d (7.5)	104.1
2″								75.1
3″							3.28–3.40, 4H, overlapped	78.3
4″								71.3
5″								77.6
6″							3.69, dd (11.9, 4.8) 3.85, dd (11.9, 1.6)	62.5
OCH_3	3.98, s	56.9	3.93, s	56.8	3.93, s	56.8		

methylene protons to C-2′/4′, and from the methyl protons to C-2′/3′, together with the results of the chemical shifts and molecular formula, implied the presence of a 1-hydroxy-2-oxobutyl moiety at the C-6 position. An (1′*R*) absolute configuration for **1** was inferred from an obviously negative Cotton effect at 330 nm in the ECD spectrum of the $Rh_2(OCOCF_3)_4$ complex in CH_2Cl_2 (Fig. 3A) [8]. Thus, the structure of integmarin A (**1**) was established as (1′*R*)-6-(1-hydroxy-2-oxobutyl)-7-methoxycoumarin, which is a coumarin with a rare butyl moiety at the C-6 position.

Integmarin B (**2**) was isolated as a pale yellow oil, and showed a protonated molecular ion $[M + H]^+$ at *m/z* 233.0814 (Calcd. for $C_{13}H_{13}O_4$, 233.0814) in the HR-ESIMS, corresponding to a molecular formula of $C_{13}H_{12}O_4$. According to the NMR data (Table 1), **1** and **2** were found to have similar structures, except that the 1-hydroxy-2-oxobutyl moiety in **1** was changed to 2-oxopropyl group [δ_H 3.76 (2H, s, H-1′), 2.14 (3H, s, H-3′); δ_C 44.6 (C-1′), 205.1 (C-2′), 29.6 (C-3′)] in **2**, which was supported by the HMBC correlations from the methylene protons to C-5/6/7/2′, and from the methyl pro-



1 **2** **3** **4**

Fig. 2 Key HMBC correlations of compounds 1–4

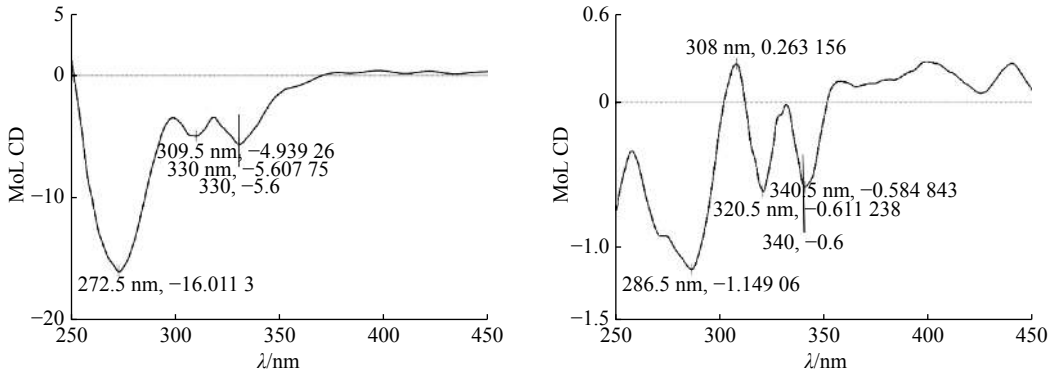


Fig. 3 $Rh_2(OCOCF_3)_4$-induced CD spectra of compounds 1 (A) and 3 (B) in CH_2Cl_2

tons to C-1′/2′ (Fig. 2). Therefore, the structure of integmarin B (**2**) was confirmed as 6-(2-oxopropyl)-7-methoxycoumarin.

