New Constituents with iNOS Inhibitory Activity from Mycelium of *Antrodia camphorata*

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Key words
- *Antrodia camphorata*
- Polyporaceae
- mycelium
- antroquinonol B
- iNOS

Abstract

In continuing our investigation on the bioactive constituents of mycelium of *Antrodia camphorata*, antroquinonol B (**1**), 4-acetyl-antroquinonol B (**2**), 2,3-(methylenedioxy)-6-methylbenzene-1,4-diol (**3**) and 2,4-dimethoxy-6-methylbenzene-1,3-diol (**4**) along with antrodin D (**5**) were isolated by the guidance of an inducible nitric oxide synthase (iNOS) inhibitory assay and identified on the basis of their spectroscopic analysis. The effect of these compounds on the inhibition of NO production in lipopolysaccharide (LPS)-activated murine macrophages was further evaluated. Compounds **4** and **5** significantly inhibited NO production without any cytotoxicity, the IC_{50} values being 32.2 ± 0.1 and 26.3 ± 1.6 µg/mL, respectively. Compounds **1** and **2** possessed greater effects on NO inhibition, with IC_{50} values of 16.2 ± 0.8 and 14.7 ± 2.8 µg/mL, respectively, but displayed cytotoxicity at considerably higher concentrations. Compound **3** showed the lowest percent cell viability of 45.5 ± 1.8% as observed in treated cells at a concentration of 16.8 µg/mL.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

The wild-collected fruiting body of *Antrodia camphorata* Wu, Ryvarden & Chang (Polyporaceae, Aphyllophorales), has long been used as a folk remedy for the treatment of diarrhea, abdominal pain, hypertension, itching of the skin, and liver cancer in Taiwan [1]. So far, the fruiting body cannot be mass cultivated for commercialization due to its extremely slow growth rate and high host specificity for the endangered tree *Cinnamomum kanehira* Hay (Lauraceae). The mycelium of *A. camphorata* has thus become industrialized as a health food for the past few years, and it was found to exhibit bioactivities in vasorelaxation [2], immunomodulation [3] and in protection of oxidative damage [4], and was active as an anticancer agent and against hepatitis B virus [5]. Besides, all the tested extracts of *A. camphorata* exerted anti-inflammatory effects by suppression of inflammatory mediators such as NO through reducing inducible nitric oxide synthase (iNOS) expression in activated macrophages or microglia [6]. However, it is still unclear which active ingredients of *A. camphorata* are responsible for regulating NO production. Overproduction of NO is involved in inflammatory pathogenesis, including carcinogenesis [7], autoimmunity [8] and atherogenesis [9]. The appropriate adjustment of NO release and compromising NO-associated tissue injuries may have significant therapeutic intervention in the progress of inflammatory processes. The present study, therefore, was designed to isolate the active principles from the mycelium of *A. camphorata*, and to assess their effects on NO production induced by lipopolysaccharide (LPS)-activated RAW 264.7 cells.

Materials and Methods

General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. ^{1}H- and ^{13}C-NMR spectra were acquired on a Bruker DMX-500 SB spectrometer. Mass spectra were obtained using a Thermo Finnigan LCQ-Duo spectrometer. IR spectra were recorded on a JASCO FT/IR 4100 spectrometer. UV spectra were measured on a Thermo Helios α spectrophotometer.

Table 1 ^{1}H-NMR spectroscopic data (CD_3OD, 500 MHz) for compounds **1–4** [δ in ppm, mult. (*J* in Hz)].

Position	1[a]	2[a]	3	4
1				
2				
3				
4	4.32 d (3.2)	5.73 d (3.1)		
5	1.68 m	1.93 m	6.15 s	6.43 s
6	2.48 dq (11.4, 6.9)	2.51 dq (10.7, 6.9)		
7	2.23	2.28	2.08 s	2.10 s
8	5.24	5.14 t (6.9)	5.83 s	3.78 s
9				3.74 s
10	2.08 m	2.06 m		
11	2.16 m	2.17 m		
12	5.25	5.29 t (6.4)		
13				
14	2.38 dd (13.9, 7.0), 2.25	2.40 dd (13.9, 7.1), 2.27		
15	4.68 m	4.70 m		
16	2.20 1.98 m	2.22 1.98 m		
17	2.73 m	2.75 m		
18				
19	1.22 d (7.3)	1.22 d (7.3)		
20	1.66 s	1.57 s		
21	1.67 s	1.67 s		
22	1.14 d (6.9)	1.17 d (6.9)		
23	3.58 s	3.61 s		
24	4.05 s	3.98 s		
-OAc		2.08 s		

[a] Signals without multiplicity were overlapped and obtained from COSY or HMQC spectra.

