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Nitric oxide inhibitory coumarins from the roots and rhizomes of *Notopterygium incisum*

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ABSTRACT

Phytochemical study on the roots and rhizomes of *Notopterygium incisum* resulted in the isolation of six new coumarins, notoptetherins A − F (**1–6**), and 20 known analogues (**7–26**). Their structures were elucidated on the basis of extensive analyses of NMR and HRMS data, and the absolute configurations of **5** and **6** were established by $Mo_2(AcO)_4$-induced CD and exciton chirality, respectively. Moreover, a biomimetic synthesis of **6** from **21** was employed to confirm its absolute configuration. In a subsequent activity screening, compounds **12** and **17** exhibited potent inhibition against LPS-induced nitric oxide production in RAW 264.7 cells with IC_{50} values of 12.7 and 10.2 μM, respectively.

1. Introduction

The roots and rhizomes of *Notopterygium incisum*, known as "Qianghuo" in traditional Chinese medicine (TCM), have been used as an anti-inflammatory, anti-pyretic, and analgesic agent [1,2]. Previous studies on this plant revealed various types of natural products, such as coumarins [3–5], polyacetylenes [6,7], terpenoids [8,9], and alkaloids [10], some of which exhibited analgesic [7], anti-inflammatory [6,11–13], cytotoxic [14,15], anti-tumor [16,17], and immunosuppressive [18] activities. Aimed to search for new bioactive components from *N. incisum*, a phytochemical investigation was carried out on its 95% aq. EtOH extract and led to the isolation of 26 coumarins, including six new ones, notoptetherins A − F (1–6). Herein, the isolation and structural elucidation of the new compounds are described, along with their nitric oxide (NO) inhibitory effects on LPS-induced RAW 264.7 cells.

2. Experimental

2.1. General experimental procedures

HRESIMS data were obtained on a Shimadzu IT-TOF LC/MS instrument. IR spectra were measured on a Thermo Scientific Nicolet Nexus 470 FT-IR spectrometer. UV spectra were recorded on a Shimadzu UV-2450 UV–visible spectrometer. 1D and 2D NMR data were acquired on Agilent VNMRS-500 and Bruker AVANCE III-400 spectrometers. Specific rotations were measured on a Rudolph Autopol IV automatic polarimeter. ECD spectra were collected on a Jasco J-810 spectrometer. Semipreparative HPLC was performed on a LC3000 apparatus equipped with a UV3000 single channel detector (Beijing Tong Heng Innovation Technology Co., Ltd., China) using a Thermo Scientific BDS-C18 column (250 × 10 mm, 5 μm). UPLC/MS analysis was performed on a Waters ACQUITY UPLC apparatus coupled with an AB Sciex Triple Quad™ 4500 triple quadrupole-linear ion trap (Qtrap) mass spectrometer using a Shimadzu InertSustain C18 column (150 × 2.1 mm, 3 μm). Column chromatography (CC) was conducted on Merck silica gel 60 (40–63 μm), Merck Li Chroprep RP-18 (40–63 μm) and Mitsubishi Diaion HP20 (≥ 0.25 mm, ≥ 90%). Thin layer chromatography (TLC) was performed on pre-coated silica gel GF_{254} plates (Qingdao, Haiyang Chemical Co. Ltd., China). Solvents used in CC and HPLC were analytical grade (Beijing Chemical Reagents Co. Ltd., Beijing, China) and HPLC grade (Thermo Fisher Scientific Inc., Waltham, MA, USA), respectively. Reagents used for chemical communications were purchased from Energy Chemical Co. Ltd. (Shanghai, China).

2.2. Plant material

The roots and rhizomes of *N. incisum* were collected in Ngawa Tibetan and Qiang Autonomous Prefecture of Sichuan Province, China, in September 2013, and were authenticated by Prof. Pengfei Tu (one of the authors in this paper). A voucher specimen (No. QH-201309) has been deposited in the Herbarium of Modern Research Center for Traditional Chinese Medicine (TCM), Peking University, Beijing.

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2.3. Extraction and isolation

The air-dried powders of the roots and rhizomes of *N. incisum* (20 kg) were extracted with 95% aq. EtOH (120 L) by refluxing for thrice, each for 2 h. The combined ethanol extracts were concentrated *in vacuo* to obtain 12.6 kg crude extract which was successively partitioned with petroleum ether (PE, 6 L × 3), $CHCl_3$ (6 L × 3) and *n*-BuOH (6 L × 3) to give the corresponding portions. The $CHCl_3$ extract (1570 g) was subjected to silica gel CC and eluted with PE/acetone (1:0 → 0:1) to afford seven fractions, Frs. C1–C7. Separation of the Fr. C3 (11.2 g) by silica gel CC using PE/EtOAc solvent system (7:1) led to four subfractions, Frs. C3A–C3D. Fr. 3B (414 mg) was loaded onto an ODS column eluting with the mixture of MeOH/H_2O (75:25) to give two fractions, both of which were subjected to silica gel CC (PE/EtOAc, 15:1) to afford **22** (31.7 mg) and **10** (23.1 mg). Fr. C5 (53 g) was chromatographed on a silica gel column eluting with a gradient of PE/EtOAc (50:1 → 1:1) to give six fractions, Frs. C5A–C5F. Fr. C5B (2.3 g) was loaded onto an ODS column and eluted with MeOH/H_2O (60:40 → 100:0) to yield five subfractions, Frs. C5BI − C5BV. Fr. C5BII (72.1 mg) was purified by silica gel CC (PE/acetone, 15:1) to afford **8** (26.5 mg). Fr. C5BIII (91.7 mg) was subjected to silica gel CC (PE/acetone, 15:1) to obtain **12** (31.3 mg). Fr. C5BIV (53.3 mg) was loaded onto a silica gel column eluting with PE/EtOAc (8:1) to give **4** (19.8 mg) and **23** (12.7 mg). Fr. C5BV (83.2 mg) was purified by silica gel CC (PE/EtOAc, 15:1) to afford **9** (12.1 mg) and **13** (22.3 mg). Separation of Fr. C5C (3.4 g) by ODS CC (MeOH/H_2O, 60:40 → 100:0) led to five fractions, Frs. C5CI − C5CV. Fr. C5CI (33.7 mg) was purified using semi-preparative HPLC (MeOH/H_2O, 55:45, 2 mL/min) to give **15** (12.3 mg, t_R 30.2 min). Fr. C5CII (56.7 mg) was applied to semipreparative HPLC (MeCN/H_2O, 45:55, 2 mL/min) to yield **17** (16.7 mg, t_R 36.8 min) and **18** (13.7 mg, t_R 38.7 min). Fr. C5CIII (55.3 mg) was purified by semi-preparative HPLC (MeCN/H_2O, 45:55, 2 mL/min) to afford **16** (30.1 mg, t_R 36.3 min). Fr. C5CIV (79.3 mg) was subjected to silica gel CC (PE/acetone, 30:1 → 10:1) to obtain **21** (32.7 mg) and **24** (14.7 mg). Fr. C5CV (47.6 mg) was loaded onto a silica gel column eluting with a mixture of PE/acetone (10:1) to yield **26** (39.4 mg). Fr. C5E (4.5 g) was chromatographed over an ODS column eluting with MeOH/H_2O (60:40 → 100:0) to give four fractions, Frs. C5EI − IV. Fr. C5EII (730 mg) was subjected to ODS CC (MeCN/H_2O, 85:15) to yield three fractions, Frs. C5EIIa − C5EIIc. Fr. C5EIIb (121 mg) was loaded onto a silica gel column eluting with a gradient of PE/EtOAc (5:1 → 3:1) to obtain four subfractions, and the first subfraction was followed by semipreparative HPLC (MeCN/H_2O, 85:15, 2 mL/min) to afford **1** (3.1 mg, t_R 23.1 min), **2** (2.4 mg, t_R 24.8 min), and **3** (1.3 mg, t_R 27.3 min). Fr. C6 (154 g) was treated with PE/CH_2Cl_2 (7:1) to give **14** (53 g) as while crystals. Separation of Fr. C7 (973 g) by silica gel CC (PE/EtOAc, 3:1 → 1:3) led to four fractions, Frs. C7A − C7D. Fr. C7A (340 mg) was chromatographed on an ODS column eluting with MeOH/H_2O in gradient (50:50 → 70:30) and followed by semipreparative HPLC (MeCN/H_2O, 35:65, 2 mL/min) to give **5** (13.7 mg, t_R 25.6 min). Fr. C7B (716 g) was subjected to silica gel CC (PE/acetone 10:1) to afford **7** (13 g) and **11** (24 g). A solution of Fr. C7D (21 g) in CH_2Cl_2 was filtrated to obtain **19** (2.3 g), and the filtrate was concentrated under reduced pressure and subjected to ODS CC (MeOH/H_2O, 75:25 → 100:0) to yield five fractions, Frs. C7DI − C7DV. Fr. C7DI (35.7 mg) was purified by silica gel CC (PE/EtOAc, 3:1) to give **25** (11.9 mg). Fr. C7DIII (93.6 mg) was loaded onto an ODS column eluting with MeOH/H_2O (60:40) to afford three subfractions, and the second subfraction was purified using semipreparative HPLC (MeCN/H_2O, 55:45, 2 mL/min) to obtain **6** (34.2 mg, t_R 14.9 min). Separation of the *n*-BuOH extract (1356 g) by macroporous adsorption resin (EtOH/H_2O, 10:90 → 90:10) led to five fractions, Frs. B1–B5. White crystals of **20** (437 g) were obtained from Fr. B3 (593 g) by recrystallization in EtOH.