Integmarin C (**3**) was purified as a white amorphous powder, $[\alpha]_D^{25}$ +44 (*c* 0.1, MeOH). Its HR-ESIMS data showed a pseudomolecular ion at *m/z* 291.0877 $[M - H]^-$ (Calcd. for $C_{15}H_{15}O_6$, 291.0869), together with its ^{13}C NMR data, indicating that the molecular formula of **3** was $C_{15}H_{16}O_6$. Interpretation of the NMR data (Table 1) suggested that the structure of **3** is similar to that of hydroxyosthole epoxide [9]. The main difference between these two compounds was that the 2, 3-epoxy-1-hydroxy-3-methylbutyl moiety in hydroxyosthole epoxide was replaced by 1, 4-dihydroxy-2, 3-epoxy-3-methylbutyl group in **3** [δ_H 4.99 (1H, d, *J* = 7.7 Hz, H-1′), 3.81 (2H, s, H-4′), 3.06 (1H, d, *J* = 7.7 Hz, H-2′), 1.36 (3H, s, H-5′)], which was linked to C-6 deduced from the HMBC correlations from H-1′ to C-5/7/3′ and from H-2′ to C-6/4′/5′ (Fig. 2). In addition, the large coupling constant between H-1′ and H-2′ (*J* = 7.7 Hz) indicated that **3** is a *threo* isomer [7, 9]. The NOESY correlations of H-1′/H-4′ and H-2′/H-5′ (Fig. 4) suggested that these two groups of protons are located in the different sides of the epoxy ring. The (1′*R*) absolute configuration was assigned by a negative Cotton effect at 340 nm in the ECD spectrum of the $Rh_2(OCOCF_3)_4$ complex in CH_2Cl_2 (Fig. 3B) [8]. Thus, the absolute configuration of **3** was assigned as (1′*R*, 2′*R*, 3′*S*). In summary, the structure of integmarin C (**3**) was established as (1′*R*, 2′*R*, 3′*S*)-6-(1, 4-dihydroxy-2, 3-epoxy-3-methylbutyl)-7-methoxycoumarin, a novel coumarin with a highly oxidized prenyl group.

Integmaside A (**4**) was obtained as a pale yellow amorphous powder, $[\alpha]_D^{25}$ –48 (*c* 0.1, MeOH), with a molecular formula of $C_{20}H_{22}O_{11}$, as deduced from the ^{13}C NMR and HR-ESIMS (*m/z* 437.1079 $[M - H]^-$, Calcd. for $C_{20}H_{21}O_{11}$, 437.1084) data. Its MS and NMR data (Table 1) were extremely similar to those of peucedanoside A, an angular bis-dihydrofuranocoumarin glycoside from *Peucedanum praeruptorum* [10]. However, **4** was deduced to be a linear bis-dihydrofuranocoumarin glycoside based on the ^1H NMR data [δ_H 7.66 (1H, s, H-5), 6.84 (1H, s, H-8)] and the HMBC correlations of H-3′ with C-5/7 and H-2′ with C-6 (Fig. 2). Acid

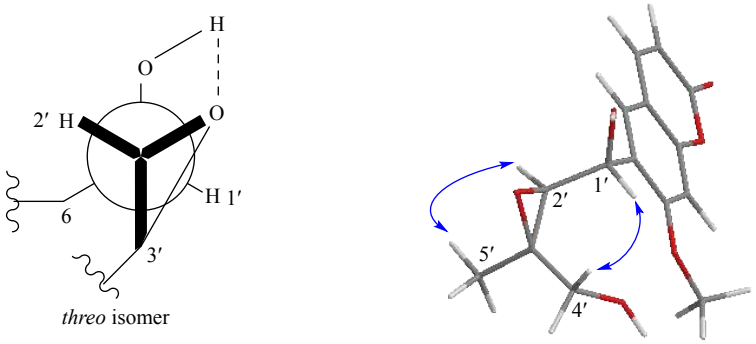


Fig. 4 Newman projection of the most dominant conformation and the NOE correlations of 3

hydrolysis of **4** gave glucose and a *β*-D configuration was determined from its larger coupling constant (J = 7.5 Hz) [10, 11] and from the HPLC analysis of its chiral derivatives (Fig. S24, Supplementary data). The HMBC correlation from H-1″ to C-5′ (Fig. 2) indicated that the glucosyl unit was attached to C-5′.

The coupling constant between H-2′ and H-3′ (J = 6.6 Hz) indicated its *cis* configuration [10]. The NOESY correlations of H-2′/H-3′, H-6′ and H-5′/H-6′ determined their *cis* configurations. The acid hydrolysate was extracted by CH_2Cl_2 and then purified by HPLC to afford the aglycone unit (**4a**), whose absolute configuration was assigned as (2′*S*, 3′*R*, 4′*S*, 5′*R*) by comparison of its experimental and calculated ECD data (Fig. 5). Based on the data mentioned above, the structure of integmaside A (**4**) was defined as below.

Compounds **1**–**4** were evaluated for their inhibitory effects on NO production stimulated by lipopolysaccharide in BV-2 cells, cytotoxic activities against HepG2, HTC-116, Hela, and PANC-1 cells, and inhibitory effects on the apoptosis in PC12 cells induced by 6-hydroxydopamine. However, none of these compounds showed obviously biological activities below 40 μmol·L^{-1}.

