Three New Abietane-Type Diterpenoids from *Callicarpa macrophylla* Vahl.

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**Abstract:** Three new abietane-type diterpenoids, named callicapoic acid M3 (1), callicapoic acid M4 (2) and callicapoic acid M5 (3), were isolated from the *Callicarpa macrophylla* Vahl. Their structures were established by spectroscopic techniques (IR, UV, MS, 1D and 2D NMR). All the isolated three compounds were evaluated for inhibitory activity on NO production in LPS-activated RAW 264.7 macrophage cells by using MTT assays. Compounds 1, 2 and 3 showed potent inhibitory activity, with inhibition rates of 34.47–40.13%.

**Keywords:** *Callicarpa macrophylla* Vahl.; abietane-type diterpenoids; NMR; anti-inflammatory activity

1. Introduction

The genus *Callicarpa* belongs to the family Verbenaceae, with about 190 species widely distributed throughout the tropical and subtropical regions of Asia and Oceanica and parts of America [1,2]. Many *Callicarpa* species are used in Chinese folk medicine for various indications [2]. Compounds that represent a variety of different classes have been reported, including diterpenoids, triterpenoids, flavonoids, phenolic acids, volatile oils and so on [2–5]. Currently, *Callicarpa macrophylla* Vahl. is used as a folk medicine in China’s Yunnan Province, were the root, the stem, and the leaves are all used in medicine. *Callicarpa macrophylla* Vahl. has a bitter taste, slightly acrid and flat, with clinical actions that eliminate stasis to activate blood circulation and stop bleeding, and it also has detumesence and analgesic actions [6].

![Figure 1. The structures of compounds 1 to 3.](image)

Past phytochemical studies on *Callicarpa macrophylla* Vahl. Have revealed the presence of pentacyclic triterpenes, sterols [6], and diterpenes [7,8]. Additionally, some of the literature has reported that diterpenoid compounds in *Callicarpa* showed potent anti-inflammatory activities [9,10]. Being interested in finding more biologically active substances from this folk medicine, we further...
underwent an investigation to explore its phytochemical composition. As a result, three new abietane-type diterpenoids 1–3, named callicapoic acids M 3–5 (Figure 1) were isolated from the dried whole herb of Callicarpa macrophylla Vahl. This paper deals with their structural elucidation and anti-inflammatory activity against RAW 264.7 macrophage cells line determined by means of MTT assays.

2. Results and Discussion

Compound 1 (callicapoic acid M3), obtained as white amorphous powder (from acetone), gave the molecular formula C20H36O3, as deduced from the HR-ESI-MS peak at m/z 325.1958 [M + H]+. Its IR spectrum showed hydroxyl (3401 cm⁻¹) and carboxyl group (1700 cm⁻¹) absorptions, and the UV spectrum showed the presence of an aromatic moiety with maxima at 211 and 250 nm. The 1H-NMR spectrum of 1 (Table 1) revealed an isopropenyl group [δH 2.11 (3H, s), 5.02 (1H, s), 5.32 (1H, s), one methyl group [δH 1.16 (3H, s)], a pair of methylene protons [δH 3.48, 4.16 (each 1H, d, J = 5.9 Hz)] bearing an oxygen function, and a 1,2,4 substitution pattern for the aromatic C ring was easily recognized from inspection of other 1H-NMR signals [δH 7.21, 8.25 (each 1H, d, J = 8.2 Hz) and 7.12 (1H, s)] and of other C ring aromatized compounds [11–13].

The 13C-NMR spectrum of 1 (Table 1) confirmed the presence of a benzene ring, in addition to two methyl, six methylene, one methine, one carboxyl, two olefinic carbon signals, and two quaternary carbon signals, as well as an additional methylene carbon (δC 71.4) attached to an oxygen function. From this information, compound 1 was inferred to be an abietane-type diterpene by comparison with the literature identification data of similar typical abietanes, like the triptobenzenes A–K isolated from T. wilfordii var. Regelii [14] and T. hypoglaucaum [15].