2.3.1. Notoptetherin A (1)

Colorless oil, UV (MeOH) λ_{max} (log ε) 308 (3.76), 259 (3.81), 251 (3.86), 220 (3.95), 207 (3.96) nm; IR (KBr) ν_{max} 2960, 2919, 2850, 1739, 1625, 1607, 1580, 1456, 1356, 1201, 1153, 1128, 1019 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 475.2094 $[M + Na]^+$ (calcd for $C_{27}H_{32}O_6Na$, 475.2097).

2.3.2. Notoptetherin B (2)

Colorless oil, UV (MeOH) λ_{max} (log ε) 310 (4.04), 258 (4.09), 251 (4.13), 221 (4.22), 207 (4.23) nm; IR (KBr) ν_{max} 2959, 2919, 2872, 2851, 1736, 1626, 1580, 1457, 1359, 1153, 1128, 1073 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 477.2243 $[M + Na]^+$ (calcd for $C_{27}H_{34}O_6Na$, 477.2253).

2.3.3. Notoptetherin C (3)

Colorless oil, UV (MeOH) λ_{max} (log ε) 308 (3.68), 260 (3.72), 252 (3.74), 220 (3.79), 206 (3.85) nm; IR (KBr) ν_{max} 2957, 2917, 2850, 1730, 1624, 1455, 1375, 1262, 1121, 1020 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 477.2260 $[M + Na]^+$ (calcd for $C_{27}H_{34}O_6Na$, 477.2253).

2.3.4. Notoptetherin D (4)

Colorless oil, UV (MeOH) λ_{max} (log ε) 310 (4.15), 259 (4.22), 250 (4.26), 220 (4.37), 206 (4.39) nm; IR (KBr) ν_{max} 2973, 2919, 1736, 1625, 1579, 1456, 1355, 1201, 1128, 1070 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 405.1674 $[M + Na]^+$ (calcd for $C_{23}H_{26}O_5Na$, 405.1678).

2.3.5. Notoptetherin E (5)

Colorless oil, $[\alpha]_D^{25}$ + 13.2 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 309 (3.94), 250 (4.05), 219 (4.19), 207 (4.20) nm; $Mo_2(OAc)_4$-induced CD (DMSO) 304 ($\Delta\varepsilon$ + 0.31) nm; IR (KBr) ν_{max} 3349, 2926, 2854, 1742, 1593, 1444, 1367, 1217, 1125, 1030 cm^{-1}; ^1H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m/z* 393.1299 $[M + Na]^+$ (calcd for $C_{21}H_{22}O_6Na$, 393.1314).

2.3.6. Notoptetherin F (6)

White amorphous powder, $[\alpha]_D^{25}$ + 186.3 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 323 (4.58), 235 (4.17), 205 (4.74) nm; ECD (*c* 0.6, MeOH) 342 ($\Delta\varepsilon$ + 11.35), 300 ($\Delta\varepsilon$ − 6.02), 246 ($\Delta\varepsilon$ + 2.19) nm; IR (KBr) ν_{max} 3335, 2926, 2855, 1738, 1616, 1454, 1366, 1217, 1156, 1117, 1029 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ_H: 7.73 (1H, d, *J* = 9.6 Hz, H-4), 7.40 (1H, d, *J* = 16.0 Hz, H-7″), 7.38 (1H, d, *J* = 8.2 Hz, H-5), 7.07 (1H, d, *J* = 2.0 Hz, H-2″), 6.95 (1H, dd, *J* = 8.1, 2.0 Hz, H-6″), 6.80 (1H, d, *J* = 8.2 Hz, H-6), 6.78 (1H, d, *J* = 8.1 Hz, H-5″), 6.14 (1H, d, *J* = 16.0 Hz, H-8″), 6.04 (1H, d, *J* = 9.6 Hz, H-3), 5.63 (1H, dd, *J* = 10.2, 7.3 Hz, H-2′), 5.40 (1H, brs, H-4′a), 5.36 (1H, brs, H-4′b), 4.99 (1H, d, *J* = 13.2 Hz, H-5′a), 4.72 (1H, d, *J* = 13.2 Hz, H-5′b), 3.89 (3H, s, H_3-OCH_3), 3.66 (1H, dd, *J* = 16.0, 10.2 Hz, H-1′a), 3.36 (1H, dd, *J* = 16.0, 7.3 Hz, H-1′b). ^{13}C NMR (125 MHz, CD_3OD) δ_C: 168.3 (C-9″), 165.2 (C-7), 163.0 (C-2), 152.6 (C-8a), 150.8 (C-4″), 149.4 (C-3″), 146.9 (C-7″), 146.0 (C-4), 145.0 (C-3′), 130.5 (C-5), 127.4 (C-1″), 124.3 (C-6″), 116.5 (C-5″), 115.9 (C-4′), 114.7 (C-8), 114.6 (C-8″), 114.6 (C-4a), 112.4 (C-3), 111.6 (C-2″), 108.0 (C-6), 86.7 (C-2′), 64.7 (C-5′), 56.5 (C-OCH_3), 33.4 (C-1′). HRESIMS *m/z* 419.1134 $[M - H]^-$ (calcd for $C_{24}H_{19}O_7$, 419.1131).

