Sesquiterpenoids from *Tussilago farfara* and Their Inhibitory Effects on Nitric Oxide Production

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Key words
- *Tussilago farfara*
- Asteraceae
- sesquiterpenoid
- nitric oxide inhibition

Abstract

Tussilagone (TSL) and its allied sesquiterpenoids were considered as the main active principles of the flower buds of *Tussilago farfara*, which has been widely used in China as an antitussive herbal medicine. Six new bisabolane-type sesquiterpenoids, tussfararins A–F (**1**–**6**), along with 12 known sesquiterpenoids, were isolated from the flower buds of *T. farfara*. Structures of the new compounds were elucidated by extensive spectroscopic analysis. The biological analysis showed that compounds **1, 3, 6**, and **7** inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells with IC_{50} values of 13.6–24.4 μM.

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Introduction

Sesquiterpenoids were abundant in the Asteraceae family and exhibited interesting chemical diversities and important biological activities, which made them attractive targets for phytochemical, pharmacological, and synthetic researches [1]. As a kind of perennial herb, *Tussilago farfara* L. (Asteraceae) is mainly distributed in China, Europe, and Northern Africa [2]. The spear leaves of *T. farfara* are consumed as vegetable in China and European countries [3,4], while the flower buds are widely used as folk medicine for the treatment of coughs, bronchitis, and asthmatic disorders in China [5]. A great number of compounds have been identified from this species, including sesquiterpenoids [6], triterpenoids [7], pyrrolizidine alkaloids [8], and chlorogenic acids [9]. Among them, tussilagone (TSL) and its allied sesquiterpenoids were considered to be the main active components of the flower buds of *T. farfara*, which possessed potent anti-inflammation and antidiabetic activities [10–12]. In the present study, six new bisabolane-type sesquiterpenoids, tussfararins A–F (**1**–**6**), together with 12 known sesquiterpenoids (**Fig. 1**), (4*R*, 6*E*)-2-acetoxy-8-angeloyloxy-4-hydroxybisabola-2,6,10-trien-1-one (**7**) [13], 1*β*-(3-ethyl-*cis*-crotonoyloxy)-8-angeloyloxy-3*α*,4*α*-epoxybisabola-7(14),10-dien-2-one (**8**) [10], 1*β*,8-bisangeloyloxy-3*α*,4*α*-epoxybisabola-7(14),10-dien-2-one (**9**) [10], (1*R*,3*R*,4*R*,5*S*,6*S*)-1-acetoxy-8-angeloyloxy-3,4-epoxy-5-hydroxybisabola-7(14),10-dien-2-one(**10**) [14], (1*R*,3*R*,4*R*,5*S*,6*S*)-1,5-diacetoxy-8-angeloyloxy-3,4-epoxybisabola-7(14),10-dien-2-one (**11**) [14], 7*β*-(3-ethyl-*cis*-crotonoyloxy)-1*α*-(2-methylbutyryloxy)-3(14)-dehydro-*Z*-notonipetrane (**12**) [12], tussilagone (**13**) [12], 14-acetoxy-7*β*-(3-ethyl-*cis*-crotonoyloxy)-1*α*-(2-methylbutyryloxy)-notonipetranone (**14**) [6], 7*β*-(3-ethyl-*cis*-crotonoyloxy)-14-hydroxy-notonipetranone (**15**) [6], *β*-oploplenone (**16**) [15], 2,2-dimethyl-6-acetylhromanone (**17**) [16], (−)-cryptomerion (**18**) [14], were isolated from the flower buds of *T. farfara*. Herein, the isolation and structure elucidation of the new sesquiterpenoids and the inhibitory effects of active compounds on NO production are reported.

Results and Discussion

Tussfararin A (**1**) was obtained as colorless oil. Its molecular formula, $C_{24}H_{32}O_8$ with nine degrees of unsaturation, was established by HR-EI-MS at *m*/*z* 448.2096 $[M]^+$ (calcd. for $C_{24}H_{32}O_8$, 448.2097). The IR spectrum showed the presence of carbonyl (1730 cm^{-1}) and double bond (1648 cm^{-1}) groups. The ^{13}C-NMR spectra exhibited 24 carbon signals attributed to eight quaternary carbons (four car-