In the HMBC spectrum of 1 (Figure 2), the methyl proton signal (δH 2.11) of the isopropenyl group correlated with the carbon signals at δC 138.3 (C-13), 142.9 (C-15), and 111.7 (C-16), and the proton signal δH 8.25 (H-12) correlated with signals at δC 142.9 (C-15), 147.2 (C-9), and 126.0 (C-14). In turn, the proton signal δH 7.12 (H-14) correlated with the signals at δC 142.9 (C-15), 147.2 (C-9), 123.1 (C-12), and 31.6 (C-7). From these observations, the location of the isopropenyl group at C-13 was inferred. Further, the proton signals at δH 3.48, 4.16 (H₂-18) correlated with the signals at δC 32.0 (C-3), 49.9 (C-4), 47.7 (C-5), and 181.1 (C-19), implied the hydroxyl group in 1 could be assigned to position C-18. In the NOESY spectrum, the proton signals at δH 3.48, 4.16 (H₂-18) showed correlations with the proton signal at δH 1.69 (H-5), and showed no correlation with the methyl proton signals at δH 1.16 (H₃-20). Thus, the configuration of the hydroxy methylene at C-4 was confirmed to be α, and the methyl at C-10 was confirmed to be β. The above evidence allowed identification of compound 1 as 18-hydroxy-8,11,15-abietatetraen-19-oic acid.

Callicapoic acid M4 (2) was isolated as a white amorphous powder (from acetone). Its molecular formula was established as C₂₀H₃₆O₃ by the HR-ESI-MS signal at m/z 329.1927 [M + Na]+. The 1D (Table 1) and 2D NMR data of 2 showed the presence of an abietane-type diterpene skeleton, which indicated its structure be similar to that of 1, except for a side chain isopropyl group, suggested by the appearance of characteristic resonances at δH 1.21 (3H, s), 1.22 (3H, s), and 2.82 (1H, m); and δC 24.0, 24.0 and 33.4. In the HMBC spectrum (Figure 2), the methine proton signal (δH 2.82) of the isopropyl group correlated with C-12 (δ 124.1), C-13 (δ 145.8) and C-14 (δ 126.8). In turn, the proton signals at δH 6.99 (H-12) and 6.87 (H-14) correlated with the methine signal at δC 33.4 (C-15). From these observations, the location of the side chain of isopropyl group at C-13 was inferred. The relative configuration of 2 was established by a NOESY experiment, and 4-CH₂OH, 10-CH₃ were found to be the same as those of 1. Accordingly, compound 2 was identified as 18-hydroxy-8,11,13-abietatetraen-19-oic acid (Figure 1) and named callicapoic acid M4 (2). Compound 2 is the C-4 epimer of a known compound described in the literature [16].
Callicapoic acid M5 (3), a white amorphous powder, possessed a molecular formula of C_{21}H_{26}O_{5} according to its HR-ESI-MS signal at m/z 359.1873 [M + H]^+ . The 1D (Table 1) and 2D NMR data of 3 clearly revealed the presence of an abietane-type diterpene skeleton, and also indicated its structure be similar to that of 1 except for the two side chains. The differences between them were that the isopropenyl was replaced by an acetyl at C-13, and the hydroxyl at C-18 was acetylated. The first difference had characteristic resonances at δ_H 2.56 (3H, s); and δ_C 26.7, 198.3 which implied the presence of an acetyl, while HMBC correlations from protons of methyl at δ_H 2.56 to C-13 (δ 134.8) suggested that...
acetyl was connected to C-13. The second difference in the characteristic resonances at δ_H 2.06 (3H, s); and δ_C 20.9, 171.0 also implied the presence of an acetyl, HMBC correlations from the methylene protons at δ_H 4.10 and 4.49 (H-18) to C-21 (δ 171.0) suggested that the C-18 hydroxyl was acetylated. The relative configuration of 10-CH_3 was established as β-oriented, 4-CH_2O- was confirmed to be α-oriented by a NOESY experiment, the same as those of 1 and 2. Therefore, compound 3 was thus identified as 15-acetyl-19-carbethoxy-8,11,13-abietatetraen-18-oic acid (Figure 1).

