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ENT-3,4-*SECO*-LABDANE DITERPENOIDS FROM *CALLICARPA NUDIFLORA* LEAVES WITH ANTI-INFLAMMATORY ACTIVITY

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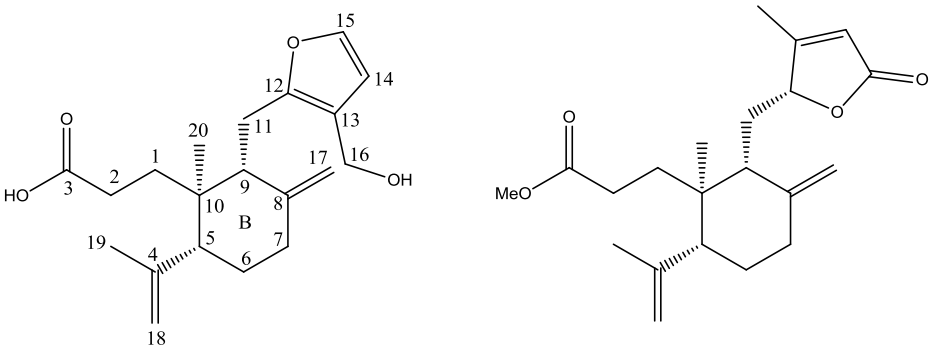
Abstract – Two new *ent*-3,4-*seco*-labdane diterpenoids, *ent*-3,4-*seco*-16-hydroxy-12,15-epoxy-4(18),8(17),12,14-labdatetraen-3-oic acid (**1**) and *ent*-3,4-*seco*-12*R*,15-epoxy-4(18),8(17),13-labdatrien-3-oic acid (**2**), were isolated from the leaves of *Callicarpa nudiflora*. Their structures were elucidated by extensive spectroscopic analysis and the biosynthetic pathways leading to these compounds were proposed. The two compounds both exerted potent inhibitory activity against the production of nitric oxide (NO) in RAW264.7 stimulated by lipopolysaccharide (LPS).

Callicarpa nudiflora grows mainly in southern China, India and Malaya and is one of popular Li medicine in Hainan Province.[1,2] In previous work, *C. nudiflora* had been found to exhibit anti-inflammatory, haemostatic, antibacterial, cytotoxic and immuno-enhancing activities.[2] Therefore, the bioactive constituents and their mechanism of *C. nudiflora* are of our interest.

Prophase chemical studies on this species have led to the isolation of diterpenoids, triterpenoids, flavonoids, phenolic acids, volatile oils and so on.[2-5] During the course of our investigations, we found two new diterpenoids whose structures are based on the *ent*-3,4-*seco*-labdane skeleton. In the current study, we present details of the isolation and structural determination of compounds **1** and **2**, as well as a proposed biosynthetic pathway leading to all compounds isolated. A pharmacological investigation was also carried out on inhibitory effect of the compounds on nitric oxide (NO) production in LPS-activated

RAW264.7 macrophage cells.

