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Flavidanolides A and B from *Isodon flavidus*

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ABSTRACT

A phytochemical investigation on *Isodon flavidus* led to the isolation of flavidanolide A (**1**), a rearranged diterpenoid featuring a six/seven/five-membered tricyclic skeleton, together with flavidanolide B (**2**), an uncommon heterodimeric diterpenoid consisting of a norabietane and a *seco*-isopimarane monomeric units. Their structures were elucidated by extensive spectroscopic data and single-crystal X-ray diffraction analyses. Their plausible biosynthetic routes were also proposed. In the bioassay, flavidanolide B was found to exhibit good inhibitory effect against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW264.7 cells comparable to positive control pyrrolidinedithiocarbamate ammonium (PDTC), which provided evidence for the medicinal value of *I. flavidus* as a folk medicine for treating inflammatory diseases.

Diterpenoids with unique skeletons and various bioactivities have been constantly found in plants [1,2], especially *Isodon* species [3], indicating that this genus is a valuable plant source to identify lead molecules for drug discovery. Our research group has been dedicated to search for new diterpenes from the *Isodon* plants in Guizhou Province [4–8]. In our recent studies, several diterpenoids with unique skeletons have been reported. For example, amethinol A, possesses a six/five/seven-membered tricyclic system [9]; fladin A, a novel diterpenoid, contains an unprecedented cyclic ether group formed between C-4 and C-9 [10]; rubesanolides A and C, two novel abietanes, were identified with rare γ-lactone units formed between C-9 and C-20 for the former [11], and between C-8 and C-20 for the latter [12].

As a continuous part of the project to discover novel active compounds from *I. flavidus*, two novel diterpenoids (**1** and **2**) have been isolated from the twigs and leaves of this plant (Fig. 1). Compound **1** represents an unprecedented diterpenoid with a novel six/seven/five-membered carbon skeleton, and compound **2** is a heterodimeric diterpenoid belonging to a *seco*-isopimarane coupled with a norabietane unit featuring an unique ester linkage between C-9 and C-3′. The compounds have been evaluated for their antiinflammatory activities based on the inhibitory assays of nitric oxide (NO) production and tumor necrosis factor-α (TNF-α) expression in lipopolysaccharide (LPS)-induced RAW264.7 cells.

Compound **1**, colorless crystals, was deduced to have the molecular formula of $C_{20}H_{30}O_5$ from its high resolution electrospray ionization mass spectrometry (HR-ESI-MS) (*m/z* 373.1985 $[M+Na]^+$, calcd. for 373.1991), which is calculated to have 6 unsaturated degrees. The infrared (IR) spectrum displayed absorption bands at 3474 cm^{-1} belonging to hydroxy groups and 1730 and 1702 cm^{-1} belonging to carbonyl groups. The ^{13}C nuclear magnetic resonance (NMR), distortionless enhancement by polarization transfer (DEPT)-135° and DEPT-90° spectra of **1** (Table 1) exhibited 20 carbon signals, characterizing as four methyl carbons, six aliphatic methylene carbons, one oxymethine carbon, three non-oxymethine carbons, one ketone carbonyl carbon, one carboxylic carbon, and four aliphatic quaternary carbons including two oxygenated ones.

Starting with the two singlet methyl signals at δ_H 0.83 and 0.88, other key substructures of **1** could be established from the analysis of the 2D spectral data (Fig. 2A). In the ^1H–^1H correlation spectroscopy (COSY) spectrum, the correlation signals among the protons at δ_H 1.62/1.49, 2.10/1.87 and 1.45/1.24, the correlations among δ_H 1.80, 2.52/1.99 and 4.81, the correlations among δ_H 3.38 and 2.26/1.53, and the correlations among δ_H 1.73, 0.94 and 0.95 indicated the presences of the segments of a -$CH_2CH_2CH_2$-, a -$CHCH_2CH$-, a -$CHCH_2$-, and a -$CH(CH_3)_2$, respectively. Further analysis of the heteronuclear multiple bond correlation (HMBC) spectral data connected these segments together to form the diterpene skeleton. The -$CH_2CH_2CH_2$- segment is shown to have HMBC to the quaternary carbon at δ_C

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Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for **1** and **2** in CD_3OD (δ in ppm, J in Hz).