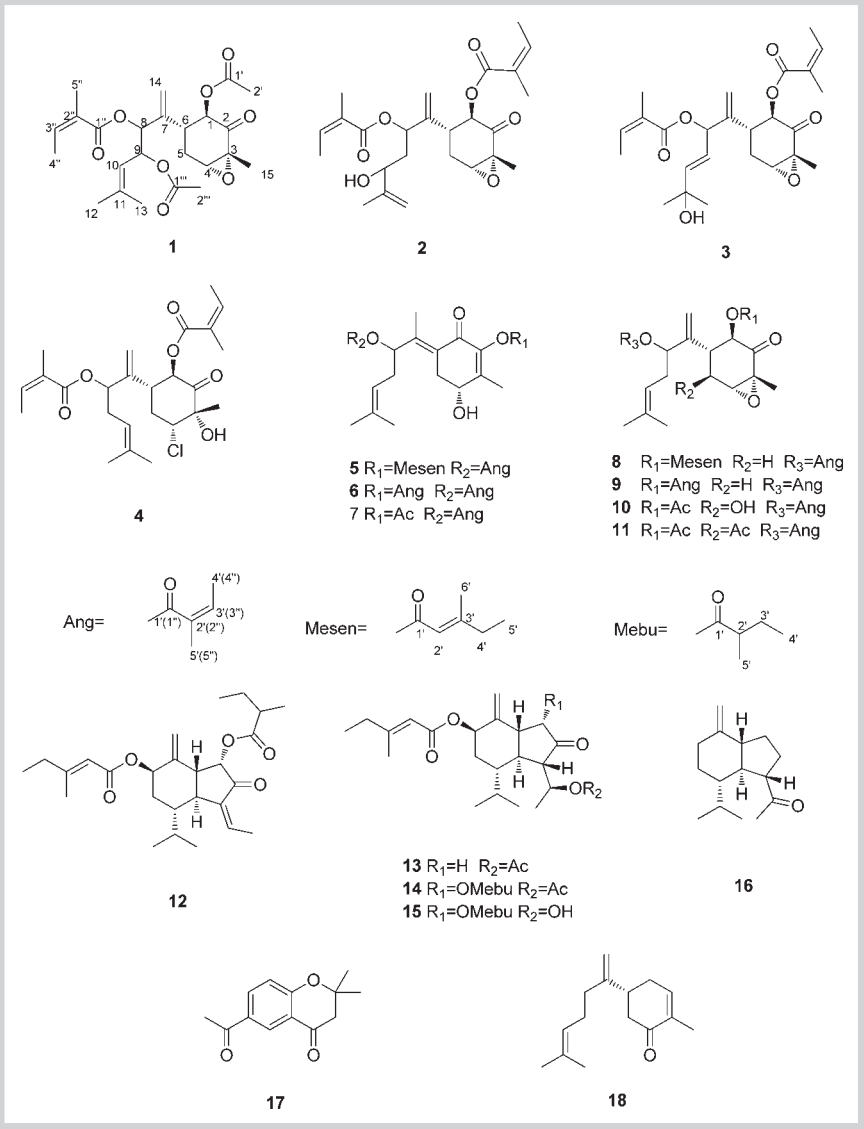

Fig. 1 Chemical structures of compounds **1**–**18**.

bonyl, three olefinic, and one oxygenated carbons), seven methines (four oxygenated and two olefinic ones), two methylenes (one olefinic carbon), and seven methyl groups. Further analysis of the 1D and 2D NMR data of **1** revealed some characteristic signals that were assigned to one angeloyloxy group [δ_C 167.3 (C-1″), 128.7 (C-2″), 140.6 (C-3″), 16.2 (C-4″), 20.8 (C-5″)], two acetoxy groups [δ_C 171.5 (C-1′), 21.1 (C-2′), 171.4 (C-1‴), and 20.3 (C-2‴)], one trisubstituted double bond [δ_C 120.5 (C-10) and 141.5 (C-11)], one terminal double bond [δ_C 146.4 (C-7) and 116.6 (C-14)], and one carbonyl carbon [δ_C 202.9 (C-2)]. The presence of a trisubstituted epoxide was inferred from the chemical shift [δ_H 3.55 (1H, d, J = 4.3 Hz, H-4), δ_C 62.6 (C-3) and 66.0 (C-4)] and the degree of unsaturation. The above spectroscopic analysis suggested that compound **1** is a sesquiterpenoid with the bisabolane skeleton containing one epoxy, one angeloyloxy, and two acetoxy groups. The HMBC correlations from H-1 (δ_H 5.64, d, J = 13.0 Hz), H-4, H-6 (δ_H 3.01, ddd, J = 13.0, 11.4, 7.4 Hz), and H_3-15 (δ_H 1.40, s) to C-2 (δ_C 202.9) suggested that the carbonyl group was located at C-2. The terminal double bond was inferred to be located at C-7 and C-14 by the HMBC correlations of H-14a (δ_H 5.33, br s) and H-14b (δ_H 5.21, br s) with C-6 (δ_C 45.2), C-7, and C-8 (δ_C 75.6). The ^{1}H-^{1}H COSY correlation of H-9 (δ_H 5.64 dd, J = 9.2, 5.9 Hz) with H-10 (δ_H 5.18, br d, J = 9.2 Hz), along with the HMBC correlations of H_3-12 (δ_H 1.74 d, J = 1.2 Hz) and H_3-13 (δ_H 1.75 d, J = 1.2 Hz) with C-10 (δ_C 120.5) and C-11 (δ_C 141.5) established the trisubstituted double bond at C-10 and C-11. In addition, the HMBC correlations of H-1, H-9, H-8 (δ_H 5.36 d, J = 5.9 Hz) with three carbonyl groups at δ_C 171.5, 171.4, and 167.3, respectively, implied that the two *O*-acetyl groups were attached to C-1 and C-9, and the angeloyloxy group was at C-8.