Table 2 ^{13}C-NMR spectroscopic data (CD_3OD, 125 MHz) for compounds **1–4** (δ in ppm).

Position	1[a]	2[a]	3[a]	4[a]
1	199.6 s	199.1 s	133.2 s	143.5 s
2	137.5 s	138.3 s	137.4 s	137.5 s
3	164.7 s	160.6 s	134.5 s	139.0 s
4	67.6 d	70.4 d	134.7 s	142.5 s
5	45.7 d	44.2 d	112.7 d	110.7 d
6	41.5 d	42.5 d	121.5 s	115.3 s
7	28.2 t	28.0 t	15.8 q	15.7 q
8	123.2 d	122.5 d	102.2 t	60.8 q
9	138.1 s	138.8 s		57.5 q
10	40.6 t	40.5 t		
11	27.4 t	27.1 t		
12	129.3 d	129.2 d		
13	131.8 s	131.7 s		
14	45.9 t	45.9 t		
15	78.9 d	78.8 d		
16	35.7 t	35.6 t		
17	35.1 d	35.0 d		
18	182.7 s	182.7 s		
19	16.0 q	16.0 q		
20	16.2 q	16.2 q		
21	16.5 q	16.5 q		
22	12.9 q	13.1 q		
23	60.8 q	61.1 q		
24	59.1 q	60.3 q		
$\underline{C}H_3CO$-		20.9 q		
$CH_3\underline{C}O$-		171.4 s		

[a] Multiplicities were obtained from DEPT experiments.

Fermentation of *Antrodia camphorata*

A. camphorata Wu, Ryvarden & Chang (strain *AC* Hsuehshan), isolated and identified by Mr. Maw-Jer Wang, the manager of a private Chiu-Chyy Biotechnology Laboratory at Taipei County, was inoculated into Erlenmeyer flasks (1.0 L), each containing 10.0 g glucose (Merck), 2.0 g Bacto yeast extract (BD), 10.0 g Difco agar (BD), a small amount of multivitamin (Wyeth) and deionized water (total volume of 500 mL). Solid culture was conducted under sterile conditions at 28 °C for 4 weeks.

Extraction and isolation

The mycelium together with the medium from the above fermentation was dried and ground into a powder (350.0 g), and extracted three times with 1 L MeOH by stirring (4800 rpm) at room temperature for 6 h. The crude extract was then filtered and concentrated by rotatory evaporation (38.0 g). Subsequently, this residue was redissolved in 65 : 35 MeOH : H_2O, and partitioned two times with equal volumes of *n*-hexane and EtOAc, successively, to give three fractions soluble in *n*-hexane (5.6 g), EtOAc (12.1 g) and aqueous MeOH (22.4 g). The EtOAc soluble fraction was redissolved in 25 mL MeOH, and applied onto a Sephadex LH-20 column (3 cm i.d. × 65 cm; GE Healthcare Bio-sciences AB) eluted by MeOH with a flow rate of 2.5 mL/min. Each subfraction (18 mL) collected was checked for its compositions by TLC (Si 60 F_{254}, 0.2 mm; Merck) using EtOAc/acetic acid/H_2O (85 : 10 : 10, v/v/v) for development, and observation under UV 254 nm was used in the detection of compounds with similar chromophores. Subsequently, subfractions containing similar compounds were combined to give two major portions I (#subfr. 7–12, 8.0 g) and II (#subfr. 13–21, 4.1 g). All the above fractionated samples were subjected to biological tests, and among them, portions I and II with significant biological activities were further chromatographed on HPLC. HPLC of portion I on a semi-preparative reversed-phase column (Thermo BDS Hypersil C18; 10 × 250 mm) with acetonitrile/H_2O (1 : 1, v/v) as eluent, 2 mL/min, afforded **1** (3.4 mg), **2** (10.5 mg) and **5** (12.0 mg). HPLC of portion II on the same column with acetonitrile/H_2O (1 : 4, v/v) as eluent, 2 mL/min, afforded **3** (16.5 mg) and **4** (11.0 mg).