2.4. Chemical transformation

2.4.1. Synthesis of (5′S) hydroxyangenomalin (28)

According to the procedures described previously [19,20] with slight modifications, to a solution of **21** (10 mg, 0.044 mmol, 1.0 equiv) in CH_2Cl_2 (5 mL) was added SeO_2 (2.4 mg, 0.022 mmol, 0.5 equiv), potassium *tert*-butanolate (TBHP, 20 μL, 0.11 mmol, 2.5 equiv, from 5.5 M toluene solution), and AcOH (0.8 mL). The mixture was stirred at room temperature for 24 h and filtered to remove the SeO_2. The filtrate was then washed with 10 mL brine and dried with $MgSO_4$, filtered and concentrated *in vacuo* to afford the residue, which was purified by

preparative TLC using PE/EtOAc (3:1, R_f = 0.4) to give the product **28** (2.5 mg, 23% yield) and the starting material **21** (4.8 mg, recovered in 48%). $[\alpha]_D^{25}$ + 186.1 (*c* 0.06, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 7.64 (1H, d, *J* = 9.5 Hz), 7.28 (1H, d, *J* = 8.3 Hz), 6.77 (1H, d, *J* = 8.3 Hz), 6.22 (1H, d, *J* = 9.5 Hz), 5.52 (1H, t, *J* = 9.0 Hz), 5.31 (1H, brs), 5.30 (1H, brs), 4.29 (2H, m), 3.59 (1H, dd, *J* = 16.1, 9.8 Hz), 3.30 (1H, dd, *J* = 16.1, 8.2 Hz); HRESIMS *m/z* 245.0815 $[M + H]^+$ (calcd for $C_{14}H_{13}O_4$, 245.0814).

2.4.2. Synthesis of trans-4-O-tertbutyldimethylsilylferulic acid (30)

According to a procedure reported previously [21], to a 50 mL round-bottom flask charged with **29** (100 mg, 0.52 mmol, 1.0 equiv) in 20 mL CH_2Cl_2 was added *tert*-butyldimethylsilyl chloride (TBSCl, 194 mg, 1.3 mmol, 2.5 equiv) and *N*,*N*-diisopropylethylamine (DIPEA, 270 μL, 1.5 mmol, 3.0 equiv). The resulting mixture was stirred at room temperature for 5 h and then quenched by adding 5 mL water and diluted with 40 mL EtOAc. The organic phase was washed with 20 mL 1.0 M aqueous HCl and 15 mL brine, dried by $MgSO_4$ and concentrated to afford the bis-TBS product as yellowish oil. The intermediate was dissolved in a mixture of 25 mL of THF/H_2O (5:1) followed by adding K_2CO_3 (178 mg, 1.3 mmol, 2.5 equiv) in a single portion. The resultant slurry was stirred vigorously for 2 h at room temperature and then diluted with 40 mL of EtOAc. The organic phase washed with 20 mL water, 20 mL 1.0 M aqueous HCl and 15 mL brine, dried ($MgSO_4$) and concentrated to obtain a white solid, which was further dried in high vacuum at 80 °C for 6 h to drive off the residual TBSOH to give **30** as white solid (156 mg, 98% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 7.72 (1H, d, *J* = 15.8 Hz), 7.06 (1H, dd, *J* = 8.5, 2.1 Hz), 7.05 (1H, brs), 6.86 (1H, d, *J* = 8.5 Hz), 6.31 (1H, d, *J* = 15.8 Hz), 3.85 (3H, s), 1.00 (9H, s), 0.18 (6H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C: 172.4, 151.4, 148.2, 147.3, 128.1, 122.9, 121.3, 115.0, 111.2, 55.6, 25.8, 18.6, −4.4.

2.4.3. Synthesis of 4″-O-tertbutyldimethylsilynotoptetherin F (31)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCl, 2.7 mg, 0.014 mmol, 1.5 equiv) and catalytic amount of 4-dimethylaminopyridine (DMAP) were added to a solution of **30** (4.4 mg, 0.014 mmol, 1.5 equiv) in CH_2Cl_2 (2 mL) followed by dropping the solution of **28** (2.3 mg, 0.0094 mmol, 1.0 equiv) in 0.5 mL of CH_2Cl_2. The mixture was allowed to stirred overnight at room temperature and directly purified by preparative TLC (PE/EtOAc, 3:1, R_f = 0.5) to afford the product **31** (2.8 mg, 56% yield). $[\alpha]_D^{25}$ + 187.3 (*c* 0.05, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 7.59 (1H, d, *J* = 9.5 Hz), 7.55 (1H, d, *J* = 15.6 Hz), 7.26 (1H, overlapped), 6.99 (2H, m), 6.85 (1H, d, *J* = 8.6 Hz), 6.78 (1H, d, *J* = 8.3 Hz), 6.23 (1H, d, *J* = 15.6 Hz), 6.16 (1H, d, *J* = 9.5 Hz), 5.54 (1H, t, *J* = 8.9 Hz), 5.41 (1H, brs), 5.38 (1H, brs), 4.89 (1H, d, *J* = 13.4 Hz), 4.80 (1H, d, *J* = 13.4 Hz), 3.85 (3H, s), 3.64 (1H, dd, *J* = 16.1, 9.9 Hz), 3.38 (1H, dd, *J* = 16.1, 7.7 Hz), 1.01 (9H, s), 0.18 (6H, s); HRESIMS *m/z* 535.2159 $[M + H]^+$ (calcd for $C_{30}H_{35}O_7Si$, 535.2152).

2.4.4. Synthesis of notoptetherin F (6)

To a solution of **31** (2.5 mg, 0.0047 mmol, 1.0 equiv) in 2 mL of THF stirred at room temperature, the 1.0 M solution of *tetra-n*-butylammonium fluoride (TBAF) in THF (10 μL, 2.1 equiv) was added dropwise. The reaction was monitored by TLC and upon completion, the product (1.4 mg, 73% yield) was purified by preparative TLC (PE/EtOAc, 1:1, R_f = 0.4). $[\alpha]_D^{25}$ + 183.1 (*c* 0.04, MeOH); ECD (*c* 0.04 MeOH) 342 ($\Delta\varepsilon$ + 10.12), 300 ($\Delta\varepsilon$ − 5.06), 253 ($\Delta\varepsilon$ + 2.51) nm; ^{1}H NMR (400 MHz, CD_3OD) δ_H: 7.75 (1H, d, *J* = 9.5 Hz), 7.41 (1H, d, *J* = 15.9 Hz), 7.41 (1H, d, *J* = 8.3 Hz), 7.09 (1H, d, *J* = 1.9 Hz), 6.96 (1H, dd, *J* = 8.2, 1.9 Hz), 6.82 (1H, d, *J* = 8.3 Hz), 6.79 (1H, d, *J* = 8.2 Hz) 6.16 (1H, d, *J* = 15.9 Hz), 6.06 (1H, d, *J* = 9.5 Hz), 5.66 (1H, dd, *J* = 10.3, 7.3 Hz), 5.42 (1H, brs), 5.38 (1H, brs), 5.01 (1H, d, *J* = 13.2 Hz), 4.73 (1H, d, *J* = 13.2 Hz), 3.69 (1H, dd, *J* = 16.1, 10.3 Hz), 3.39 (1H, dd, *J* = 16.1, 7.3 Hz); HRESIMS *m/z* 419.1133 $[M - H]^-$ (calcd for $C_{24}H_{19}O_7$, 419.1131).