1			**2**					
No.	δ_H (J in Hz)	δ_C	No.	δ_H (J in Hz)	δ_C	No.	δ_H (J in Hz)	δ_C
1β	2.10 d (13.9)	29.1 CH_2	1	5.77 t (4.1)	123.6 CH	1′a	1.70 m	32.9 CH_2
1α	1.87, td (13.6, 4.8)					1′b	1.66 d (4.8)	
2β	1.62 qt (14.0, 3.7)	18.9 CH_2	2β	2.13 m	23.1 CH_2	2′a	2.35 m	30.3 CH_2
2α	1.49 overlap		2α	2.11 m		2′b	2.22 m	
3β	1.45 overlap	41.4 CH_2	3β	1.39 overlap	31.0 CH_2	3′	–	171.6 C
3α	1.24 td (13.6, 4.0)		3α	1.16 dd (13.2, 3.8)				
4	–	35.5 C	4	–	30.9 C	4′	–	147.5 C
5α	1.80 dd (10.8, 5.3)	44.7 CH	5α	1.55 d (5.9)	43.2 CH	5′α	2.19 m	49.7 CH
6β	1.99 overlap	24.1 CH_2	6β	1.67 d (5.2)	25.1 CH_2	6′β	2.23 m	29.3 CH_2
6α	2.52 dd (15.1,10.7)		6α	1.26 dd (13.4, 3.9)		6′α	1.81 m	
7α	4.81 d (7.2)	80.9 CH	7β	1.81 m	34.8 CH_2	7′	5.37 d (3.9)	121.5 CH
			7α	1.51 m				
8	–	82.4 C	8	–	74.8 C	8′	–	136.1 C
9	–	204.9 C	9	–	84.5 C	9′α	1.87 m	44.4 CH
10	–	62.0 C	10	–	138.7 C	10′	–	37.6 C
11	3.38 dd (12.1, 5.3)	56.8 CH	11β	2.37 m	20.7 CH_2	11′β	1.39 overlap	21.0 CH_2
			11α	2.21 m		11′α	1.55 m	
12β	1.53 dd (12.4, 5.2)	36.5 CH_2	12β	1.58 m	29.8 CH_2	12′β	1.39 overlap	36.3 CH_2
12α	2.26 t (12.3)		12α	1.51 m		12′α	1.51 m	
13	–	83.7 C	13	–	73.8 C	13′	–	37.1 C
14β	1.97d (13.9)	52.1 CH_2	14β	1.58 m	38.9 CH_2	14′β	1.94 dd (13.9, 2.4)	46.5 CH_2
14α	2.10d (13.9)		14α	1.75 d (14.4)		14′α	1.99 d (14.0)	
15	1.73 sept (6.8)	39.3 CH	15	1.58 m	38.3 CH	15′	5.81 dd (17.5, 10.7)	150.2 CH
16	0.94 d (6.8)	17.2 CH_3	16	0.92 d (6.9)	16.9 CH_3	16′a	4.94 dd (17.5, 1.2)	109.5 CH_2
						16′b	4.88 d (10.7, 1.2)	
17	0.95 d (6.8)	17.8 CH_3	17	0.93 d (6.9)	16.9 CH_3	17′	0.88 s	21.6 CH_3
18	0.88 s	31.4 CH_3	18	0.81 s	27.2 CH_3	18′a	4.87 d (5.7)	114.1 CH_2
						18′b	4.80 d (5.7)	
19	0.83 s	20.6 CH_3	19	0.87 s	28.1 CH_3	19′	1.82 s	23.7 CH_3
20		177.7 C				20′	0.92 s	17.1 CH_3

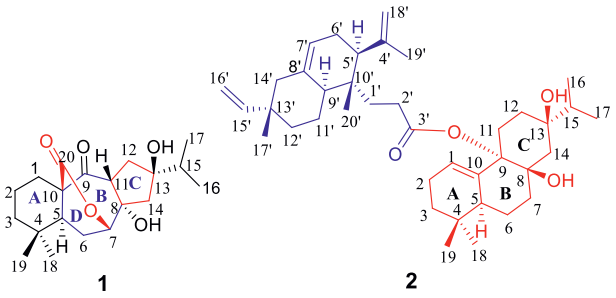


Fig. 1. Structures of **1** and **2**.