The relative configuration of **1** was determined by the coupling pattern and ROESY experiment. The coupling pattern and the constants for H-6 (ddd, $J_{1,6}$ = 13.0 Hz, $J_{5\alpha,6}$ = 11.4 Hz, $J_{5\beta,6}$ = 7.4 Hz,) suggested that H-1, H-5*α* and H-6 were *β*-axially oriented and the side chain at C-6 was *α*-equatorially oriented [14]. The small coupling constant (4.3 Hz) between H-4 and H-5*β* indicated that the epoxide ring at C-3 and C-4 was *α*-oriented [14], which was further confirmed by the ROESY correlations (**Fig. 2**) of H-4/H-5*β* and H-4/H_3-15. Therefore, the structure of **1** was determined as shown, and named as 1*β*,9-diacetoxy-8-angeloyloxy-3*α*,4*α*-epoxybisabola-7(14),10-dien-2-one.

Tussfararin B (**2**) was isolated as colorless oil, $[\alpha]_D^{22.6}$ – 46.6 (*c* 0.17, MeOH). Its molecular formula was determined to be $C_{25}H_{34}O_7$ by HR-EI-MS at *m*/*z* 446.2307 $[M]^+$ (calcd. for $C_{25}H_{34}O_7$, 446.2305),

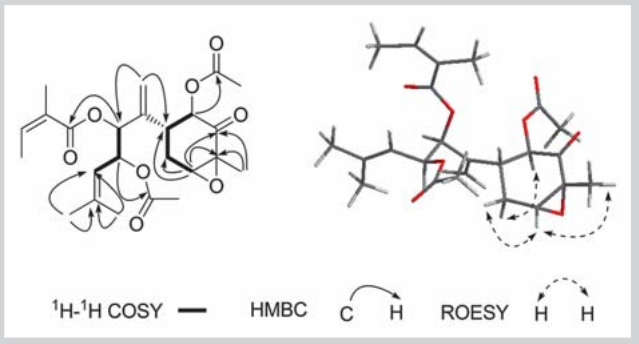

Fig. 2 Key 2D NMR correlations of **1**. (Color figure available online only.)

indicating nine degrees of unsaturation. The ^{1}H- and ^{13}C-NMR data of **2** (**Table 1**) were similar to those of **9** [10], with the main difference being that an oxygenated methine in **2** instead of a sp^2 methine in **9**. The HMBC correlations from H-10 (δ_H 4.10, t, J = 6.7 Hz) to C-8 (δ_C 72.0), C-9 (δ_C 40.6), C-11 (δ_C 147.9), C-12 (δ_C 17.5), and C-13 (δ_C 112.7), as well as the ^{1}H–^{1}H COSY correlations of H_2-9 with H-10 confirmed that the hydroxy group was connected to C-10. Another difference between these two compounds was that the methyl group of C-13 in **9** was replaced by a terminal double bond [δ_C 147.9 and 112.7] between C-11 and C-13 in **2**, which was deduced from the HMBC correlations of H-13a (δ_H 4.86, br s) and H-13b (δ_H 4.83, br s) with C-9, C-11, and C-12. In the ROESY spectrum, H-4 (δ_H 3.55, d, J = 4.2 Hz) showed correlations to H-5β (δ_H 2.64, ddd, J = 15.6, 7.5, 4.2 Hz) and H_3-15 (δ_H 1.41, s), revealing H-4 and H_3-15 to be β-oriented. The coupling pattern analysis further suggested that the rest of the relative configurations of **2** were the same as those of **1**. Accordingly, the structure of **2** was established as 1β,8-bisangeloyloxy-10-hydroxy-3α,4α-epoxybisabola-7(14),11(13)-dien-2-one.

Tussfararin C (**3**) exhibited the same molecular formula $C_{25}H_{34}O_7$ as that of **2**, as established by HR-EI-MS at m/z 446.2308 $[M]^+$ (calcd. for $C_{25}H_{34}O_7$, 446.2305), requiring nine degrees of unsaturation. The ^{1}H- and ^{13}C-NMR data of **3** showed a close structural resemblance to **2**, differing in the locations of a double bond [δ_H (5.65, dd, J = 15.6, 7.0 Hz, H-9 and 5.92, d, J = 15.6 Hz, H-10), δ_C 124.8 (C-9), 143.5 (C-10)] and a hydroxy group. The HMBC correlations from H-9 to C-8 (δ_C 75.6), C-10, and C-11 (δ_C 70.9) and from H-10 to C-8, C-9, C-11, C-12 (δ_C 29.7), and C-13 (δ_C 29.6), along with the ^{1}H–^{1}H COSY correlations of H-8/H-9/H-10 supported the occurrence of the double bond at C-9 and C-10. Moreover, the HMBC correlations observed from the proton signals of H-9, H-10, H_3-12 (δ_H 1.27, s), and H_3-13 (δ_H 1.27, s) to C-11 indicated that the hydroxy group was located at C-11. The double bond between C-9 and C-10 was determined as E configuration based on the big coupling constant (15.6 Hz) of H-9 with H-10. The remaining relative configurations of **3** were assigned as being the same as those of **2** according to the coupling pattern and ROESY experiment. Thus, the structure of **3** was proposed as 1β,8-bisangeloyloxy-11-hydroxy-3α,4α-epoxybisabola-9(10),7(14)-diene-2-one.