2.5. Liquid chromatography and mass spectrometry

The powders (80 mesh, 100 mg) of the roots and rhizomes of *N. incisum* were extracted with 10 mL of MeOH by ultrasonification at 40 kHz for 30 min. Then 1.5 mL of the MeOH extract was filtrated through a 0.22 μm membrane and 5 μL of the filtrate was injected into the UPLC device eluting with MeCN/H_2O in gradient (60:40 → 100:0) within 20 min at 0.4 mL/min. ESIMS data were acquired in the positive ion mode and the ion source parameters were set as follows: source temperature 550 °C; ion spray voltage (IS) 4500 V; ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were at 55, 55, and 35 psi, respectively; the collision gas (CAD) was high.

2.6. Bioassay

2.6.1. Cell culture

The murine macrophage RAW 264.7 cells were purchased from Beijing Union Medical University (Beijing, China) and maintained in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2.

2.6.2. NO assay

NO production and cell viability were measured as the procotols described previously [22,23]. For detailed procedures, see Supplementary data.

3. Results and discussion

The 95% aq. EtOH extract of the roots and rhizomes of *N. incisum* was suspended in water and successively partitioned with PE, $CHCl_3$, and *n*-BuOH. The resultant $CHCl_3$ and *n*-BuOH fractions were subjected to various CC and reverse phase HPLC to yield compounds **1–26** (Fig. 1).

Notoptetherin A (**1**) was obtained as a colorless oil. The molecular formula of **1** was established as $C_{27}H_{32}O_6$ by the positive HRESIMS ion at *m/z* 475.2094 $[M + Na]^+$ (calcd for $C_{27}H_{32}O_6Na$, 475.2097), corresponding to 12 indices of hydrogen deficiency. The ^{1}H NMR signals of two pairs of *ortho*-coupled olefinic doublets at δ_H 8.27 (1H, d, *J* = 9.7 Hz), 6.29 (1H, d, *J* = 9.7 Hz) and 7.79 (1H, d, *J* = 2.3 Hz), 7.16 (1H, d, *J* = 2.3 Hz) and a phenyl singlet at δ_H 7.21 (1H, s) (Table 1) indicated that **1** was a linear furocoumarin with a side-chain substituted at C-5 [24], which was confirmed by the HMBC correlation from H_2–1′ (δ_H 5.04) to C-5 (δ_C 150.4) and the NOE enhancement between H-10 (δ_H 7.16) and H_2–1′ (δ_H 5.04). Analysis of the ^{13}C NMR data (Table 2) in combination with the HSQC correlations revealed that the residue substituted at C-5 was composed of 16 carbon resonances attributable to one bisubstituted double bond (δ_C 139.0 and 127.2), one trisubstituted double bond (δ_C 143.3 and 121.1), two sp^3 oxygenated tertiary carbons (δ_C 90.8 and 80.5), six sp^3 methylenes (δ_C 70.9, 43.2, 38.0 × 2, and 25.5 × 2), and four methyls (δ_C 16.8, 24.7, and 25.6 × 2). The aforementioned spectroscopic data suggested that **1** was a derivative of notoptol (**7**) [4,24] with an additional C_6 ring moiety accounting for the remaining one index of hydrogen deficiency, which was deduced to be a 1-methylcyclopentyl hydroxyl group [25] by the ^{1}H−^{1}H COSY and HMBC correlations (Fig. 2). By comparison with the NMR data of notoptol (**7**) [4,24] and 1-methylcyclopentanol [25], the deshielded resonances of C-7′ (δ_C 80.5 for **1** and δ_C 70.6 for **7**) and C-1″ (δ_C 90.8 for **1** and δ_C 79.7 for 1-methylcyclopentanol) in **1** indicated that these two moieties were linked via a peroxy bond between C-7′ and C-1″, which was supported by the molecular composition and the similar C-1″ resonance value to 1-methylcyclopentyl hydroperoxide (δ_C 92.1) [25]. In the 1D NOE experiment, when irradiation of H_2–1′ (δ_H 5.04), H_3–9′ (δ_H 1.65) showed an enhancement, indicating a 2′*E*-configuration for the double bond (Fig. 2). Meanwhile, the 5′*E*-configuration was determined from the coupling constant of 15.8 Hz between H-5′ and H-6′. Thus, the structure of notoptetherin A (**1**) was determined as shown.