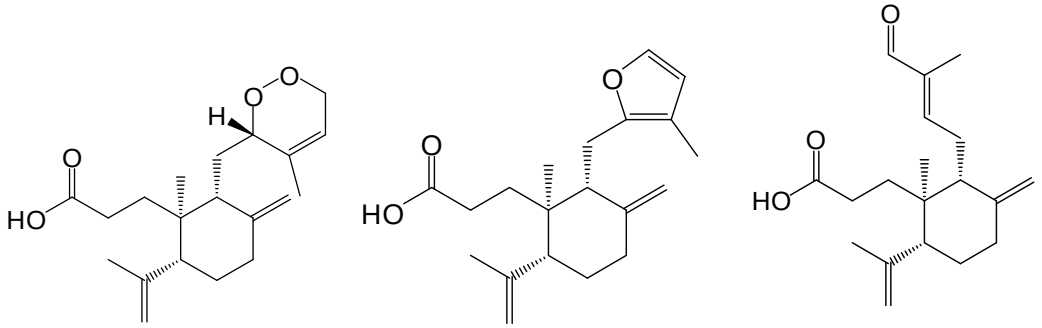


1 **2**

Figure 1. The structures of compounds **1** and **2**

The air-dried leaves of *C. nudiflora* were extracted exhaustively with water. The aqueous extract was eluted over a macroporous resin column with water, 60% EtOH, and 90% EtOH, successively. The 90% EtOH eluate was concentrated to dryness, and was subjected to repeated column chromatography and semi-preparative HPLC to give *ent*-3,4-*seco*-16-hydroxy-12,15-epoxy-4(18),8(17),12,14- labdatrien-3-oic acid (**1**) and *ent*-3,4-*seco*-12*R*,15-epoxy-4(18),8(17),13-labdatrien-3-oic acid (**2**). The structures of **1** and **2** were shown in **Figure 1**, and their ^{1}H and ^{13}C NMR spectroscopic data were listed in **Table 1**. The *in vitro* anti-inflammatory activity of **1** and **2** were measured. Meanwhile, the plausible biosynthetic pathways for formation of the two isolated metabolites were postulated (**Figure 6**), see below.

Compound **1**, obtained as yellow oil, had a molecular formula of $C_{20}H_{28}O_4$ as determined by HR-ESI-MS *m/z* 331.1898 $[M-H]^-$ (calcd. for 331.1909). Its IR spectrum indicated the presence of carboxylic acid (2922 cm^{-1}), vinyl (1722, 855 cm^{-1}) and ether (1204 cm^{-1}) groups. The ^{1}H NMR and ^{13}C NMR (**Table 1**) spectra revealed the presence of an isopropenyl group [δ_H 1.73 (3H, s), 4.72 (1H, s), 4.88 (1H, s), δc 23.5, 113.6, 147.1], two vinylidene signals [δ_H 4.62 (1H, s), 4.76 (1H, s), δc 108.0, 147.2], a methyl bounded to a quaternary carbon [δ_H 0.74 (3H, s), δc 17.1], and a carboxylic acid [δc 174.8]. The remaining signals were at higher field and were deduced to five methylenes, two methines, and one quaternary carbon from the DEPT spectra data. The above features indicated that compound **1** had a 3, 4-*seco*-labdane skeleton which similar to *ent*-3,4-*seco*-12,15-dioxo-4,8,13-labdatrien-3-oic acid (**Figure 2**).[6] However, there are some differences in the chemical shifts for the side chain. A disubstituted furan ring was derived from the signals at δ_H 7.39 (1H, d, J = 1.7 Hz) and 6.31 (1H, d, J = 1.7 Hz); δc 150.3, 140.1, 119.9 and 111.6.[7] The HMBC correlation (**Figure 3**) from H-11 (δ_H 2.83) to C-12 (δc 150.3) placed the furan ring at C-11. Finally, the characteristic resonances [δ_H 4.31 (2H, d, J = 3.7 Hz), δc 54.7] implied the appearance of a vinylic primary alcohol and the HMBC correlations from H-16 (δ_H 4.31) to C-12 (δc 150.3), C-13 (δc 119.9) and C-14 (δc 111.6) indicated the vinylic primary alcohol attached at C-13.



3 **4** **5**

Figure 2. The structures of *ent*-3,4-*seco*-12,15-dioxo-4,8,13-labdatrien-3-oic acid (**3**) and other known compounds (**4**, **5**) relating to compounds **1** and **2**

The relative configuration of **1** was readily determined by NOESY experiment (**Figure 3**). The side chain, the vinylidene group, H_3-20 and the isopropenyl moiety were in the same α-orientation due to correlations of H-9/H-7β, H-11/H-17, H_3-20/H-11, H_3-20/H-18. Therefore, it was proposed that ring B has a chair conformation.