Nitric oxide (NO) plays an important role in the inflammatory process [17]. The inhibition of NO release may be effective as a therapeutic agent in the inflammatory diseases [18]. Therefore, compounds 1 to 3 were tested for the inhibitory activity against the production of NO in RAW 264.7 stimulated by lipopolysaccharide (LPS). The anti-inflammatory assay was carried out according to the procedure described previously. The results were summarized in Table 2 and indicated that all the three compounds could significantly inhibit NO introduction in LPS-activated RAW 264.7 macrophage cells.

### Table 2. Anti-inflammatory effects of compounds 1–3 from Callicarpa macrophylla Vahl. on LPS-induced RAW264.7 macrophages.

| Compound | Conc. (µM) | NO Inhibitory Rate (%) | Cell Viability (%) |
|----------|-----------|------------------------|-------------------|
| 1        | 50        | 37.89 ± 3.28 a         | 94.26 ± 7.78      |
| 2        | 50        | 34.47 ± 4.35 a         | 93.58 ± 2.16      |
| 3        | 50        | 40.13 ± 2.45 a         | 94.76 ± 3.91      |
| Z, Z’-6,6’,7,7’,3’α-Diligustilide b | 50 | 69.37 ± 6.08 a | 108.50 ± 1.90 |

a The three 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.

### 3. Experimental

#### 3.1. General Procedures

Optical rotations were determined on a 241 MC polarimeter (Perkin-Elmer, Waltham, MA, USA). UV spectra were obtained on Perkin Elmer Lambda 35 UV/VIS Spectrometer. IR spectra were recorded on a IFS 55 spectrophotometer (Bruker, Billerica, MA, USA). The NMR data were recorded on a Bruker AV-600 spectrometer. The HR-ESI-MS data were obtained on a LCT Premier XE time-of-flying mass spectrometer (Waters, Milford, MA, USA). Chromatography was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Group Co. Ltd., Qingdao, China) and ODS (30–50 µm; Tianjin Mical Reagent Co., Tianjin, China). Prep. HPLC was performed on a system comprised of a L-7110 pump and a L-7420 UV spectrophotometric detector set at 203 nm (Hitachi, Tokyo, Japan). A YMC C_18 reversed-phase column (5 µm, 10 × 250 mm; flow rate 2.0 mL/min) was used.

#### 3.2. Plant Material

Dried whole herb of Callicarpa macrophylla Vahl. were collected in Yunnan Province, China, in August 2011 and identified by Jincai Lu (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University). A voucher specimen (No. 20110801) was deposited in the Research Department of Natural Medicine, Shenyang Pharmaceutical University.

#### 3.3. Exaction and Isolation

Dried whole herb of Callicarpa macrophylla Vahl. (4.5 kg) was extracted with 50 L 95% EtOH (×3) under reflux conditions for three hours to give a crude extract, which was suspended in H_2O and successively extracted with petroleum ether (PE), CHCl_3 and EtOAc to yield a PE-soluble fraction (17.2 g), a CHCl_3-soluble fraction (261.7 g) and an EtOAc-soluble fraction (55.3 g). A part of the CHCl_3-soluble fraction (70 g) was subjected to column chromatography (CC, silica gel, gradient of PE-acetone 100:1–0:100) to afford 46 fractions (numbered 1–46). Fractions 30–36 (5.2 g) were separated by CC (ODS, MeCN–H_2O 30:70, MeCN–H_2O 40:60) to yield fraction ods40, which was
further subjected by semi-preparative reversed-phase HPLC (MeOH/H$_2$O with 0.2% HCO$_2$H, (62:38, v/v) as mobile phase, flow rate 3.0 mL/min, wavelength 203 nm), to afford 1 (10.8 mg), 2 (16.4 mg) and 3 (11.2 mg), respectively.