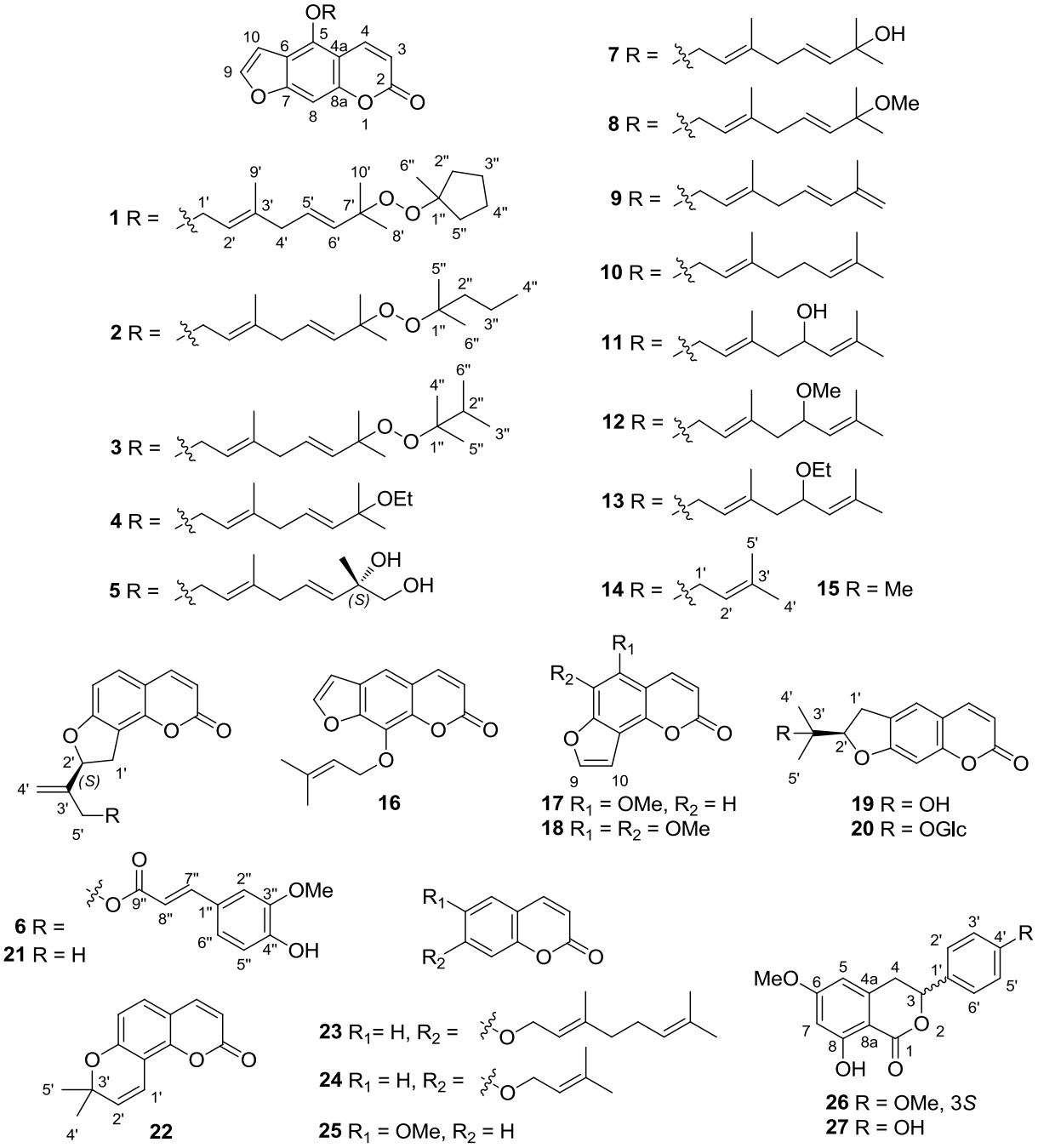


Fig. 1. Structures of compounds **1–27.**

Notoptetherin B (**2**), isolated as a colorless oil, had a molecular formula of $C_{27}H_{34}O_6$ determined by the positive HRESIMS ion at *m/z* 477.2243 $[M + Na]^+$ (calcd for $C_{27}H_{34}O_6Na$, 477.2253), processing 11 indices of hydrogen deficiency. The NMR data of **2** (Tables 1 and 2) were similar to those of **1**, except that the 1-methylcyclopentyl hydroxyl group located at C-7′-OH in **1** was substituted by a 2-methyl-2-pentanyl hydroxyl group in **2**, which was supported by the $^1H-^1H$ COSY correlations of H_2–2″/H_2–3″/H_3–4″ and the HMBC correlations of H_2–3″/C-1″ and H_3–5″ (H_3–6″)/C-1″and C-2″ (Fig. 2). The connection between C-7′ and C-1″ via a peroxy bond was confirmed by the molecular composition and the C-1″ resonance (δ_C 81.5 for **2** and δ_C 80.1 for *tert*-butyl 2-methyl-2-pentanyl peroxide [26]). Therefore, the structure of notoptetherin B (**2**) was established.

Notoptetherin C (**3**) was obtained as a colorless oil with a molecular formula of $C_{27}H_{34}O_6$ determined by the HRESIMS ion at *m/z* 477.2260 $[M + Na]^+$ (calcd for $C_{27}H_{34}O_6Na$, 477.2253). The ^1H and ^{13}C NMR data of **3** (Tables 1 and 2) were similar to those of **1** and **2** except for the different substituent group attached at C-7′-OH, which was deduced to be a 2,3-dimethyl-2-butanyl hydroxyl group based on the $^1H-^1H$ COSY correlations of H-2″/H_3–3″ (H_3–6″) and the HMBC correlations from H_3–3″ (H_3–6″) to C-1″ and C-2″, from H-2″ to C-1″ and C-4″ (C-5″), and from H_3–4″ (H_3–5″) to C-1″. The presence of the peroxy bond between C-7′ and C-1″ was established by the molecular composition and the C-1″ resonance (δ_C 83.9 for **3** and δ_C 85.1 for 2,3-dimethyl-2-butanyl hydroperoxide [25]). Thus, the structure of notoptetherin C (**3**) was characterized unequivocally.

Peroxy natural products usually possess hydroperoxy groups or peroxide bridges generated intramolecularly [27,28]. However, acyclic

Table 1
^{1}H NMR data of compounds **1–5** (500 MHz, δ in ppm, J in Hz).

Position	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[a]
3	6.29, d (9.7)	6.28, d (9.8)	6.29, d (9.7)	6.25, d (9.8)	6.28, d (9.8)
4	8.27, d (9.7)	8.27, d (9.8)	8.26, d (9.7)	8.13, d (9.8)	8.26, d (9.8)
8	7.21, s	7.21, s	7.21, s	7.12, s	7.19, s
9	7.79, d (2.3)	7.79, d (2.4)	7.80, d (2.3)	7.59, d (2.3)	7.79, d (2.4)
10	7.16, d (2.3)	7.16, d (2.4)	7.16, d (2.3)	6.94, d (9.3)	7.16, dd (2.4, 1.1)
1′	5.04, d (6.9)	5.04, d (7.0)	5.04, d (7.0)	4.95, d (6.8)	5.03, d (6.9)
2′	5.59, t (6.9)	5.59, t (7.0)	5.60, t (7.0)	5.55, t (6.8)	5.61, t (6.9)
4′	2.74, d (6.8)	2.74, d (6.8)	2.74, d (6.8)	2.78, d (5.9)	2.78, d (6.7)
5′	5.48, dt (15.8, 6.8)	5.48, dt (15.7, 6.8)	5.48, dt (15.8, 6.8)	5.46, dd (15.8, 5.9)	5.65, dt (15.6, 6.7)
6′	5.59, d (15.8)	5.59, d (15.7)	5.59, d (15.8)	5.51, d (15.8)	5.57, d (15.6)
8′	1.24, s	1.24, s	1.24, s	1.25, s	3.35, d (2.7)
9′	1.65, s	1.65, s	1.65, s	1.67, s	1.67, s
10′	1.24, s	1.24, s	1.24, s	1.25, s	1.21, s
2″a	1.85, m	1.45, m	1.85, m		
b	1.38, m				
3″a	1.66, m	1.32, m	0.85, d (6.9)		
b	1.55, m				
4″a	1.66, m	0.87, t (7.3)	1.09, s		
b	1.55, m				
5″a	1.85, m	1.13, s	1.09, s		
b	1.38, m				
6″	1.30, s	1.13, s	0.85, d (6.9)		
OCH_2CH_3				3.31, q (7.0)	
OCH_2CH_3				1.13, t (7.0)	

[a] measured in CD_3OD.
[b] measured in $CDCl_3$.

Table 2
^{13}C NMR data of compounds 1–5 (125 MHz, δ in ppm).