Tussfararin D (**4**) was shown to have the molecular formula $C_{25}H_{35}O_6Cl$ by HR-EI-MS m/z 466.2119 at $[M]^+$ (calcd. for $C_{25}H_{35}O_6Cl$, 466.2122), implying eight degrees of unsaturation. The existence of chlorine atom was deduced by the appropriate ^{13}C-NMR chemical shift at δ_C 66.4 (C-4) and HR-EI-MS analysis. The ^{1}H- and ^{13}C-NMR data were similar to those of 1β,8-bisangeloyloxy-3α,4α-epoxybisabola-7(14),10-dien-2-one (**9**) [10], except for a hydroxy group at C-3 (δ_C 78.5) and a chlorine atom at C-4 (δ_C 66.4) in **4** instead of the 3,4-epoxy group in **9** (**Table 1**). This was supported by the ^{1}H–^{1}H COSY correlation of H-4 (δ_H 4.45, t, J = 3.5 Hz) with H_2-5 (δ_H 2.30, dt, J = 15.0, 3.5 Hz and 2.76, ddd, J = 15.0, 12.6, 3.5 Hz) and the HMBC correlations of H-4 and H_3-15 (δ_H 1.46, s) with C-2 (δ_C 202.3) and C-3 (δ_C 78.5). The ROESY correlations from H-4 to H-6 and H_3-15 suggested that the H_3-15 was β-oriented. Consequently, the structure of **4** was elucidated as 1β,8-bisangeloyloxy-3α-chlorine-4α-hydroxybisabola-7(14),10-diene-2-one.

Tussfararin E (**5**) was isolated as colorless oil. Its molecular formula was determined to be $C_{26}H_{36}O_6$ by HR-EI-MS at m/z 444.2523 $[M]^+$ (calcd. for $C_{26}H_{36}O_6$, 444.2512), accounting for nine degrees of unsaturation. In the ^{1}H-NMR spectrum (DMSO-d_6), two methyl groups at δ_H (1.06 t, J = 7.4 Hz, H_3-5′ and 2.12 br s, H_3-6′), one olefinic proton at δ_H 5.87 (br s), and one of the methylene protons at δ_H 2.24 (q, J = 7.4 Hz, H_2-4′) suggested the presence of one 3-ethyl-*cis*-crotonoyloxy group, while two methyl groups at δ_H (1.93 br d, J = 7.2 Hz, H_3-4″ and 1.85 d, J = 1.5 Hz, H_3-5″) and one olefinic proton at δ_H 6.17 (qq, J = 7.2, 1.5 Hz) indicated the presence of an angeloyloxy group. In addition, one hydroxy proton at δ_H 5.70 (d, J = 6.5 Hz) was also observed in the ^{1}H-NMR spectrum. The ^{13}C-NMR and DEPT spectra showed 26 carbon resonances including eight methyls, three methylenes, five methines (two oxygenated and three olefinic ones), and ten quaternary carbons (three carbonyl and seven olefinic carbons). These spectroscopic data indicated **5** to be a bisabolane-type sesquiterpenoid with one angeloyloxy, one 3-ethyl-*cis*-crotonoyloxy, and one hydroxy group. Its NMR data were similar to those of **7**, except that the 3-ethyl-*cis*-crotonoyl group in **5** is replacing the acetyl group in **7** [13]. The HMBC correlations of OH-4 with C-3 (δ_C 149.0), C-4 (δ_C 67.2), and C-5 (δ_C 36.9), as well as the ^{1}H-^{1}H COSY correlation of OH-4 and H-4, allowed the OH group to be at C-4. Based on the HMBC correlation from H-8 (δ_H 5.59, t, J = 7.4 Hz) to C-1″ (δ_C 168.5), angeloyloxy group was positioned at C-8 (**Fig. 3**). In turn, the 3-ethyl-*cis*-crotonoyloxy group should be located at C-2, which was further supported by the ROESY correlation between H_3-15 and the H_3-6′ of the 3-ethyl-*cis*-crotonoyloxy group. A positive Cotton effect was observed at 244 nm ($\Delta\varepsilon$ = + 2.18) in the CD spectrum, which indicated the absolute configuration at C-4 was R [13]. Therefore, the structure of **5** was determined to be (4R, 6E)-2-(3′-ethyl-*cis*-crotonoyloxy)-8-angeloyloxy-4-hydroxybisabola-2,6,10-trien-1-one.

Tussfararin F (**6**) had a molecular formula of $C_{25}H_{34}O_6$ with nine degrees of unsaturation, determined by the HR-EI-MS at m/z 430.2361 $[M]^+$ (calcd. for $C_{25}H_{34}O_6$, 430.2355). The ^{1}H- and ^{13}C-NMR spectroscopic data (**Table 2**) of **6** were similar to those of **5**. The key difference was that 3-ethyl-*cis*-crotonoyloxy group at C-2 in **5** was replaced by an angeloyloxy group [δ_H 6.16 (1H, qq, J = 7.2, 1.4 Hz, H-3′), 1.96–2.02 (3H, m, H-4′), 1.97–2.01 (3H, m, H-5′), δ_C 166.7 (C-1′), 128.1 (C-2′), 141.5 (C-3′), 16.0 (C-4′), 20.7 (C-5′)] at C-2 in **6**. This deduction was further confirmed by comparing their ^{13}C NMR data of the dienone unit. The absolute configuration at C-4, determined by the CD spectrum, was the same as those of **5**. Thus, the structure of **6** was determined to be (4R, 6E)-2,8-bisangeloyloxy-4-hydroxybisabola-2,6,10-trien-1-one.