**Callicapoic Acid M3 (1).** White amorphous powder, $[\alpha]_{D}^{20} +57.0$ (MeOH, c 0.37). UV (MeOH) $\lambda_{\text{max}}$: 211, 250 nm. IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 3401, 2930, 1700 and 1561. $^1$H-NMR and $^{13}$C-NMR spectral data are shown in Table 1. HR-ESI-MS $m/z$: 315.1958 (C$_{20}$H$_{27}$O$_3$ $^+$, [M + H]$^+$, calc. 315.1960).

**Callicapoic Acid M4 (2).** White amorphous powder, $[\alpha]_{D}^{20} +22.2$ (MeOH, c 0.10). UV (MeOH) $\lambda_{\text{max}}$: 205, 266 nm. IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 3426, 2923, 1630 and 1384. $^1$H-NMR and $^{13}$C-NMR spectral data are shown in Table 1. HR-ESI-MS $m/z$: 339.1927 (C$_{20}$H$_{28}$NaO$_3$ $^+$, [M + Na]$^+$, calc. 339.1936).

**Callicapoic acid M5 (3).** White amorphous powder, $[\alpha]_{D}^{20} +41.2$ (MeOH, c 0.24). UV (MeOH) $\lambda_{\text{max}}$: 209, 258 nm. IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$): 3411, 2927, 1723, 1658 and 1351. $^1$H- and $^{13}$C-NMR spectral data are shown in Table 1. HR-ESI-MS $m/z$: 359.1873 (C$_{21}$H$_{27}$O$_5$ $^+$, [M + H]$^+$, calc. 359.1858).

### 3.4. Anti-Inflammatory Assay

The anti-inflammatory activities of compounds 1 to 3 were evaluated using LPS-induced RAW 264.7 cells. RAW 264.7 macrophages cells (8 $\times$ 10$^4$ cells/well) were suspended in 100 $\mu$L of DMEM supplemented with 10% fetal bovine serum, and precultured in 96-well microplates and 5% CO$_2$ in air for 12 h at 37 $^\circ$C, then test compounds (50 $\mu$mol/L) were cultured, and were treated with or without 1 $\mu$g/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 $\mu$L, 5 mg/mL in PBS) solution was added to the wells. After 4 h of culturing, the medium was removed and DMSO 100 $\mu$L/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’-$\alpha$-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%). MTT experiments were repeated three times. The NO inhibitory ratio (%) was calculated by the following formula:

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

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### Author Contributions:
Zhen-Hui Wang designed the experiments and wrote the paper; Chao Niu and De-Jun Zhou performed the experiments; Ji-Chuan Kong and Wen-Kui Zhang modified the paper. All authors read and approved the manuscript.

### Conflicts of Interest:
The authors declare no conflict of interest.

### References
1. Liu, M.S. *Introduction of Li Nationality Medicine; People’s Medical Publishing House: Beijing, China, 2008; pp. 74–78.*
2. Wang, Y.J.; Yang, Y.F.; Gao, D. Advances in studies on chemical constituents in plants of *Callicarpa Linn.* and their bioactivities. *Chin. Tradit. Herb. Drugs* 2008, 39, 133–138.
3. Dong, L.; Liu, M.S.; Wang, J.H. Lipophilic compounds of *Callicarpa nudiflora* Hook. Et Arn. *Chin. J. Med. Chem.* 2009, 19, 371–374.
4. Dong, L.; Wang, J.H.; Liu, M.S. Phenolic acid constituents of leaves from *Callicarpa nudiflora* Hook. Et Arn. *J. Shenyang Pharm. Univ.* 2010, 27, 290.
5. Cai, J.P.; Dong, L.; Guan, W.W.; Liu, M.S. Research progress of *Callicarpa nudiflora*. Drugs Clin. **2012**, 27, 60–63.