Position	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[a]
2	163.2, C	163.2, C	163.2, C	161.3, C	163.2, C
3	113.1, CH	113.1, CH	113.1, CH	112.7, CH	113.1, CH
4	141.5, CH	141.5, CH	141.5, CH	139.6, CH	141.5, CH
4a	109.1, C	109.1, C	109.1, C	107.6, C	108.9, C
5	150.4, C	150.4, C	150.4, C	148.9, C	150.4, C
6	116.1, C	116.3, C	116.3, C	114.3, C	116.1, C
7	159.7, C	159.7, C	159.7, C	158.2, C	159.7, C
8	95.0, CH	95.0, CH	95.0, CH	94.3, CH	94.9, CH
8a	153.8, C	153.8, C	153.8, C	152.7, C	153.8, C
9	146.9, CH	146.9, CH	146.9, CH	145.0, CH	146.9, CH
10	106.2, CH	106.2, CH	106.2, CH	105.1, CH	106.2, CH
1′	70.9, CH_2	70.9, CH_2	70.9, CH_2	69.8, CH_2	70.9, CH_2
2′	121.1, CH	121.0, CH	121.1, CH	119.8, CH	121.0, CH
3′	143.3, C	143.3, C	143.3, C	141.8, C	143.2, C
4′	43.2, CH_2	43.2, CH_2	43.2, CH_2	42.5, CH_2	43.3, CH_2
5′	127.2, CH	127.2, CH	127.1, CH	126.1, CH	127.3, CH
6′	139.0, CH	139.0, CH	139.1, CH	138.9, CH	138.0, CH
7′	80.5, C	80.5, C	80.5, C	74.6, C	73.9, C
8′	25.6, CH_3	25.5, CH_3	25.5, CH_3	26.5, CH_3	70.9, CH_2
9′	16.8, CH_3	16.8, CH_3	16.8, CH_3	16.8, CH_3	16.8, CH_3
10′	25.6, CH_3	25.5, CH_3	25.5, CH_3	26.5, CH_3	24.6, CH_3
1″	90.8, C	81.5, C	83.9, C		
2″	38.0, CH_2	43.0, CH_2	36.1, CH		
3″	25.5, CH_2	18.3, CH_2	17.9, CH_3		
4″	25.5, CH_2	15.1, CH_3	22.0, CH_3		
5″	38.0, CH_2	25.1, CH_3	22.0, CH_3		
6″	24.7, CH_3	25.1, CH_3	17.9, CH_3		
OCH_2CH_3				57.8, CH_2	
OCH_2CH_3				16.2, CH_3	

[a] measured in CD_3OD.
[b] measured in $CDCl_3$.

dialkyl peroxides [29–34], especially those with two different alkyl groups [35,36], such as notoptetherins A-C (**1–3**), are rare examples from natural source. A putative biosynthetic pathway to **1–3** is proposed to be derived from compounds **7** or **10** and volatile C_6 alkanes, including methylcyclopentane [37], 2-methylpentane [38], and 2,3-dimethylbutane [39] via a S_N1-type nucleophilic substitution by the hydroperoxy group (Scheme. S1, Supplementary Data). The hydroperoxy intermediate **iv** could be formed by oxidation of **10** followed by trapping the generated vinyl free radicals **i**/**ii** with molecule oxygen (O_2). Nucleophilic substitution of cations **A**, produced by oxidation of the corresponding C_6 alkanes, with **iv** afforded the dialkyl peroxides **1–3**. On the other hand, **1–3** could also be formed by reaction of hydroperoxy intermediate **B** with cation **v**. The hydroperoxy intermediate **B** could come from the C_6 alkanes via a process similar to that for the formation of **iv**, while the counterpart cation **v** could be obtained from **7**.

Notoptetherin D (**4**), obtained as a colorless oil, had a molecular formula of $C_{23}H_{26}O_5$ according to its sodium adduct ion at m/z 405.1674 $[M + Na]^+$ (calcd for $C_{23}H_{26}O_5Na$, 405.1678) in the HRESIMS spectrum. The NMR data of **4** (Tables 1 and 2) were similar to those of **7** [4,24] except for the presence of an additional ethyl group (δ_H 3.31, q, J = 7.01 Hz, δ_C 57.8 and δ_H 1.13, t, J = 7.01 Hz, δ_C 16.2), indicating that **4** was an ethyl etherificated derivative of notoptol (**7**). This deduction was confirmed by the HMBC correlation from the protons of ethoxy group (δ_H 3.31) to C-7′ (δ_C 74.6) (Fig. S1, Supplementary Data). To clarify whether compound **4** was an artificial product, the MeOH extract of *N. incisum* was analyzed by using UPLC/MS. The chromatographic peak at 6.01 min with a sodium additive ion peak at m/z 405.2 was consistent with the case of **4** under the same analytical conditions (Fig. S66, Supplementary Data). This indicated that compound **4** occurred naturally.

Notoptetherin E (**5**) was isolated as a colorless oil. Its molecular formula was deduced as $C_{21}H_{22}O_6$ from a sodium additive ion peak at m/z 393.1299 $[M + Na]^+$ (calcd for $C_{21}H_{22}O_6Na$, 393.1314) in the HRESIMS spectrum, 16 mass units more than that of notoptol (**7**) [4,24]. Analyses of the NMR data (Tables 1 and 2) indicated that **5** was 8′-hydroxynotoptol, which was confirmed by the HMBC correlations from H_2–8′ to C-6′, C-7′, and C-10′, from H-6′ to C-8′, and from H_3–10 to C-8′ (Fig. S1, Supplementary Data). To establish the absolute configuration of the 7′,8′-diol moiety, the in situ $Mo_2(OAc)_4$-induced circular dichroism (ICD) method [40–42] was employed. Compound **5** was mixed with $Mo_2(OAc)_4$ in anhydrous DMSO to form a metal complex, which gave a positive Cotton effect at 304 nm (Fig. 3), suggesting the (7′*S*) configuration for **5**.