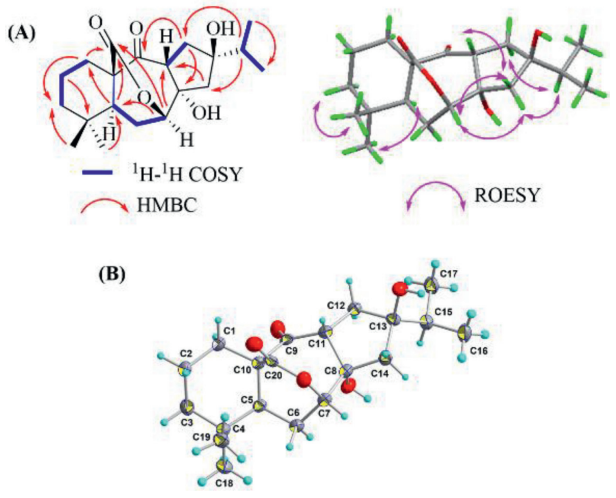


Fig. 2. 2D NMR correlations and X-ray data of **1**. (A) Key 2D NMR correlations of **1**. (B) Plot of X-ray crystallographic data for **1** (Displacement ellipsoids are drawn at the 30% probability level).

35.5 (C-4), which is then linked to the -$CHCH_2CH$- segment. The -$CHCH_2CH$- segment is found to have HMBC with the oxytertiary carbon at δ_C 82.4 (C-8), which is connected to the -$CHCH_2$- segment. The presence of HMBC of the -$CHCH_2$- segment to the oxy-tertiary carbon at δ_C 83.7 (C-13), and C-13 to the isopropyl group [-$CH(CH_3)_2$] further determined the connections of these segments as -$CH_2CH_2CH_2$-C$(CH_3)_2$-CH-CH_2-CH(O)-C(O)-CH-CH_2-C(O)-CH-$(CH_3)_2$. The segment of -$CH_2CH_2CH_2$- also showed HMBC with the quaternary carbon at δ_C 62.0 (C-10) and the ketone carbon at δ_C 204.9 (C-9). The carbonyl carbon C-9 is linked to the -$CHCH_2$- segment by observing HMBC between the segment and C-9. The presence of HMBC between the -$CHCH_2$- segment and the methylene group (-CH_2-) of C-14, between the -CH_2- and C-8, and between the -$CHCH_2CH$- segment and the carboxylic carbon at δ_C 177.7 (C-20) finally made all the connections of the carbons present in the diterpenoid. C-20 is connected to C-7 to form a lactone ring through the observation of HMBC of H-7 (δ_H 4.81). Taken together, the planar structure of **1** was assigned. In the rotating frame Overhauser effect spectroscopy (ROESY) spectrum (Fig. 2A), the presence of the correlation signals of H-11 with H-14β, and H-15 with H-12α/H-14α, indicated the same orientation of H-11 and 13-OH.

To completely determine its absolute configuration, **1** was crystallized in methanol to afford a crystal of the monoclinic space group $P2_12_12_1$, which was analyzed by X-ray crystallography. The final refinement on the Cu Kα data resulted in a Flack parameter of 0.02 (5) [13], allowing an unambiguous assignment of the absolute structure of **1** (Fig. 2B). The six chiral centers, C-5, C-7, C-8, C-10, C-11 and C-13, were thus determined as *S*, *S*, *S*, *R*, *R* and *R*, respectively. Accordingly, compound **1** was identified as an unprecedented diterpenoid bearing a novel six/seven/five-membered carbon skeleton that has not been reported before. We thus designate this type of diterpene structure as 'flavidane' and report **1** as the first example of flavidanes, which was given the trivial name flavidanolide A.