Experimental

General experimental procedures

UV spectra were obtained on a Shimadzu UV-2450 spectrophotometer (Shimadzu Co., Japan). IR spectra were recorded on a Thermo Nicolet Nexus 470 FT-IR spectrometer (MA, USA). NMR spectra were acquired on a Varian INOVA-500 NMR spectrometer (Varian Co., USA) or Bruker Avance-400 FT NMR spectrometer (Bruker Co., Switzerland), using CD_3OD as solvent and the chemical shifts were referenced to the solvent residual peak. HR-ESIMS was performed on a Waters Xevo G2 Q-TOF mass spectrometer (Waters Co., Milford, MA, USA). The specific rotations were measured in methanol using an Autopol VI polarimeter (Rudolph, USA). Silica gel (200–300 mesh, Qingdao Marine Chemical, Co., Ltd., China) and MCI GEL CHP20 (Mitsubishi Chemical Holdings, Japan) were used for open column chromatography (CC). Semipreparative RP-HPLC was performed on an Agilent 1260 system with a Zorbax Eclipse XDB-C_{18} (250 mm × 9.4 mm, 5 μm) column. All the solvents used for isolation were of analytical grade and the solvents used for HPLC were of HPLC grade.

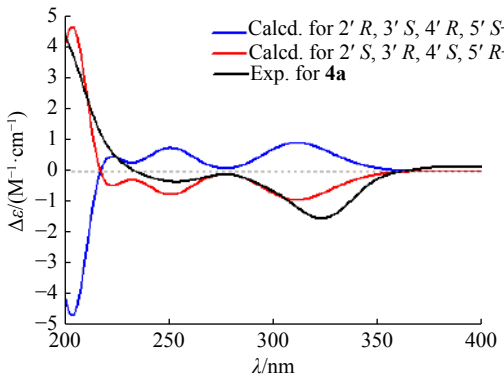


Fig. 5 Calculated and experimental ECD data of compound 4a

Plant material

The dry stems and leaves of *Micromelum integerrimum* (Buch.-Ham.) Roem. (Rutaceae) were collected in Oct., 2017 from Pingbian County, Yunnan Province, China and identified by Prof. TU Peng-Fei from Peking University (Beijing, China). A voucher specimen (No. XYM201710) was deposited at the Herbarium of Peking University Modern Research Center for Traditional Chinese Medicine.

Extraction and isolation

Air-dried leaves and stems of *M. integerrimum* (6.4 kg) were extracted with 95% aqueous EtOH (70 L × 2 h) at 80 °C for three times to afford a crude extract. The extract was evaporated under reduced pressure and the residue (560 g) was suspended in water and then partitioned sequentially with CH_2Cl_2 and EtOAc. The CH_2Cl_2-soluble extract (175 g) was subjected to silica gel CC, eluting with petroleum ether−acetone (10 : 1, 8 : 1, 5 : 1, 3 : 1, 1 : 1, and 0 : 1 gradient system, *V/V*) to give five fractions (Frs. A–E). Fr. B (26 g) was separated into three fractions (B1–B3) *via* Sephadex LH-20 CC eluting with CH_2Cl_2−MeOH (1 : 1, *V/V*). Fr. B2 (7 g) was further separated over ODS eluting with MeOH−H_2O (50 : 50–100 : 0 gradient system, *V/V*) to obtain six fractions (B2a–B2f). Compounds **1** (13.8 mg, t_R 8.0 min) and **2** (15.2 mg, t_R 9.8 min) were yielded from Fr. B2b (400 mg) using semipreparative RP-HPLC with MeCN−H_2O (40 : 60, 3 mL·min^{-1}) over 20 min. Separation of the Fr. D (41 g) by Sephadex LH-20 CC eluting with CH_2Cl_2−MeOH (1 : 1, *V/V*) yielded three subfractions, D1–D3. Subfraction D2 (13 g) was fractioned by ODS CC (MeOH−H_2O, 30 : 70–100 : 0 gradient system, *V/V*) to obtain nine subfractions (D2a–D2i). Compound **3** (5.7 mg, t_R 12.9 min) was isolated from Fr. D2f (180 mg) using semipreparative RP-HPLC with a mobile phase of MeCN−H_2O (20 : 80, *V/V*, 3 mL·min^{-1}). EtOAc-soluble extract (20 g) was chromatographed over a MCI CC eluting with MeOH–H_2O (20 : 80–100 : 0 gradient system, *V/V*) to obtain seven fractions (Frs. 1–7). Fr. 5 was further purified by semipreparative RP-HPLC using a mobile phase of MeCN–H_2O (15 : 85–25 : 75 gradient system, *V/V*, 3 mL·min^{-1}) to afford **4** (19.6 mg, t_R 16.0 min).