Selected isolates (purity > 93%, **1**–**7, 10, 14** and **16**) were tested for their inhibitory effects on the NO production in LPS-stimulated RAW 264.7 cells with MG 132 as a positive control [17]. As a potent inhibitor of the proteasome, MG 132 decreases LPS-stimulated NO production in RAW 264.7 cells by inhibiting NF-κB sig-

Table 1 ^{1}H and ^{13}C NMR spectroscopic data for compounds **1**–**4**.

	1[a]		**2**[b]		**3**[a]		**4**[c]	
	δ_H multi (*J*)	**δ_C**	**δ_H multi (*J*)**	**δ_C**	**δ_H multi (*J*)**	**δ_C**	**δ_H multi (*J*)**	**δ_C**
1	5.64 d (13.0)	76.2 d	5.80 d (13.0)	75.5 d	5.79 d (12.6)	75.5 d	6.08 d (12.6)	75.9 d
2		202.9 s		203.1 s		202.9 s		202.3 s
3		62.6 s		62.8 s		62.6 s		78.5 s
4	3.55 d (4.3)	66.0 d	3.55 d (4.2)	65.8 d	3.53 d (4.3)	65.7 d	4.45 t (3.5)	66.4 d
5α	2.11 dd (15.6, 11.4)	32.2 t	2.16 dd (15.6, 11.4,)	32.6 t	2.21 dd (15.6, 11.1)	31.7 t	2.30 dt (15.0, 3.5)	35.7 t
5β	2.62 ddd (15.6, 7.4, 4.3)		2.64 ddd (15.6, 7.5, 4.2)		2.53 ddd (15.6, 7.4, 4.3)		2.76 ddd (15.0, 12.6, 3.5)	
6	3.01 ddd (13.0, 11.4, 7.4)	45.2 d	2.93 ddd (13.0, 11.4, 7.5)	46.4 d	2.96 ddd (12.6, 11.1, 7.4)	46.4 d	3.07 td (12.6, 3.5)	41.9 d
7		146.4 s		149.9 s		148.1 s		148.8 s
8	5.36 d (5.9)	75.6 d	5.23 t (6.8)	72.0 d	5.77 d (7.0)	75.6 d	5.22 dd (7.7, 4.9)	75.3 d
9a	5.64 dd (9.2, 5.9)	72.7 d	1.89–1.94 m (2H)	40.6 t	5.65 dd (15.6, 7.0)	124.8 d	2.47ddd (15.0, 7.7, 4.9)	33.1 t
9b							2.38 dt (15.0, 7.7)	
10	5.18 br d (9.2)	120.5 d	4.10 t (6.7)	73.8 d	5.92 d (15.6)	143.5 d	5.13 t (7.7)	120.4 d
11		141.5 s		147.9 s		70.9 s		134.8 s
12	1.74 d (1.2)	26.0 q	1.72 br s	17.5 q	1.27 s	29.7 q	1.62 s	18.0 q
13a	1.75 d (1.2)	18.9 q	4.86 br s	112.7 t	1.27 s	29.6 q	1.65 s	25.8 q
13b			4.83 br s					
14a	5.33 br s	116.6 t	5.26 br s	113.5 t	5.32 br s	115.4 t	5.31 s	112.4 t
14b	5.21 br s		5.09 br s		5.15 br s		5.19 s	
15	1.40 s	15.0 q	1.41 s	15.3 q	1.38 s	15.0 q	1.46 s	21.2 q
1′				168.4 s		168.3 s		167.1 s
2′				129.1 s		128.7 s		128.6 s
3′			6.06 qq (7.2, 1.5)	139.2 d	6.08 qq (7.2, 1.4)	139.2 d	6.06–6.14 m	138.6 d
4′			1.91dq (7.2, 1.5)	20.9 q	1.91 dq (7.2, 1.4)	1 6.1 q	1.90–1.99 m	15.9 q
5′			1.82 dq (1.5, 1.5)	16.2 q	1.83 dq (1.4, 1.4)	20.7 q	1.84–1.89 m	20.7 q
1′′		167.3 s		168.0 s		170.9 s		166.9 s
2′′		128.7 s		129.3 s		128.9 s		128.3 s
3′′	6.19 qq (7.3, 1.4)	140.6 d	6.12 qq (7.2, 1.5)	139.9 d	6.13 qq (7.2, 1.4)	139.9 d	6.06–6.14 m	138.4 d
4′′	1.97 dq (7.3, 1.4)	16.2 q	1.95 dq (1.5, 1.5)	21.0 q	1.94 dq (7.2, 1.4)	16.1 q	1.90–1.99 m	15.1 q
5′′	1.88 dq (1.4,1.4)	20.8 q	1.87 dq (1.5, 1.5)	16.3 q	1.87 dq (1.4, 1.4)	20.8 q	1.84–1.89 m	20.7 q
1-$COCH_3$	2.01 s	21.1 q 171.5 s						
9-$COCH_3$	1.95 s	20.3 q 171.4 s						