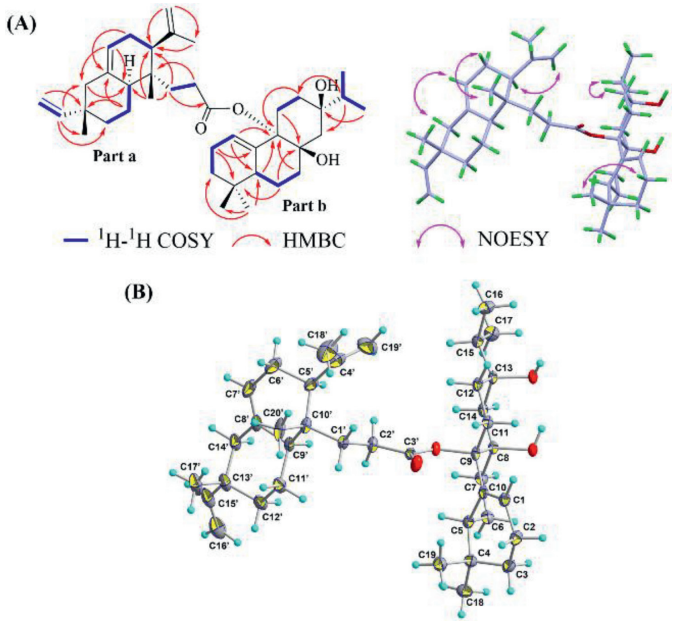


Fig. 3. 2D NMR correlations and X-ray data of **2**. (A) Key 2D NMR correlations of **2**. (B) Plot of X-ray crystallographic data for **2** (Displacement ellipsoids are drawn at the 30% probability level).

Compound **2** was obtained as colorless crystals. Its molecular formula of $C_{39}H_{60}O_4$ was deduced from the HR-ESI-MS (*m/z* 615.4379 $[M+Na]^+$, calcd for $C_{39}H_{60}O_4Na$, 615.4384). The IR spectral data indicated the presence of hydroxyl (3324 cm^{-1}) and ester carbonyl (1745 cm^{-1}) groups in **2**. The ^{1}H NMR spectrum (Table 1) coupled with the analysis of the ^{1}H–^{1}H COSY spectral data (Fig. 3A) exhibited the characteristic proton signals for five singlet methyls (δ_H 0.81, 0.87, 0.88, 0.92, 1.82), two olefinic protons from their corresponding trisubstituted double bond counterparts [(δ_H 5.37 (d), 5.77 (t)], three olefinic protons from a vinyl group [(δ_H 4.88 (d), 4.94 (d), 5.81 (dd)], two olefinic protons from a methylene group [(δ_H 4.80 (d), 4.87 (d)], and seven aliphatic protons from an isopropyl group [(δ_H 0.92 (dd, 3H); 0.93 (dd, 3H); 1.58 (m, 1H)]. The ^{13}C NMR and DEPT spectra (Table 1) revealed that **2** contains 39 carbon signals, which consist of one ester carbonyl carbon (δ_C 171.6), eight olefinic carbons (δ_C 114.1, 109.5, 121.5, 123.6, 136.1, 138.7, 147.5, 150.2), three aliphatic oxyquaternary carbons (δ_C 73.8, 74.8, 84.5), seven methyl carbons (δ_C 16.9 × 2, 17.1, 21.6, 23.7, 27.2, 28.1), thirteen methylene carbons (δ_C 20.7, 21.0, 23.1, 25.1, 29.3, 29.8, 30.3, 31.0, 32.9, 34.8, 36.3, 38.9, 46.5), four methine carbons (δ_C 38.8, 43.2, 44.4, 49.7), and three aliphatic quaternary carbons (δ_C 30.9, 37.1, 37.6). The aforementioned structure information indicated **2** as a diterpenoid dimer, which could comprise an isopimarane unit (part a, Fig. 3A), and a norabietane unit (part b, Fig. 3A) due to the presence of the characteristics vinyl and isopropyl groups.