Antroquinonol B (**1**): Colorless oil; $[\alpha]_D^{23}$: + 46.1 (*c* 0.5, MeOH); UV (MeOH): λ_{max} (log ε) = 267 (4.1) nm; IR (ZnSe): ν_{max} = 3421, 1769, 1662, 1622, 1455, 1356, 1235 cm^{-1}; ^{1}H-NMR data: see **Table 1**; ^{13}C-NMR data: see **Table 2**; ESI-MS: *m*/*z* = 443 [M + Na]$^+$; HR-ESI-MS: *m*/*z* = 443.2412 [M + Na]$^+$; calcd. for $C_{24}H_{36}O_6$ + Na^+: 443.2410.

4-Acetylantroquinonol B (**2**): Colorless oil; $[\alpha]_D^{23}$: + 113.2 (*c* 0.5, MeOH); UV (MeOH): λ_{max} (log ε) = 263 (4.0) nm; IR (ZnSe): ν_{max} = 1771, 1747, 1671, 1628, 1456, 1363, 1233 cm^{-1}; ^{1}H-NMR data: see **Table 1**; ^{13}C-NMR data: see **Table 2**; ESI-MS: *m*/*z* = 485 [M + Na]$^+$; HR-ESI-MS: *m*/*z* = 485.2518 [M + Na]$^+$; calcd. for $C_{26}H_{38}O_7$ + Na^+: 485.2515.

2,3-(Methylenedioxy)-6-methylbenzene-1,4-diol (**3**): Colorless crystals; mp 135–136 °C; UV (MeOH): λ_{max} (log ε) = 329 (3.5) nm; IR (ZnSe): ν_{max} = 3338, 1645, 1610, 1505, 1223, 1089, 1018 cm^{-1}; ^{1}H-NMR data: see **Table 1**; ^{13}C-NMR data: see **Table 2**; ESI-MS: *m*/*z* = 169 [M + H]$^+$; HR-ESI-MS: *m*/*z* = 169.0496 [M + H]$^+$; calcd. for $C_8H_8O_4$ + H^+: 169.0501.

2,4-Dimethoxy-6-methylbenzene-1,3-diol (**4**): Colorless crystals; mp 130–131 °C; UV (MeOH): λ_{max} (log ε) = 286 (3.5) nm; IR

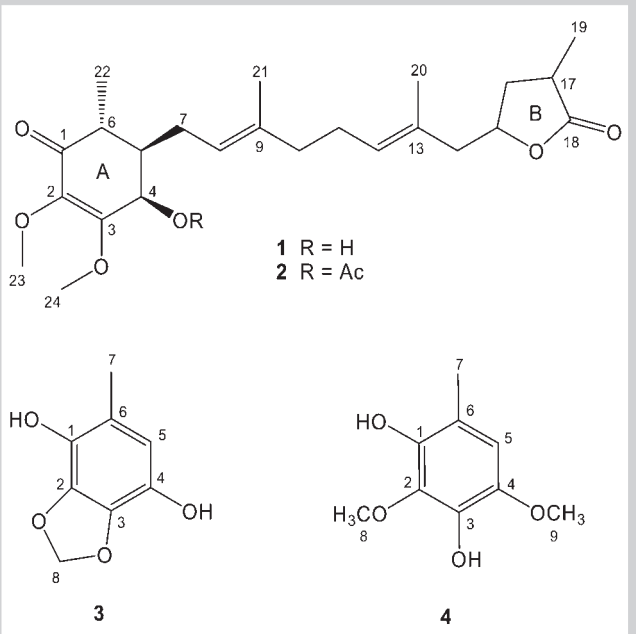

Fig. 1 Chemical structures of compounds **1**–**4** identified in this report.

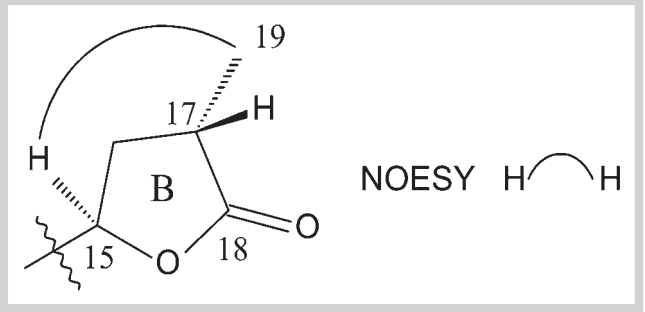

Fig. 2 One key NOESY correlation in ring B of **1**.