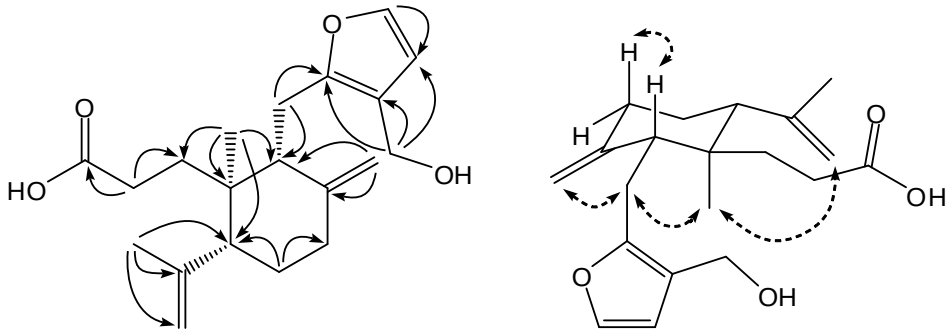


Figure 3. Key HMBC ( ) and NOESY ( ) correlations of compound **1**

It was found that compound **1** had a similar CD spectrum (**Figure 4**) with methylcallicarpate,[8] which indicated that compound **1** possessed the same stereochemistry at C-5, 9, 10 with methylcallicarpate (5*R*,9*R*,10*R*). Hence, the absolute configuration at C-5, 9, 10 of compound **1** was elucidated as *R*, *R*, and *R*. In addition, the stereochemistry of compound **1** was *ent* according to *ent*-CPP (9*R*,10*R*).[9] Based on the above results, the structure of compound **1** was elucidated as *ent*-3,4-*seco*-16-hydroxy-12,15-epoxy-4(18),8(17),12,14-labdatetraen-3-oic acid.

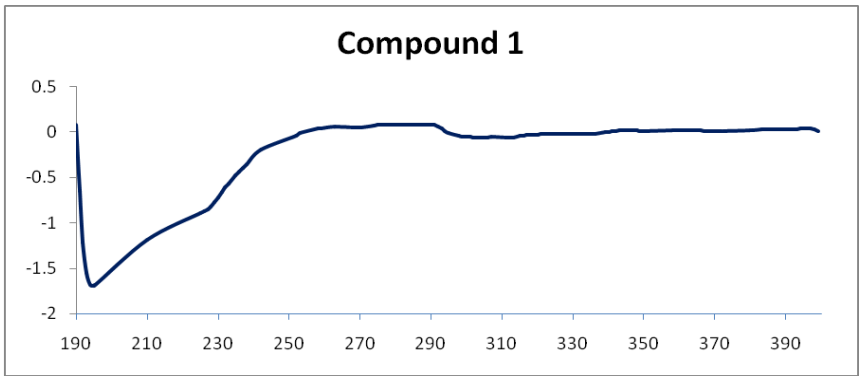


Figure 4. The CD spectrum of compound **1**

Compound **2** was obtained as yellow oil. Its molecular formula was determined as $C_{21}H_{30}O_4$ by HR-ESI-MS *m/z* 345.2062 $[M-H]^-$ (calcd. for 345.2066). A primary analysis of the NMR data (**Table 1**) showed that **2** has the same 3,4-*seco*-labdane skeleton as **1** and differs only in the side chain attached at C-11. It is obvious a *β*-methyl-*α,β*-unsaturated-*γ*-lactone [δ_H 2.12 (3H, d, J = 0.4 Hz), 5.79 (1H, t, J = 1.6 Hz), δc 13.9, 83.2, 116.6, 169.0, 172.5] existed on the side chain moiety based on the HMBC correlations (**Figure 5**) from H-12 (δ_H 4.91) to C-11 (δc 28.3), C-13 (δc 169.0) and C-14 (δc 116.6) and correlations from H-16 (δ_H 2.12) to C-12 (δc 83.2), C-13 (δc 169.0) and C-14 (δc 116.6).[10] The IR spectrum of **2** exhibited absorption at 1762 cm^{-1} 1642 cm^{-1} also confirmed the existence of the *α,β*-unsaturated-*γ*-lactone moiety.

The relative configuration of **2** was established by NOESY spectroscopic analysis (**Figure 5**). In the NOESY spectrum, correlations of H-5/H-9, H-17/H-7*α*, H-17/H-11, H-11/H_3-20 and H_3-20/H_3-19 placed the side chain, the vinylidene group, H_3-20 and the isopropenyl moiety were in the same *α*-orientation. Hence, the ring B has the same chair conformation as compound **1**. The configuration of C-12 was determined as *R* because of the negative cotton effect observed at 220nm (Δε -1.18).[11] The above evidence allowed identification of compound **2** as *ent*-3,4-*seco*-12*R*,15-epoxy-4(18),8(17),13-labdatrien-3-oic acid.