6. Pan, P.; Sun, Q.S. The chemical composition of *Callicarpa macrophylla* Vahl. Et Arn. J. Shenyang Pharm. Univ. **2006**, 23, 565–567.

7. Talapatra, S.K.; Polley, M.; Talapatra, B. Terpenoids and Related Compounds. Part 32. Calliphyllin, a New Diterpene from the Leaves of *Callicarpa macrophylla*. Cheminform **1995**, 26, 40. [CrossRef]

8. Goel, M.K.; Kukreja, A.K.; Singh, A.K.; Khanuja, S.P.S. In vitro plant growth promoting activity of phyllocladane diterpenoids isolated from *Callicarpa macrophylla* Vahl. in shoot cultures of *Rauwolfia serpentine*. Nat. Prod. Commun. **2007**, 2, 799–802.

9. Xu, J.; Li, S.; Sun, X.C.; Ma, J.; Liu, F.; Tong, L.; Lee, D.; Ohizumi, Y.; Tuerhong, M.; Guo, Y.Q. Diterpenoids from *Callicarpa kwangtungensis* and their NO inhibitory effects. *Fitoterapia* **2016**, 113, 151–157. [CrossRef] [PubMed]

10. Zhang, L.; Dong, L.; Huang, J.; Liu, M.S.; Li, G.Y.; Zhang, C.; Zhang, K.; Wang, J.H. 3,4-seco-Labdane diterpenoids from the leaves of *Callicarpa nudiflora* and their inhibitory effects on nitric oxide production. *Fitoterapia* **2013**, 83, 218–223. [CrossRef] [PubMed]

11. Lee, C.K.; Fang, J.M.; Cheng, Y.S. Abietanes from leaves of *Juniperus chinensis*. *Phytochemistry* **1994**, 35, 983–986. [CrossRef]

12. Su, W.C.; Fang, J.M.; Cheng, Y.S. Diterpenoids from leaves of *Cryptomeria japonica*. *Phytochemistry* **1996**, 41, 255–261.

13. Cambie, R.C.; Mathai, K.P. The structure of pododacric acid. *Aust. J. Chem.* **1971**, 24, 1251–1256. [CrossRef]

14. Shishido, K.; Nakano, K.; Wariishi, N.; Tateishi, H.; Omodani, T.; Shibuya, M.; Goto, K.; Ono, Y.; Takaishi, Y. *Tripterygium wilfordii* var. *regelii* which are interleukin-1 inhibitors. *Phytochemistry* **1994**, 35, 731–737.

15. Duan, H.Q.; Kawazoe, K.; Bando, M.; Kido, M.; Takaishi, Y. Di- and triterpenoids from *Tripterygium hypoglaucum*. *Phytochemistry* **1997**, 46, 535–543. [CrossRef]

16. Shmidt, E.N.; Khan, V.A.; Isaeva, Z.A.; Drebushchak, T.D.; Dubovenko, Z.V.; Kemertelidze, E.P.; Pentegovka, V.A. Terpenoids of the oleoresins of picea orientalis and abies normanniana growing in the Caucasus. *Khimiya Prirodnykh Soedinenii* **1982**, 2, 189–194.

17. Calixto, J.B.; Otuki, M.F.; Santos, A.R. Anti-inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kB (NF-kB). *Planta Med.* **2003**, 69, 973–983. [PubMed]

18. Lee, H.J.; Kin, N.Y.; Jang, M.K.; Son, H.J.; Kim, K.M.; Sohn, D.H.; Ryu, J.H. A sesquiterpene, dehydrocostus lactone, inhibits the expression of inducible nitric oxide synthase and TNF-α in LPS-activated macrophages. *Planta Med.* **1999**, 65, 104–108. [CrossRef] [PubMed]

**Sample Availability:** Samples of the compounds are available from the authors.

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