*Integmarin A (**1**)*

Pale yellow oil; $[\alpha]_D^{25}$ –84 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 221 (3.78), 327 (3.69) nm; IR (KBr) ν_{max} 3434, 2938, 2873, 1728, 1619, 1383, 1359, 1275, 1208, 1135, 1013, 825 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR-ESIMS: *m/z* 263.0917 $[M + H]^+$ (Calcd. for $C_{14}H_{15}O_5$, 263.0919).

*Integmarin B (**2**)*

Pale yellow oil; UV (MeOH) λ_{max} (log ε) 223 (4.06), 328 (4.01) nm; IR (KBr) ν_{max} 3415, 3080, 1739, 1716, 1623, 1564, 1383, 1275, 1130, 1018, 897, 830 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HR-ESIMS: *m/z* 233.0814 $[M + H]^+$ (Calcd. for $C_{13}H_{13}O_4$, 233.0814).

*Integmarin B (**3**)*

White amorphous powder; $[\alpha]_D^{25}$ +44 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 222 (3.89), 331 (3.87) nm; IR (KBr) ν_{max} 3412, 2931, 1719, 1619, 1561, 1383, 1275, 1209, 1137, 1040, 1014, 908, 825 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-ESIMS: *m*/*z* 291.0877 $[M - H]^-$ (Calcd. for $C_{15}H_{15}O_6$, 291.0869).

*Integmaside A (**4**)*

Pale yellow amorphous powder; $[\alpha]_D^{25}$ –48 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 224 (3.77), 330 (3.81) nm; IR (KBr) ν_{max} 3400, 2932, 1720, 1630, 1572, 1128, 1076, 1013, 826 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-ESIMS: *m*/*z* 437.1079 $[M - H]^-$ (Calcd. for $C_{20}H_{21}O_{11}$, 437.1084).

*Determinations of ECD Data of the in situ formed transition metal complexes of **1** and **3***

A 1 : 2 mixture of the secondary alcohol-$Rh_2(OCOCF_3)_4$ for **1** and **3** was respectively subjected to ECD measurement at a concentration of 0.1 $mg \cdot mL^{-1}$ in anhydrous CH_2Cl_2. The first ECD spectrum was recorded immediately after mixing, and its time evolution spectrum was monitored until a stationary state was achieved (about 10 min after mixing). The initial ECD spectrum was subtracted, and the observed sign of the E band at ca. 350 nm in the induced ECD spectrum was correlated to the absolute configuration of the secondary alcohol.

*Determination of absolute configurations of aglycone and sugar unit of **4***

Compound **4** (2.0 mg) was dissolved in 4 mL CF_3COOH (4 $mol \cdot L^{-1}$) at 80 °C for 3 h. After cooling to room temperature, the crude product was extracted with CH_2Cl_2 (2 mL) for three times, and the aqueous residue was concentrated to complete dryness. The CH_2Cl_2-soluble extract was further purified by RP-HPLC using a mobile phase of MeCN–H_2O (5 : 95–100 : 0 gradient system, *V*/*V*, 1 $mL \cdot min^{-1}$) to afford the aglycone unit (**4a**, 0.3 mg, t_R 11.5 min), which was identified by LC-MS (*m*/*z* 277 $[M + H]^+$, Fig. S25, Supporting Information). The absolute configuration was assigned by comparison of its experimental and calculated ECD data. Moreover, D-glucose standard (2 mg) and the dried aqueous residue were separately dissolved in 0.5 mL anhydrous pyridine, and L-cysteine methyl ester hydrochloride (1.0 mg) was then added. Each reaction mixture was warmed at 60 °C for 1 h. After cooling, 2-methylphenyl isothiocyanate (5 μL) was added in both reaction solutions, and the reactions were reacted at 60 °C for 1 h. Afterwards, these two final reaction products were diluted with anhydrous pyridine, and analyzed by HPLC under the following conditions: an Agilent 1260 chromatograph equipped with a Waters C_{18} column (250 mm × 4.6 mm, 5 μm); column temperature: 35 °C; mobile phase: MeCN : H_2O (25 : 75), 0–50 min; flow rate: 0.8 $mL \cdot min^{-1}$; and UV detection wavelength: 250 nm [12].

Supplementary material

Supplementary material is available on requrest from the corresponding author.

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