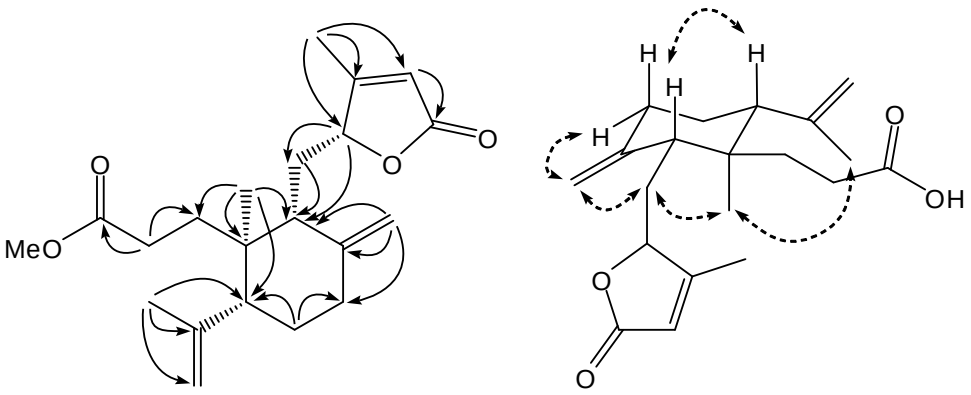


Figure 5. Key HMBC ( ) and NOESY ( ) correlations of compound **2**

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) data for compound **1** in DMSO-d_6 and ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for compound **2** in $CDCl_3$

Position	**1**		**2**	
	δ_H (J in Hz)	δc	δ_H (J in Hz)	δc
1a	1.62 (o[a])	32.5	1.72 (o[a])	32.2
1b	1.62 (o[a])	-	1.72 (o[a])	-
2a	2.44 (o[a])	27.5	2.41 (o[a])	27.2
2b	2.28 (o[a])	-	2.41 (o[a])	-
3	-	174.8	-	174.5
4	-	147.1	-	146.6
5	2.37 (dd, 12.7, 3.4)	49.5	2.27 (dd, 12.4, 4.0)	50.5
6α	1.64 (o[a])	29.8	1.71 (o[a])	30.1
6β	1.48 (o[a])	-	1.66 (o[a])	-
7α	2.22 (o[a])	36.8	2.40 (o[a])	37.6
7β	1.95 (ddd, 25.3, 13.0, 4.3)	-	2.06 (o[a])	-
8	-	147.2	-	147.6
9	2.46 (o[a])	45.9	2.39 (o[a])	44.9
10	-	40.8	-	41.2
11a	2.83 (dd, 15.7, 11.5)	21.4	1.99 (o[a])	28.3
11b	2.59 (br d, 15.4)	-	1.36 (dd, 13.2, 11.6)	-
12	-	150.3	4.91 (d, 10.8)	83.2
13	-	119.9	-	169.0
14	6.31 (d, 1.7)	111.6	5.79 (t, 1.6)	116.6
15	7.39 (d, 1.7)	140.1	-	172.5
16a	4.31 (d, 3.7)	54.7	2.12 (d, 0.4)	13.9
16b	4.31 (d, 3.7)	-	-	-
17a	4.76 (s)	108.0	4.99 (s)	107.1
17b	4.62 (s)	-	4.49 (s)	-
18a	4.88 (s)	113.6	4.88 (s)	113.9
18b	4.72 (s)	-	4.70 (s)	-
19	1.73 (s)	23.5	1.73 (s)	23.5
20	0.74 (s)	17.1	0.72 (s)	17.5
21-OMe	-	-	3.64 (s)	51.7

[a] Overlapping peaks

A plausible biosynthetic pathway for compounds **1** and **2** was proposed as illustrated in **Figure 6**. Both the two compounds might be generated from the mediate bicyclization of geranylgeranyl pyrophosphate (GGPP) to an *ent*-labdadienyl/copalyl diphosphate (CPP) intermediate, which represents a chair-chair-"antipodal/enantiomeric" conformation.[9] Ring A opened between C-3 and C-4 after a few steps.