[a] recorded in methanol-d_4, 400 MHz for δ_H, 100 MHz for δ_C; [b] recorded in methanol-d_4, 600 MHz for δ_H, 150 MHz for δ_C; [c] recorded in acetone-d_6, 400 MHz for δ_H, 100 MHz for δ_C; δ in ppm and *J* in Hz

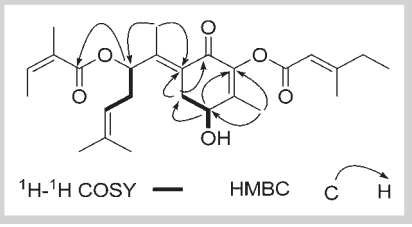

Fig. 3 Key ^{1}H-^{1}H COSY and HMBC correlations of **5**.

naling pathway [18]. The results showed that compounds **1, 3, 6**, and **7** exhibited moderate inhibitory activity against NO production (**Table 3**). Simultaneously measured with MTT assay, cell viability demonstrated that the inhibitory effect of the active compounds on NO production was not caused by cytotoxicity. Interestingly, compounds **6** and **7**, belonging to bisabolane-type sesquiterpenoids with dienone at C-1 to C-3, C-6, and C-7, showed relatively high activities than other compounds. These results indicated that dienone moieties at C-1 to C-3, C-6, and C-7 of bisabolane-type sesquiterpenoids seem to be crucial for the

Table 2 ^{1}H and ^{13}C NMR spectroscopic data for compounds **5** and **6**.

	5[a]		5[b]		6[a]	
	δ_H multi (*J*)	δ_C	δ_H multi (*J*)	δ_C	δ_H multi (*J*)	δ_C
1		185.0 s		182.7 s		184.6 s
2		144.3 s		142.1 s		144.5 s
3		149.2 s		149.0 s		149.1 s
4	4.22 dd (4.4, 7.4)	69.3 d	4.33 m	67.2 d	4.33 dd (8.1, 4.5)	69.3 d
5α	3.05 dd (13.9, 4.4)	37.7 t	3.07 dd (13.7, 4.5)	36.9 t	3.16 dd (13.9, 4.5)	37.7 t
5β	2.65 dd (13.9, 7.4)		2.47–2.53 m		2.76 dd (13.9, 8.1)	
6		129.2 s		127.9 s		129.1 s
7		146.5 s		144.2 s		146.7 s
8	5.61 t (7.4)	74.9 d	5.59 t (7.4)	73.2 d	5.72 t (7.4)	74.9 d
9a	2.47 dt (14.5, 7.4)	32.5 t	2.46–2.53 m	31.2 t	2.57 dt (14.4, 7.4)	32.5 t
9b	2.32 dt (14.5, 7.4)		2.33 dt (14.3, 7.4)		2.41 dt (14.4, 7.4)	
10	5.04 t (7.4)	119.5 d	5.10 t (7.4)	118.7 d	5.14 t (7.4)	119.5 d
11		136.8 s		134.9 s		136.8 s
12	1.56 s	18.0 q	1.66 s	17.8 q	1.67 s	18.0 q
13	1.62 s	26.0 q	1.72 s	25.7 q	1.73 s	26.0 q
14	1.89 s	15.4 q	1.99 s	14.8 q	1.97 s	14.0 q
15	1.81 s	14.0 q	1.93 s	13.7 q	1.93 s	12.0 q
1′		166.8 s		163.3 s		166.7 s
2′	5.80 br s	113.8 d	5.87 br s	112.7 d		128.1 s
3′		165.6 s		165.1 s	6.16 qq (7.2, 1.4)	141.5 d
4′	2.19 q (7.4)	34.8 t	2.24 q (7.4)	33.1 t	1.96–2.02 m	20.7 q
5′	1.04 t (7.4)	12.3 q	1.06 t (7.4)	11.8 q	1.97–2.01 m	16.0 q
6′	2.08 br s	19.1 q	2.12 br s	18.7 q		
1′′		168.5 s		166.4 s		168.5 s
2′′		128.8 s		127.1 s		128.8 s
3′′	6.06 br q (7.2)	139.8 d	6.17 qq (7.2, 1.5)	138.5 d	6.30 qq (7.2, 1.4)	139.8 d
4′′	1.87 br d (7.2)	16.0 q	1.93 br d (7.2)	14.8 q	1.93–1.98 (m)	20.7 q
5′′	1.79 br s	20.8 q	1.85 d (1.5)	20.3 q	1.89 br s	16.0 q
4-OH			5.70 d (6.5)			

[a] recorded in methanol-d_4, 400 MHz for δ_H, 100 MHz for δ_C; [b] recorded in DMSO-d_6, 600 MHz for δ_H, 150 MHz for δ_C; δ in ppm and *J* in Hz

Table 3 Inhibition of compounds **1–7, 10, 14** and **16** on the NO production in LPS-stimulated RAW 264.7 Cells.

Compound	IC_{50} (μM)
1	24.4 ± 0.4
2	> 25
3	23.7 ± 0.4
4	–[a]
5	–
6	13.6 ± 0.1
7	17.7 ± 0.6
10	> 25
14	–
16	> 25
MG132[b]	0.1 ± 0.0

[a] not detected due to the obvious cytotoxicity at 25 μM concentration; [b] positive control

inhibition of NO production, although further evidence is needed for establishment of a clear structure-activity relationship.