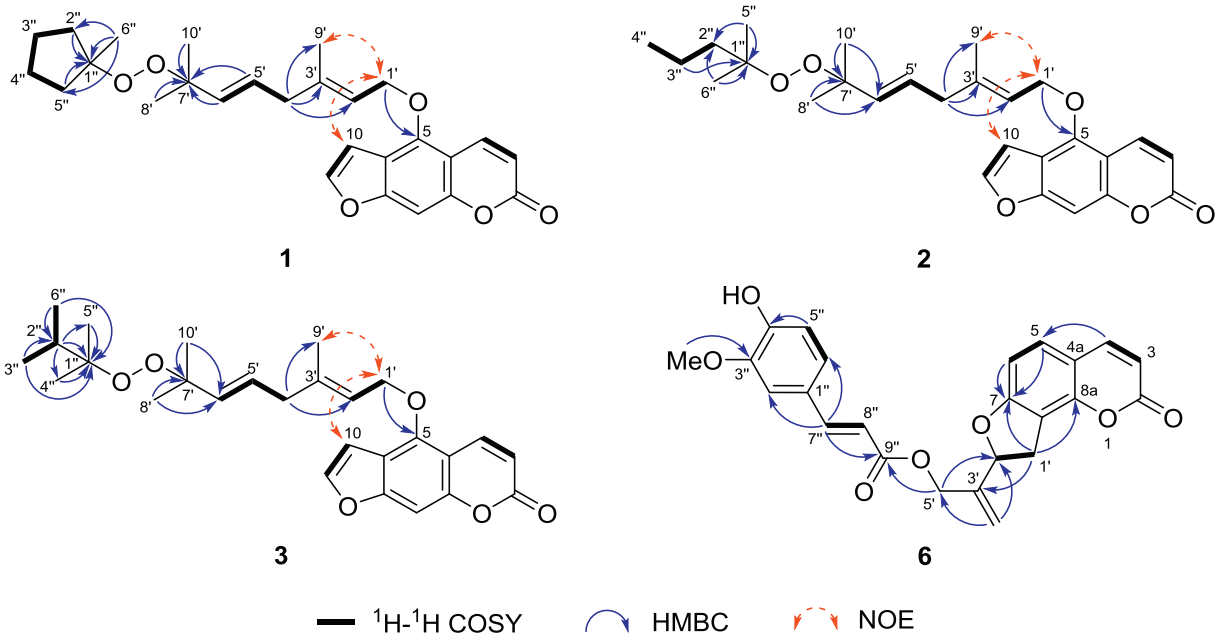


Fig. 2. Selected ^{1}H−^{1}H COSY, HMBC and NOE correlations of compounds **1–3** and **6.**

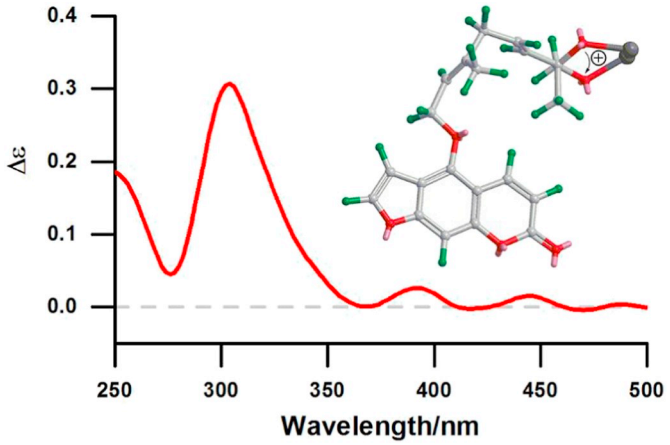


Fig. 3. $Mo_2(OAc)_4$-ICD spectrum of compound **5** in DMSO and conformations of the Mo_2^{4+} complex of compound **5**.

Notoptetherin F (**6**) was obtained as a white amorphous powder. A deprotonated ion $[M - H]^-$ at *m/z* 419.1134 from the HRESIMS data determined its molecular formula as $C_{24}H_{20}O_7$ (calcd for $C_{24}H_{19}O_7$, 419.1131). The ^{1}H NMR spectrum of **6** displayed a pair of characteristic doublets for H-3 and H-4 of a coumarin skeleton (δ_H 7.73 and 6.04, each 1H, d, J = 9.6 Hz) and two *ortho*-coupled phenyl protons at δ_H 7.38 and 6.80 (each 1H, d, J = 8.2 Hz), suggesting that **6** was a 7,8-disubstituted coumarin derivative [19]. Further inspection of the ^{1}H NMR spectrum, combining with the ^{1}H−^{1}H COSY correlations, revealed the presence of additional 13 proton signals attributable to two sp^3 geminal methylenes (δ_H 3.66, 1H, dd, J = 16.0, 10.2 Hz, 3.36, 1H, dd, J = 16.0, 7.3 Hz; 4.99 and 4.72, each 1H, d, J = 13.2 Hz), a sp^3 oxygenated methine (δ_H 5.63, 1H, dd, J = 10.2, 7.3 Hz), a trisubstituted aromatic ring with an AMX system (δ_H 7.07, 1H, d, J = 2.0 Hz, 6.95, 1H, dd, J = 8.1, 2.0 Hz, and 6.78, 1H, d, J = 8.1 Hz), a *trans*-double bond (δ_H 7.40 and 6.14, each 1H, d, J = 16.0 Hz), a terminal vinyl (δ_H 5.40 and 5.36, each 1H, brs), and a methoxy group (δ_H 3.89, 3H, s). Taking the esterified carbon resonance at δ_C 168.3 into consideration, the above mentioned NMR data indicated that **6** was an ester

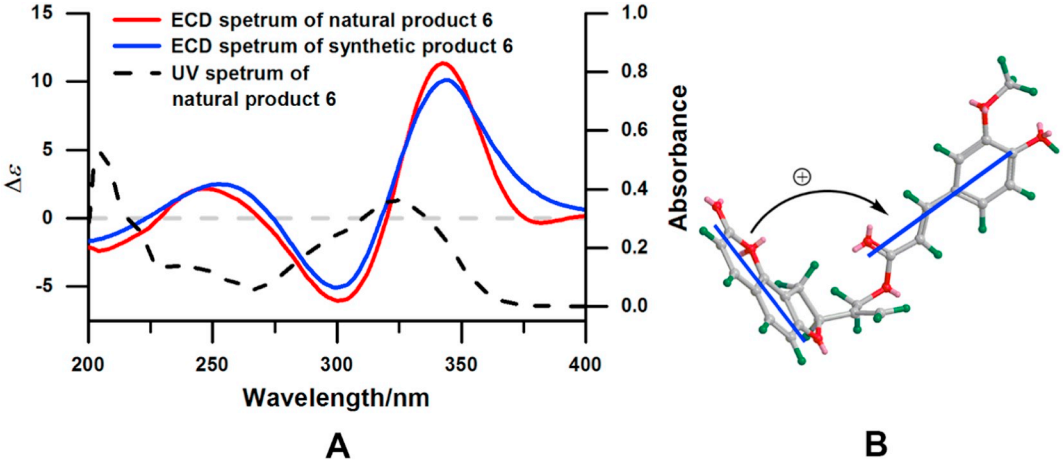
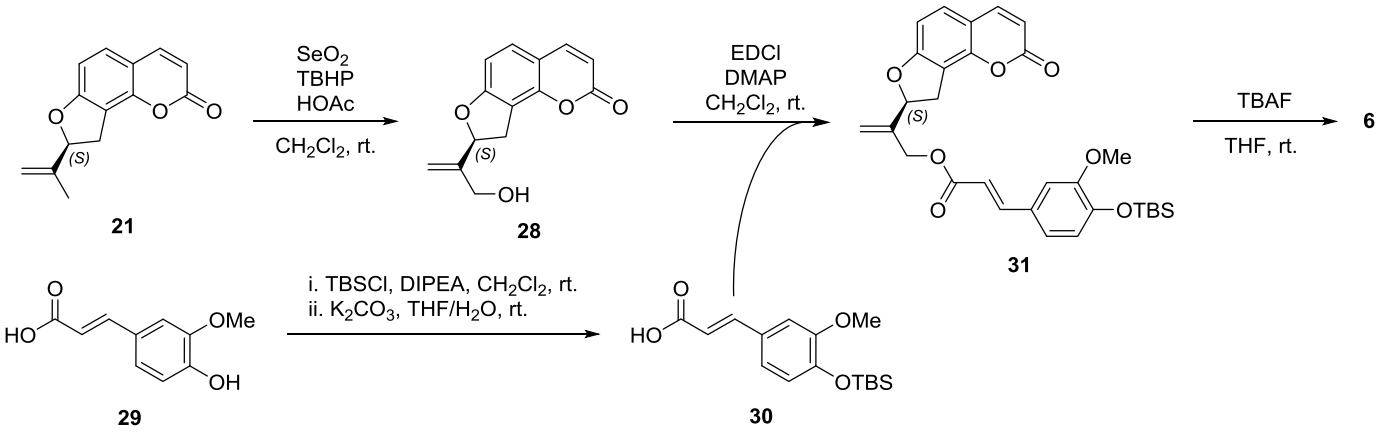


Fig. 4. ECD and UV spectra of compound **6** (A) and the positive chirality for compound **6** (B).