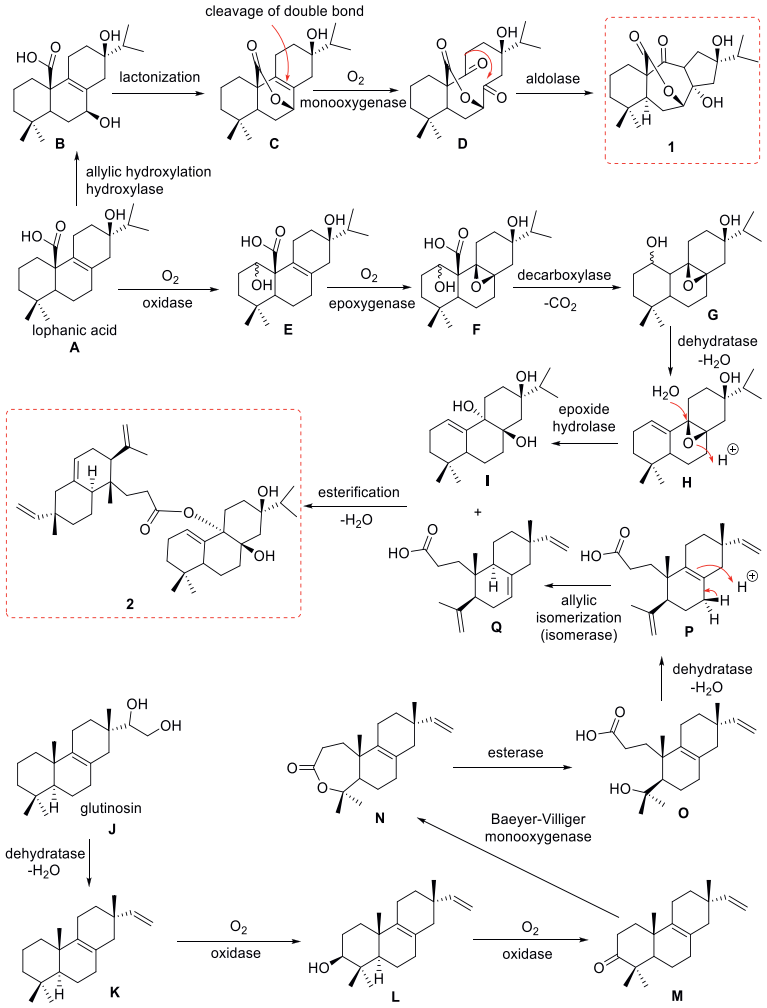
After careful comparison of the spectral data of **2** to those of the reported isopimaranes, the similar NMR pattern of 3,4-*seco*-isopimara-4(18),7,15-triene-3-oic acid [14] except for the obvious upfield shift of the carboxylic carbon (δ_C 171.6 *vs.* 181.0) is observed for **2**, indicating that the 3,4-*seco*-isopimara-4(18),7,15-triene-3-oic acid is the isopimarane unit of **2**, and the 3-oic acid is the linkage site to connect with the other abietane diterpenoid unit. The proposed isopimarane substructure was then verified by analyses of the 2D NMR correlation signals. Based on the ^{1}H–^{1}H COSY spectrum (Fig. 3A), the spin systems of H-5′/H-6′/H-7′ and H-9′/H-11′/H-12′ were established, which, in combination with the methyl and vinyl signals mentioned before, could be employed as the starting points for the structure elucidation. The presence of the key HMBC of H-1′ with C-3′, H-7′ with C-9′/C-14′, H-11′ with C-8′, H-15′ with C-12′/C-14′/C17′, H-16′ with C-13′, H-18′ with C-5′/C-19′, and H3–20′ with C-1′/C-5′/C-9′ (Fig. 3A) assigned the planar structure of the isopimarane unit as 3,4-*seco*-isopimara-4(18),7,15-triene-3-oic acid.

The planar structure of the abietane diterpenoid unit could also be determined by the 2D NMR spectral data. In addition to the characteristic isopropyl unit, the segments of $=CHCH_2CH_2$-, $-CHCH_2CH_2$- and $-CH_2CH_2$- were established based on the observation of the ^{1}H–^{1}H COSY correlation signals (Fig. 3A) among H-1 (δ_H 5.77), H-2 (δ_H 1.39, 1.16) and H-3 (δ_H 2.13, 2.11), among H-5 (δ_H 1.55), H-6 (δ_H 1.67, 1.26) and H-7 (δ_H 1.81, 1.55), and among H-11 (δ_H 2.37, 2.21) with H-12 (δ_H 1.58, 1.51), respectively. Ring A (Fig. 1) with two methyls substituted at C-4 was determined according to the presence of the key HMBC signals of H-1 with C-5 (δ_C 43.2), H-2 with C-4 (δ_C 30.9) and C-10 (δ_C 138.7), and H-18 with C-3 (δ_C 31.0), C-5 and C-19 (δ_C 28.1). The presence of HMBC signals of H-1/H-5/H-7 with C-9 (δ_C 84.5), and H-6 with C-8 (δ_C 74.8) suggested the ring B fused with ring A *via* C-5 and C-10. Based on the observation of HMBC signals of H-11 with C-8, H-1/H-12 with C-9, H-15 with C-12 (δ_C 29.8) and C-14 (δ_C 38.9), and H-16 with C-13 (δ_C 73.8), ring C was elucidated with an isopropyl group substituted at an oxyquaternary carbon C-13 and it was determined to fuse with ring B through C-8 and C-9. The planar structure of part b was subsequently established to be a 20-norabietane diterpenoid skeleton. Compared to the chemical shifts observed for the two oxy-tertiary carbons of C-8 (δ_C 74.8) and C-13 (δ_C 73.8), the ^{13}C NMR chemical signal of C-9 (δ_C 84.5) displayed an obvious downfield shift, indicating it was the connection position with the isopimarane unit. The planar structure of **2** was thus proposed as a diterpenoid dimer with parts a and b connected through an ester bond between C-9 with C-3′.