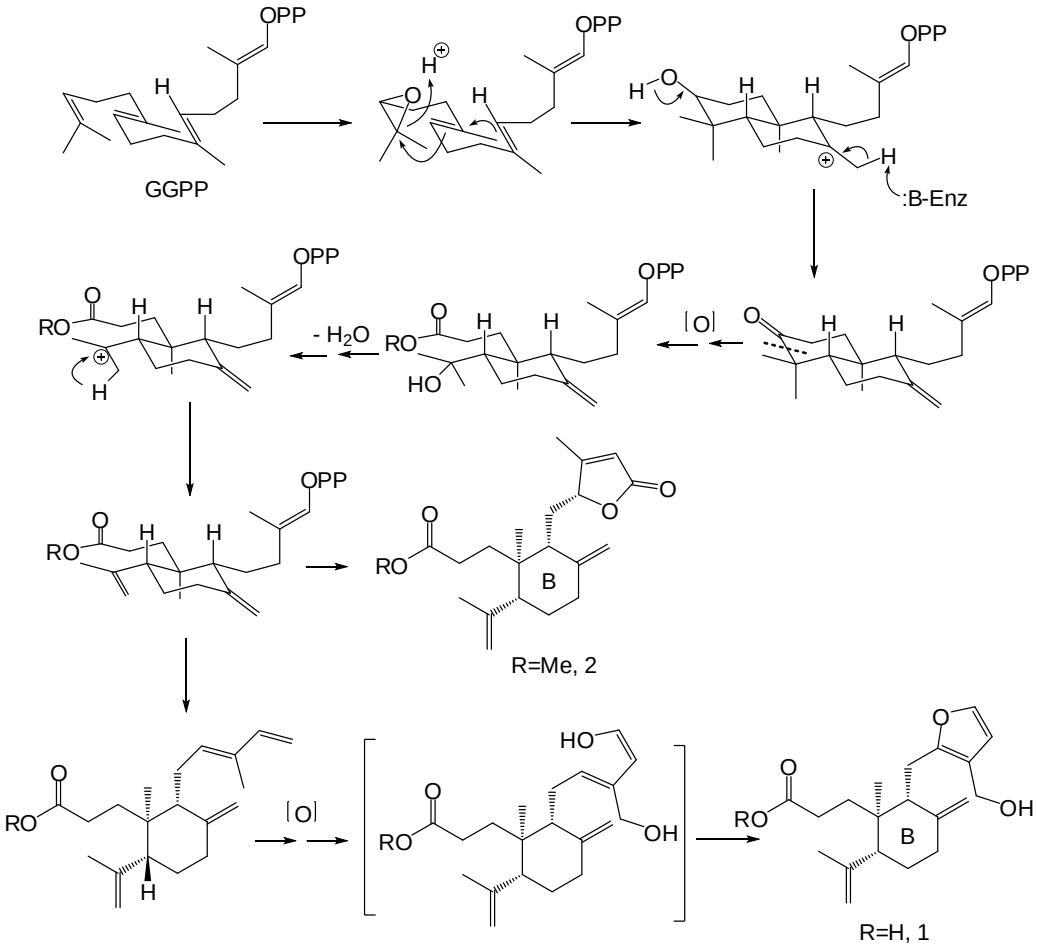


Figure 6. Proposed biosynthetic pathways of compounds **1** and **2**

Nitric Oxide (NO) plays an important role in the inflammatory process.[12] Inhibition of No release is associated with the treatment of the inflammatory diseases.[13] Therefore, Griess reaction was applied to investigate the anti-inflammatory effects of compounds **1** and **2**, with *Z*,*Z*'-6,6',7,3'α-diligustilide used as positive control. The results were summarized in **Table 2**. These results indicated that compound **1** had weak anti-inflammatory activity, however, compound **2** showed strong anti-inflammatory activity.

Table 2. Anti-inflammatory effects of compounds **1** and **2** from *Callicarpa nudiflora* on LPS-induced RAW264.7 macrophages.

Compound[a]	NO Inhibitory rate (%)	Cell Viability (%)
1	16.68±2.15[b]	85.16±1.60
2	59.81±4.55[b]	98.26±3.75
Z,*Z*'-6,6',7,3'α-diligustilide	69.37±6.08[b]	108.50±1.90

[a] Both the two compounds were tested in the same value as 50 μM.
[b] $p<0.01$, significantly different from LPS model group. Data were presented as mean ± SD of three independent experiments.

EXPERIMENTAL

General.

UV absorption spectra were measured on Shimadzu UV-1700 Spectrophotometer and Perkin Elmer Lambda 35 UV/VIS Spectrometer. IR absorption spectra were recorded on a Bruker IFS 55 Infrared spectrophotometer and Perkin Elmer Spectrum 100 FT-IR Spectrometer with KBr pellets. Optical rotations were measured with a Perkin-Elmer 241 MC polarmeter. NMR spectra were measured with 600 MHz Bruker FTNMR Ultra ShieldTM spectrometers in DMSO-d_6 and 400 MHz Varian spectrometers in $CDCl_3$. Chemical shifts (δ ppm) were given relative to TMS as internal standard. HR-ESI-MS was performed on Waters LCT Premier XE time-of -flying spectrometers. HPLC: Hitachi L-7110 pump, Hitachi L-7420 UV spectrophotometric detector at 203nm, YMC C18 reversed-phase (5μm, 10×250 mm) Flow: 2.0ml/min. Chromatography was performed on macroporous resin adsorption (HPD-100, Cangzhou Bon Adsorber Technology Co., Ltd.), silica gel (200-300 mesh; Qingdao Marine Chemical Group, Co., Qingdao, China) and ODS (30-50 μm; Tianjin Mical Reagent Co., Tianjin, China).