Scheme. 1. Chemical transformation of **6** from **21** and *trans*-ferulic acid (**29**).

Table 3
Inhibitory effects of compounds **1–26** on NO production in RAW 264.7 cells induced by LPS.

Compounds	IC_{50} (μM)[a]	Compounds	IC_{50} (μM)[a]
1	–	**14**	–
2	–	**15**	–
3	–	**16**	26.3 ± 1.2
4	20.4 ± 1.3	**17**	10.2 ± 1.2
5	49.5 ± 2.6	**18**	–
6	–	**19**	–
7	–	**20**	–
8	23.9 ± 2.9	**21**	27.0 ± 1.6
9	36.6 ± 2.1	**22**	27.9 ± 1.1
10	28.6 ± 2.3	**23**	–
11	29.1 ± 1.0	**24**	–
12	12.7 ± 0.9	**25**	–
13	18.1 ± 0.1	**26**	19.8 ± 0.5
Dexamethasone[b]	10.1 ± 0.4		

[a] Values were displayed as means ± SD from triplicate experiments. "–" meant compounds inactive (inhibition rate < 50% at 100 μM).
[b] Positive control.

comprising of an angenomalin moiety [19] and a *trans*-feruloyl group [3]. The linkage of C-5′ − *O* − C-9″ was established on the basis of the HMBC correlation from H_2–5′ (δ_H 4.99 and 4.72) to C-9″ (δ_C 168.3). Thus, the 2D structure of **6** was elucidated as *trans*-feruloylangenomalin (Fig.2). In the ECD spectrum of **6** (Fig. 4), the sequential negative and positive Cotton effects at 300 nm and 342 nm, causing by the exciton coupling between the electric transition dipoles of coumarin nucleus and the *trans*-feruloyl moiety, implied a positive chirality (+) and an *S* absolute configuration for **6**.

Even though the exciton chirality rule is nonempirical due to its well-established theoretical basis, the way to predict the sign and intensity of an exciton couplet essentially depending on the geometry of transition dipoles is not completely accurate [43]. Thus, a biomimetic synthesis of **6** from **21**, a known compound with the *S* absolute configuration assigned by comparison its specific rotation ($[\alpha]_D^{25}$ + 189.3 in MeOH) with the literature data [44] ($[\alpha]_D^{23}$ + 181.0 in EtOH), and *trans*-ferulic acid (**29**) was carried out to further confirm the absolute configuration of **6** (Scheme 1). **28** was prepared by allylic oxidation of **21** using SeO_2 and TBHP [19,20] in 23% yield with **21** recovered in 48%. Esterification of the oxidation product **28** with the mon-TBS protected *trans*-ferulic acid using EDCl in the presence of a catalytic amount of DMAP afforded **31** in a yield of 56%. **6** was easily obtained by treatment **31** with TBAF to remove the TBS group. As shown in Fig.4, the ECD spectrum of the synthetic product **6** presented the same Cotton effects as those of the natural product **6**. Moreover, both of them had the consistent specific rotation data in MeOH, $[\alpha]_D^{25}$ + 183.1 for the synthetic product and $[\alpha]_D^{25}$ + 186.3 for the natural product. Thus, the *S* absolute configuration of **6** was determined unambiguously, and the structure of **6** was defined as shown.

The other 20 known compounds were identified as notoptol (**7**) [24], methylnotoptol (**8**) [4], anhydronotoptol (**9**) [4], bergamottin (**10**) [45], notopterol (**11**) [7], methylnotopterol (**12**) [46], ethylnotopterol (**13**) [47], isoimperatorin (**14**) [47], bergapten (**15**) [48], imperatorin (**16**) [49], isobergapten (**17**) [48], pimpinellin (**18**) [48], nodakenetin (**19**) [3,50], nodakenin (**20**) [3,51], (*S*)-angenomalin (**21**) [19,44], seselin (**22**) [45], aurapten (**23**) [45], 7-*O*-prenylumbeliferone (**24**) [45], scopoletin (**25**) [52], and (*S*)-6-*O*-methylscorzocreticin (**26**) [53–56] by comparison of their physical and spectroscopic data with those in the literature. Compound **26**, reported as a synthetic racemate previously [53,54], was isolated firstly as a natural product with *S* absolute configuration. The absolute configuration of **26** was determined by comparison of its ECD spectrum with that of mellein [55,56] (Fig. S56, Supplementary Data). Since compound **26** showed a positive Cotton effect at 236 nm and a negative Cotton effect at 256 nm, similar to the case of mellein, the absolute configuration of **26** was assigned as *S*. 6-Methoxyhydrangenol (**27**) [57], an analogue of **26** isolated from *N. franchetii*, gave a specific rotation data ($[\alpha]_D^{20}$ + 11 in MeOH), which was in agreement with that of **26** ($[\alpha]_D^{25}$ + 9.1 in MeOH). Thus, compound **27** processed an *S* absolute configuration as well.

According to the clinical application of *N. incisum*, which is used in TCM for treating the common cold and inflammatory diseases [2], inhibition against LPS-induced NO production in RAW 264.7 cells was employed to evaluate the anti-inflammatory activities of compounds **1–26**, and their IC_{50} values are presented in Table 3. It could be observed that most of the furocoumarins with a linear prenyl side-chain, except for compounds **1–3**, **7**, and **14**, exhibited moderate inhibitory effects with IC_{50} values ranging from 12.7 to 49.5 μM. This indicated that compounds processing a peroxy group, such as **1–3**, or an exposed hydroxy group at C-7′, e.g. **5** and **7**, might decrease the inhibitory activity. Furthermore, compounds with an *O*-isopentenyl group at C-8 (**16**, IC_{50} = 26.3 μM) showed moderate inhibitory effects; but when the substituent group was at C-5, compounds became inactive (**14**, IC_{50} > 100 μM). Among the angular furocoumarins, **17** and **21** revealed similar NO inhibitory activities to those discussed above with IC_{50} values of 10.2 and 27.0 μM, respectively. Compound **17** exerted the most potent inhibitory effect among all the test compounds.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.10.002.

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