(ZnSe): ν_{max} = 3416, 1620, 1509, 1318, 1190, 1070 cm^{-1}; ^{1}H-NMR data: see **Table 1**; ^{13}C-NMR data: see **Table 2**; ESI-MS: *m/z* = 207 [M + Na]$^+$; HR-ESI-MS: *m/z* = 207.0630 [M + Na]$^+$; calcd. for $C_9H_{12}O_4$ + Na^+: 207.0633.

Nitrite measurement and cell viability assay

Nitrite formation and cell viability were measured with the Griess reagent and the redox indicator alamar Blue, respectively [10]. To assess the effects on LPS-induced NO production, the *n*-hexane soluble fraction, the aqueous MeOH soluble fraction, portions I – II and compounds **1**–**5**, two positive control N^{ω}-nitro-L-arginine (L-NNA, a non-selective NOS inhibitor) and aminoguanidine (a specific inhibitor of iNOS) or vehicle (0.1%, DMSO) was added in the presence of LPS (200 ng/mL) to the RAW 264.7 cells. Both inhibitors were purchased from Sigma-Aldrich Chemical Co. and the purity of each compound was more than 98%.

Statistical analyses

Comparison of the concentration and treatment dependencies was performed using ANOVA, followed by *post hoc* comparisons using the Newman-Keuls test as appropriate. The average IC_{50} was determined by data fitting with GraFit (Erithacus Software).

Supporting information

Original spectra for compounds **1, 3** and **4** are available as Supporting Information.

Results and Discussion

Among the isolated fractionated samples from crude extracts of *A. camphorata*, the EtOAc soluble fraction yielded two subfractions I and II with significant iNOS inhibitory activities, which were finally purified by HPLC to give four new compounds **1**–**4** (**Fig. 1**) together with a known alkaloid **5**. Compound **5** was identified as antrodin D, obtained previously from the same fungus [11].

The molecular formula of **1**, $C_{24}H_{36}O_6$, was established through analysis of its ^{13}C-NMR and HR-ESI-MS data. Its IR spectrum revealed the presence of a conjugated carbonyl group (1662 cm^{-1}), a γ-lactone carbonyl group (1769 cm^{-1}) and a hydroxy group (3421 cm^{-1}). The ^{1}H-NMR spectrum (**Table 1**) of **1** exhibited signals compatible with those of antroquinonol [12] except for a secondary methyl group at δ_H = 1.22 (d, *J* = 7.3 Hz, H_3-19), a methylene group at δ_H = 1.98, 2.20 (m, H_2-16) and two methine protons at δ_H = 2.73 (m, H-17) and 4.68 (m, H-15), which were also reflected in their δ_C differences of C-15 – C-19 between antroquinonol and **1** (**Table 2**). Analysis of all 2D NMR data including COSY, HSQC and HMBC (see Supporting Information) suggested that the C-15 – C-19 partial structure was replaced by a γ-lactone ring with a methyl branch at C-17 instead of a 2-methylbut-2-ene moiety in antroquinonol. The configurations of both Δ^8 and Δ^{12} bonds were *E* as evidenced from the δ_C of C-20 and C-21 at, respectively, δ_C = 16.2 and 16.5 in contrast to those of the *Z* configurations at approximately δ_C = 25.0 [13]. The relative configuration of ring A of **1** was consistent with that of antroquinonol. The isolated relative stereochemistry of ring B was elucidated with a NOESY spectrum in which a cross-peak between H-15 and H_3-19 indicated a *trans* configuration of H-15 and H-17 as shown in **Fig. 2**. Thus, **1** was concluded as the shown structure, and named antroquinonol B. When comparing the ^{1}H- (**Table 1**) and ^{13}C-NMR (**Table 2**) spectra of **2** with those of **1**, differences involved an additional acetyl group in **2** which was judged by signals of H-4 at δ_H = 5.73 (d, *J* = 3.1 Hz) in **2** instead of δ_H = 4.32 in **1**. That also corresponded to its IR spectrum, in which a hydroxy group in **1** was replaced by an acetyl group in **2**. After considering all NMR data, **2** was thus determined to be 4-acetylantroquinonol B.