Materials and Methods

General experimental procedures

Optical rotations were measured with a Horiba SEPA-300 polarimeter (Horiba). UV spectra were obtained by using a Shimadzu UV-2401A spectrophotometer (Shimadzu). A Tenor 27 spectrophotometer was used for scanning IR spectroscopy (Bio-Rad) with KBr pellets. 1D and 2D spectra were run on Bruker DRX-500, AM-400, or Avance III 600 spectrometers (Bruker) with TMS as internal standard. Chemical shift (δ) were expressed in ppm with reference to the solvent signals. ESI-MS spectrum was tested on a Bruker HCT or Esquire spectrometer (Bruker). HR-EI-MS spectrum was performed on a Waters Auto-Spec Premier P776 spectrometer (Waters). Circular dichroism (CD) spectra were detected by a digital Applied Photophysics instrument (Agilent). Middle-performance liquid chromatography (MPLC) was performed on a Lisui Ez Purifier III system including pump manager P03, detector modules P02, and fraction collector P01 (Shanghai Li Sui Chemical Engineering Co., Ltd.) and columns packed with RP-18 silica gel (40–63 μm, Merck). Semipreparative HPLC was performed on an Agilent 1260 liquid chromatography (Agilent) with a Zorbax SB-C18 (5 μm, 9.6 × 150 mm) column. Column chromatography (CC) was performed on Silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Ltd.), and Sephadex LH-20 (Amersham Pharmacia Biotech). Fractions were monitored by TLC (GF 254, Qingdao Haiyang Chemical Co., Ltd.), and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

Plant material

The plant material was purchased from Juhuacun Chinese Traditional Medicine Market, Kunming, Yunnan province, China, in February 2012, and identified by Prof. Xiao Cheng, Kunming Institute of Botany, Chinese Academy of Sciences. A voucher speci-

men (No. 20120224 t) has been deposited in Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The dried flower buds of *T. farfara* (10 kg) were extracted with 95% EtOH (50 L × 3) at room temperature for 48 h each, and concentrated in vacuo. The crude extract (610 g) was partitioned between H_2O (2.5 L) and EtOAc (6 L × 3). The EtOAc portion (143.7 g) was separated on MPLC (MCI, 15 × 110 cm, 1 kg) and eluted with MeOH-H_2O (30 : 70, 40 : 60, 50 : 50, 60 : 40, 70 : 30, 80 : 20, 90 : 10, 100 : 0, each 6 L, 60 mL/min) to yield three fractions (Fr.1–Fr.3). Compound **13** (2.4 g) was crystallized directly with MeOH from Fr.3 (50.7 g). The mother liquor of Fr.3 was further subjected to MPLC over RP-18 (6 × 25 cm, 400 g) and eluted with MeOH-H_2O gradient system (75 : 25, 80 : 20, 85 : 15, 90 : 10, 95 : 5, 0 : 100, each 2 L, 40 mL/min) to yield five subfractions (Fr.3a–Fr.3e). Subfraction 3a (3.9 g) was separated repeatedly by silica gel CC (200–300 mesh, 2.8 × 40 cm, 50 g) with petroleum ether-acetone (100 : 1, 50 : 1, 20 : 1, 9 : 1, 8 : 2, 1 : 1, each 500 mL) to afford **12** (2.3 g) and **18** (4.1 mg). Subfraction 3b (1.8 g) was separated by semipreparative HPLC at a flow rate of 3 mL/min with DAD detection at 210 nm eluting with MeOH-H_2O (82 : 28) to give compounds **8** (20.0 mg, t_R = 12.6 min), **9** (20.0 mg, t_R = 11.2 min), and **14** (3.8 mg, t_R = 15.1 min). Subfraction 3c (13.7 g) was further applied to silica gel CC (200–300 mesh, 7 × 45 cm, 200 g), eluted with chloroform to afford **17** (65.2 mg) and a mixture. The mixture was further separated by semipreparative HPLC using a solvent system of MeOH-H_2O (70 : 30, a flow rate of 4 mL/min) to give **1** (18.3 mg, t_R = 10.2 min), **2** (2.2 mg, t_R = 11.6 min), and **10** (10.6 mg, t_R = 11.9 min). Subfraction 3 d (2.1 g) was subjected to CC (200–300 mesh, 2.5 × 35 cm) on a silica gel (50 g) using a stepwise gradient of petroleum ether-acetone (200 : 1, 100 : 1, 50 : 1, 9 : 1, 4 : 1, 1 : 1, each 400 mL) to give compounds **3** (8.6 mg), **16** (27.3 mg), and a mixture, which was further separated by semipreparative HPLC using a solvent system of MeOH-H_2O (80 : 20, a flow rate of 4 mL/min) to afford compound **15** (43.2 mg, t_R = 11.8 min). Fraction 3e (2.0 g) was purified by semipreparative HPLC using a solvent system of MeOH-H_2O (75 : 25, a flow rate of 4 mL/min) to give compounds **11** (22.2 mg, t_R = 10.8 min), **7** (3.4 mg, 15.3 min), **6** (8.8 mg, t_R = 18.3 min), and **5** (12 mg, t_R = 22.6 min). Fr.2 (1.1 g) was chromatographed over Sephadex LH-20 (2.5 × 180 cm) eluted with MeOH (800 mL) and then purified by semipreparative HPLC using a solvent system of MeOH-H_2O (78 : 22, a flow rate of 4 mL/min) to give compound **4** (12.3 mg, t_R = 21.6 min).