In order to confirm the elucidated chemical structure of **2** and to determine its absolute configuration, **2** was crystallized from methanol to afford crystals suitable for X-ray analysis. The Flack number of −0.01 resulted from the final refinement on the Cu Kα data of the crystal of **2** allows an unambiguous assignment of the absolute structure of **2** (Fig. 3B). The eight chiral centers of C-5, C-5′, C-8, C-9, C-9′, C-10′, C-13, C-13′ were thus determined as *S*, *S*, *R*, *S*, *S*, *R*, *R* and *R*, respectively. Finally, the structure of **2** was established as 8β,13β-dihydyoxyl-20-norabieta-1(10)-ene-9-yl 3,4-*seco*-isopimara-4(18),7,15-triene-3-carboxylate, and given the trivial name flavidanolide B.

The plausible biosynthetic pathways of **1** and **2** were proposed in Scheme 1. For **1**, a similar biosynthetic pathway to that of amethinol A was proposed with lophanic acid (**A**) as the same precursor [9]. The difference between the two pathways is that rings B and C in **1** are formed as 5- and 7-membered rings instead of 7- and 5-membered rings for amethinol A, respectively. The cause of the difference is due to the aldol condensation with C-11 as a carbon anion is preferred for **1** instead of C-7 for amethinol A, because a lactone ring is formed between C-20 and C-7, which significantly weakens the nucleophilic strength of the C-7 anion.

For **2**, the abietane diterpenoid unit could also be derived from lophanic acid (**A**). **A** undergoes an oxidation to introduce a hydroxyl group of C-1 to produce **E**, which could be converted to **F** by epoxidation of the $\Delta^{8,9}$ double bond. The C-20 carboxylic acid could be cleaved off *via* a decarboxylation mechanism to afford **G**, which loses a H_2O molecule to form **H**. The hydrolysis of the exposide could thus produce the norditerpene unit of **2**. The *seco*-isopimarane diterpenoid unit of **2** was proposed to be derived from glutinosin, a constituent richly found in *I. flavidus* [15]. The dehydration of the two hydroxyl groups in glutinosin (**J**) forms compound **K**, which is added a hydroxyl on C-3 to produce **L** [8(9),15-isopimaradien-3β-ol from *Platycladus orientalis*] [16] by an



Scheme 1. Plausible pathway for the biogenesis of **1** and **2**.

oxidation reaction. Another oxidation further transforms **L** to **M**, which undertakes a Baeyer-Villiger reaction to yield **N** with a 7-membered lactone ring [17]. The hydrolysis of **N** opens the lactone ring to provide **O**, which loses the hydroxyl group to form a double bond between C-4′ and C-18′ for **P** by the dehydration. The other diterpene monomer unit **Q** [3,4-secoisopimara-4(18),7,15-triene-3-oic acid from *Salvia cinnabarina*] [18] is thus produced from **P** *via* an allylic isomerization to shift the $\Delta^{8,9}$ double bond to $\Delta^{7,8}$. An esterification reaction then occurs between the two diterpene monomers of **I** and **Q** to accomplish the final step of the biosynthetic pathway of compound **2**.

The antiinflammatory effects of **1** and **2** were evaluated based on the LPS induced RAW264.7 cell model [19]. As demonstrated in Table S3 (Supporting information), **2** exhibited the inhibitory effects on NO production in the concentration range of 1–10 μmol/L, comparable with those of the positive control pyrrolidinedithiocarbamate ammonium (PDTC) in the concentration range of 5–10 μmol/L. However, **2** only showed 18.98% inhibitory rate on TNF-α production in the LPS-induced cell model (Table S4 in Supporting information).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108621.

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