Analysis of the IR spectra of **3** and **4** suggested that they both contained a benzene ring bearing a hydroxy functionality as evidenced from the interpretations of their ^{1}H- (**Table 1**) and ^{13}C-NMR (**Table 2**) data. After further analysis of their 2D NMR data including HMBC and NOESY (see Supporting Information), the locations of all functionalities borne by the benzene ring were determined. Thus, **3** and **4** were identified as the shown structures, which could be intermediates or degradation products of **1** and **2** due to their quite similar skeletons as the A ring of **1** and **2**. To our knowledge, two analogues of both **3** and **4**, 4,7-dimethoxy-5-methyl-1,3-benzodioxole [14] and tetramethoxybenzoyl chloride [15], have also been isolated from the same fungus.

The *n*-hexane soluble fraction, the aqueous methanol soluble fraction and subfractions I and II were first examined for their ef-

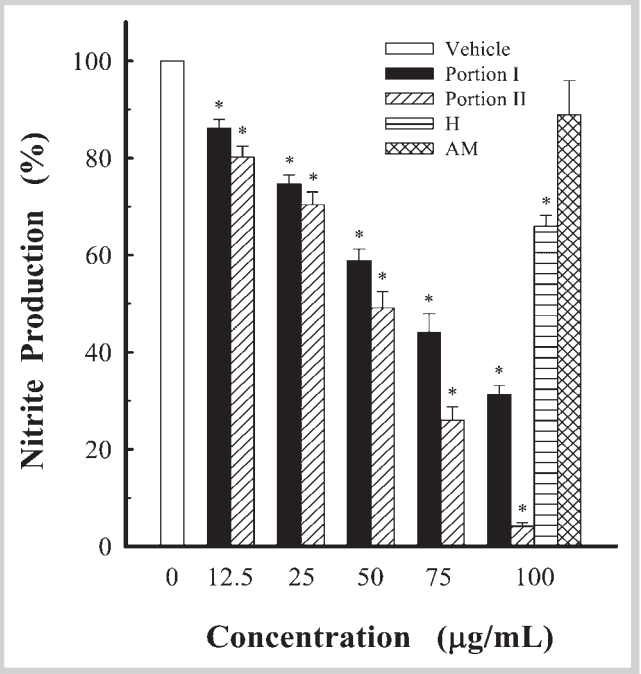

Fig. 3 The effects of H (*n*-hexane soluble) and AM (aqueous methanol soluble) fractions and portions I – II of *Antrodia camphorata* on nitrite production in LPS-activated RAW 264.7 cells. Vehicle representing 100% is equal to 36.5 ± 0.8 μM of nitrite produced in the medium per well. n = 6 in each group, * $p < 0.05$ when compared with vehicle-treated cell.