Isolates

Tussfararin A (**1**): Colorless oil; $[\alpha]_D^{22.3}$ − 58.4 (*c* 0.14, MeOH); UV (MeOH): λ_{max} (log ε) 204 (4.15) nm; IR (KBr): ν_{max} 3441, 3432, 2934, 2859, 1730, 1648, 1440, 1374, 1234, 1154, 1036 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see **Table 1**; ESI-MS: *m*/*z* 471 $[M + Na]^+$, HR-EI-MS: *m*/*z* 448.2096 $[M]^+$ (calcd. for $C_{24}H_{32}O_8$, 448.2097).

Tussfararin B (**2**): Colorless oil; $[\alpha]_D^{21.9}$ − 66.1 (*c* 0.15, MeOH); UV (MeOH):λ_{max} (log ε) 216 (4.31) nm; IR (KBr): ν_{max} 3433, 2926, 1719, 1646, 1456, 1382, 1232, 1154, 1041 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see **Table 1**; ESI-MS: *m*/*z* 469 $[M + Na]^+$, HR-EI-MS: *m*/*z* 446.2308 $[M]^+$ (calcd. for $C_{25}H_{34}O_7$, 446.2305).

Tussfararin C (**3**): Colorless oil; $[\alpha]_D^{22.6}$ − 46.6 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.32) nm; IR (KBr): ν_{max} 3434, 2975, 1719, 1647, 1457, 1438, 1381, 1232, 1154, 1041, 847 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see **Table 1**; ESI-MS: *m*/*z* 469 $[M + Na]^+$, HR-EI-MS: *m*/*z* 446.2307 $[M]^+$ (calcd. for $C_{25}H_{34}O_7$, 446.2305).

Tussfararin D (**4**): Colorless oil; $[\alpha]_D^{21.4}$ − 37.0 (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ε) 214 (4.28) nm; IR (KBr): ν_{max} 3440, 2927, 1718, 1645, 1383, 1232, 1155, 1041 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see **Table 1**; ESI-MS: *m*/*z* 489 $[M + Na]^+$, HR-EI-MS: *m*/*z* 466.2119 $[M]^+$ (calcd. for $C_{25}H_{35}ClO_6$, 466.2122).

Tussfararin E (**5**): Colorless oil; $[\alpha]_D^{21.4}$ − 33.3 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ε) 276 (3.93) nm, 221 (4.47) nm; CD (MeOH): − 6.67 (286), + 2.18 (244), + 14.66 (204); IR (KBr): ν_{max} 3442, 2925, 1715, 1644, 1440, 1381, 1232, 1128, 1042 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see **Table 2**; ESI-MS: *m*/*z* 467 $[M + Na]^+$, HR-EI-MS: *m*/*z* 444.2523 $[M]^+$ (calcd. for $C_{26}H_{36}O_6$, 444.2512).

Tussfararin F (**6**): Colorless oil; $[\alpha]_D^{21.7}$ − 53.5 (*c* 0.30, MeOH); UV (MeOH) λ_{max} (log ε) 277 (3.80) nm, 218 (4.33) nm; CD (MeOH): − 6.19 (286), + 2.10 (244), + 12.59 (204); IR (KBr): ν_{max} 3450, 2927, 1718, 1672, 1455, 1381, 1229, 1146, 1038 cm^{-1}; ^{1}H-NMR and ^{13}C-NMR spectroscopic data, see **Table 2**; ESI-MS: *m*/*z* 453 $[M + Na]^+$, HR-EI-MS: *m*/*z* 430.2361 $[M]^+$ (calcd. for $C_{25}H_{34}O_6$, 430.2355).

Determination of NO production

Murine macrophage cells RAW 264.7 were seeded into 96-well plates (2×10^5 cells/well) containing RPMI 1640 medium (Hyclone) with 10% FBS under a humidified atmosphere of 5% CO_2 at 37 °C. After 24 h incubation, cells were treated with the compounds with the maximum concentration of 50 μM in the presence of 1 μg/mL LPS for 18 h. Each compound was dissolved in DMSO and further diluted in cell culture media to obtain different concentrations. NO production was assessed by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylene diaminedihydrochloride in 5% H_3PO_4) to 100 μL supernatant from LPS or the compound-treated cells in triplicate. After 5 min incubation, the absorbance was measured at 570 nm with a 2104 Envision Multilabel PlateReader Perkin-Elmer Life Sciences, Inc.). MG132 (Sigma Aldrich, purity ≥ 99%, IC_{50} value = 0.1 μM) was used as a positive control. Cytotoxicity was determined by the MTT assay as described [19].

Supporting information

1D and 2D NMR, HRMS, and IR spectra of compounds **1**–**6**, and inhibitory effects of compounds **1, 3, 6**, and **7** on LPS-stimulated NO production in RAW 264.7 cells are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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