Plant Material.

The leaves of *C. nudiflora* Hook. Et Am. were collected in Wuzhishan county, Hainan, China, in September 2010. The plant was identified by Prof. Wei-ping Chen of Hainan Branch, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

Extraction and Isolation.

The air-dried leaves of C. *nudiflora* (6000 g) were extracted exhaustively with water (264 L). The aqueous extract was eluted over HPD-100 macroporous resin column with water, 60% EtOH, and 90% EtOH, consecutively. The 90% EtOH eluate was concentrated to dryness (60 g), and was subjected to silica gel column with a gradient of PE-acetone (200:0-0:1) to afford 29 fractions. Fraction 23 (PE-acetone 100:13) (1.8 g) was applied to ODS silica gel column with MeOH/ H_2O gradient solvent system (10:90-100:0, v/v) to afford ten subfractions, 23A-23J. Fraction 23E (MeOH/H_2O, 50:50) (655.5 mg) was separated via semi-preparative HPLC with MeCN/water (59:41, v/v) gradients to afford compound 2 (7.5 mg) and three subfractions, 23Ea-23Ec. Fraction 23Ea (227.2 mg) was separated by semi-preparative HPLC with MeOH/H_2O (0.4% HCO_2H) (70:30, v/v) as mobile phase, to afford compound 1 (5.7 mg).

***ent*-3,4-*seco*-16-Hydroxy-12,15-epoxy-4(18),8(17),12,14-labdatetraen-3-oic acid (1)**: a yellow oil; $[\alpha]_D^{20}$ -25.8 (*c* 0.16, MeCN); UV (MeCN) λ_{max} (logε) 241 nm (4.17), 316 nm (3.50), 361 nm (3.60); IR banks (KBr) ν_{max} (cm^{-1}): 2922, 1722, 1205, 1054, 1019, 855, 581 and 432; ^1H and ^{13}C NMR data (DMSO-d_6, 600 and 150 MHz, respectively) provided in **Table 1**. HR-ESI-MS (negative ion mode) *m/z* 331.1898. $[M-H]^-$ (calcd. for $C_{20}H_{27}O_4$, 331.1909).

***ent*-3,4-*seco*-12*R*,15-Epoxy-4(18),8(17),13-labdatrien-3-oic acid (2)**: a yellow oil, $[\alpha]_D^{20}$ -23.5 (*c* 0.17, MeCN); UV (MeCN) λ_{max} (logε) 203 nm (4.28), 374 nm (1.62), 380 nm (1.44); IR banks (KBr) ν_{max} (cm^{-1}): 3440, 2928, 2314, 1762, 1737, 1642, 1437, 1384, 1310, 1173, 1116 and 668; ^1H and ^{13}C NMR data ($CDCl_3$, 400 and 100 MHz, respectively) provided in **Table 1**. HR-ESI-MS (negative ion mode) *m/z* 345.2066. $[M-H]^-$ (calcd. for $C_{21}H_{29}O_4$, 345.2066).

Anti-inflammatory Assay.

The anti-inflammatory activities of compounds were evaluated using LPS-induced RAW 264.7 cells. RAW 264.7 macrophages cells (8×10^4 cells/well) were suspended in 100 μL of DMEM supplemented with 10% fetal bovine serum, and precultured in 96-well microplates at 37 °C and 5% CO_2 in air for 12 h, then test compounds (50 μmol/L) were cultured, and were treated with or without 1μg/mL LPS for 24 h. NO production in each well was assessed by measuring the accumulation of nitrite in the culture medium using Griess reagent. Cytotoxicity was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay. Briefly, after 24 h incubation with test compounds, MTT (20 μL, 5 mg/mL in PBS) solution was added to the wells. After 4 h of culturing, the medium was removed and DMSO 100 μL/well was then added to dissolve the formazan produced in the cells. The optical density of the formazan solution was measured with a microplate reader at 490 nm. *Z*,*Z*'-6,6',7,3'α-Diligustilide was used as positive control. Each test compound was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to the medium (final DMSO concentration was 0.1%).

NO inhibitory ratio (%) was calculated by the following formula:

NO inhibitory ratio (%) = $(A_{570,LPS} - A_{570,sample})/A_{570,LPS} \times 100$

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