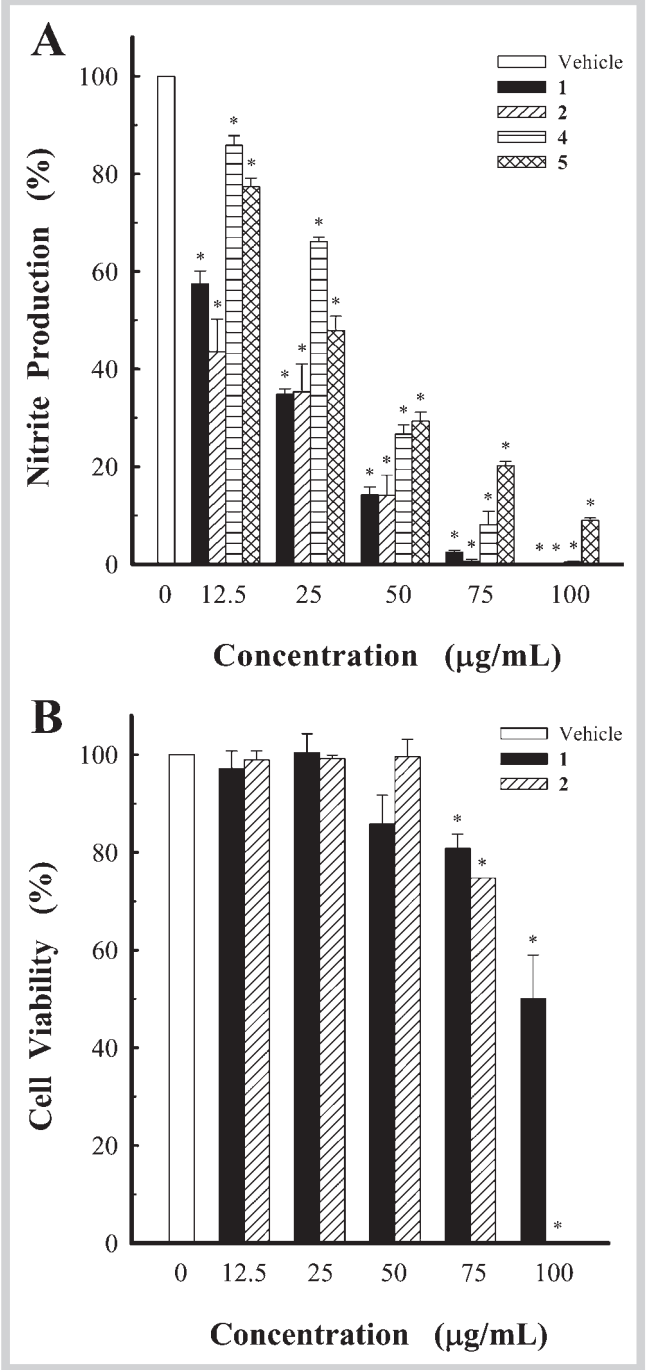

Fig. 4 The effects of the compounds isolated from *Antrodia camphorata* (12.5–100 μg/mL) on nitrite production (**A**) and cell viability (**B**) in LPS-activated RAW 264.7 cells. Vehicle representing 100% is equal to 35.0 ± 0.2 μM of nitrite produced in the medium per well. n = 6 in each group, * $p < 0.05$ when compared with vehicle-treated cell.

fect on LPS-induced NO production in RAW 264.7 cells (**Fig. 3**). Among them, subfraction II was the most potent, with an IC_{50} value of 40.8 ± 3.0 μg/mL. Cell viability testing showed that this subfraction exhibited no significant cytotoxicity at its effective concentration (data not shown). Subfraction I also concentration-dependently suppressed LPS-induced NO accumulation. Its IC_{50} was calculated as 65.0 ± 5.9 μg/mL. The percentage of surviving cells was 80.9 ± 8.1% at 100 μg/mL. The *n*-hexane soluble fraction moderately inhibited (34.0 ± 2.2%) nitrite production with 83.4 ± 3.6% cell viability. Although the aqueous methanol soluble fraction was not cytotoxic, it did not change the nitrite production. Under the same conditions, the inhibitory effects of aminoguanidine (100 μM) and L-NNA (100 μM) were 83.0 ± 1.5% and 43.5 ± 2.0%, respectively [16].

All isolates **1**–**5** significantly inhibited nitrite production induced by LPS in a concentration-dependent manner except for **3** (**Fig. 4A**). Compounds **4** and **5** did not affect cell viability at concentrations up to 100 μg/mL and showed potent inhibitory activity on nitrite production with IC_{50} values of 32.2 ± 1.0 and 26.3 ± 1.6 μg/mL, respectively. Compounds **1** and **2** showed comparably strong inhibition with IC_{50} values of 16.2 ± 0.8 and 14.7 ± 2.8 μg/mL, respectively. However, these two new compounds were cytotoxic at concentrations higher than 75 μg/mL (**Fig. 4B**). Cell survival was 49.9 ± 8.8% after treatment with **1** and no cell was alive after treatment with **2** at 100 μg/mL. Compound **3** appeared to obviously decrease nitrite production to 1.6 ± 0.1% at 16.8 μg/mL; however, this effect seemed to be related to its cytotoxicity. The percentage of surviving cells after treatment with **3** (16.8 μg/mL) was 54.5 ± 1.8%.

In summary, all five compounds isolated from the mycelium of *A. camphorata* substantially inhibited LPS-induced NO production in murine macrophages. These results provide scientific support for the traditional use of *A. camphorata*, namely treatment of inflammation. However, the further mechanism of these compounds on the inhibition of iNOS activation remains to be elucidated.

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