TWO NEW BENZOFULVENE SESQUITERPENOIDS FROM THE STEMS OF *Nicotiana tabacum* AND THEIR ANTIBACTERIAL ACTIVITY

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*Two new benzofulvene sesquiterpenoids, tabanoids A and B (**1** and **2**) as well as five known sesquiterpenoids (**3–7**) were isolated from the stem of* Nicotiana tabacum. *Their structures were determined mainly by spectroscopic methods, including extensive 1D and 2D NMR techniques. Compounds **1** and **2** were tested for their anti-methicillin-resistant* Staphylococcus aureus *(anti-MRSA) activity. The results revealed that compounds **1** and **2** showed good inhibition with IZD of 15.2 ± 2.5, and 12.8 ± 2.2 mm, respectively.*

Keywords: *Nicotiana tabacum*, sesquiterpenoids, antibacterial activity.

Sesquiterpenoids are a group of 15-carbon compounds derived from the assembly of three isoprenoid units, which are mainly found in higher plants and microorganisms [1–3]. Some of the sesquiterpenoids are interesting to drug discovery because they have biological activities that are antimicrobial [4, 5], antitumor [6, 7], antiviral [8, 9] properties, etc. In addition, they also play important ecological roles in interactions with insects and microbes in plants such as attractants, deterrents, antifeedants and phytoalexins [10, 11].

Nicotiana tabacum L. (tobacco) is a robust annual little branched herb in the Solanaceae (nightshade family) only in cultivation. Its leaves are commercially grown in many countries to be processed into tobacco [12]. In addition, they are also important to humans both for their ornamental properties and psychoactive, medicinal, and toxic values [13]. Because of its economic importance, *N. tabacum* had been studied in detail all over the world. Previous studies have led to the identification of as many as 8000 chemicals in tobacco and cigarette smoke [13, 14]. The *Nicotiana* species plants are rich in sesquiterpenoids, and the sesquiterpenoids play ecological roles such as defensive activity or hormone-like functions [13]. Moreover, sesquiterpenoids from *N. tabucum* also have strong biological activities such as cytotoxicity, antiviral, and antibacterial activities [15–18].

In recent years, gene-editing technology has widely been used in crop cultivar improvement [19], and a series of *N. tabacum* mutants had been cultivated by gene-editing technology. Because the secondary metabolism of plants would be affected by the change of gene [20, 21], the gene-editing mutants provided a new sample source for the discovery of new active metabolites. In order to discover more novel bioactivity sesquiterpenoids from *N. tabacum*, we focused on the stems of YNZY-20-06 (a mutant tobacco for gene editing with solanesol synthesis, cultivated by Yunnan Tobacco Company). which led to the isolation of two new (**1** and **2**) and five known (**3–7**) sesquiterpenoids. This paper deals with the isolation, structural elucidation, and the antibacterial activity of the preceding compounds.

TABLE 1. [1]H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds **1** and **2** (CDCl$_3$, δ, ppm, J/Hz)

C atom	1		2	
	δ$_H$	δ$_C$	δ$_H$	δ$_C$
1	–	128.0 (C)	–	127.6 (C)
2	–	137.6 (C)	–	136.9 (C)
3	6.53 (s)	117.0 (CH)	6.70 (s)	117.6 (CH)
4	–	144.7 (C)	–	149.0 (C)
5	–	143.3 (C)	–	142.2 (C)
6	7.17 (s)	112.6 (CH)	7.16 (s)	112.8 (CH)
7	–	158.0 (C)	–	158.2 (C)
8	–	123.7 (C)	–	124.0 (C)
9	6.80 (s)	128.9 (CH)	6.98 (s)	129.1 (CH)
10	6.21 (d, J = 9.8)	135.3 (CH)	6.22 (d, J = 9.8)	135.2 (CH)
11	3.09 (m)	28.7 (CH)	3.10 (m)	28.6 (CH)
12, 13	1.23 (d, J = 6.8)	24.9 (CH$_3$)	1.24 (d, J = 6.8)	24.6 (CH$_3$)
14	4.54 (s)	61.5 (CH$_2$)	1.94 (s)	22.7 (CH$_3$)
15	2.18 (s)	18.3 (CH$_3$)	2.17 (s)	18.4 (CH$_3$)
7-OMe	3.79 (s)	56.0 (CH$_3$)	3.79 (s)	56.1 (CH$_3$)

1, 2

1: R = OH; **2:** R = H

3

4

5

6

7
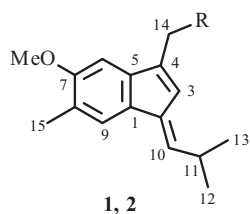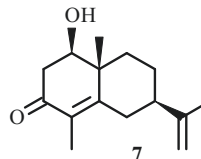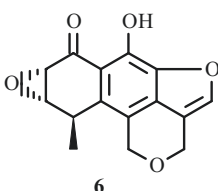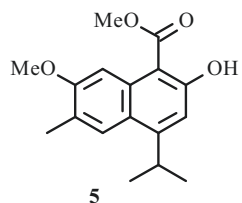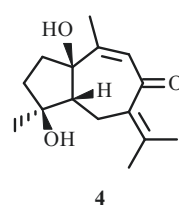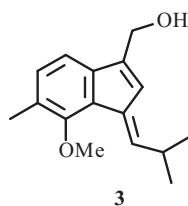
A 95% aq. ethanol extract prepared from the stems of tobacco was subjected repeatedly to column chromatography and preparative HPLC to afford two new sesquiterpenoids, tabanoids A and B (**1** and **2**), together with five known sesquiterpenoids (**3–7**). The ^{1}H and ^{13}C NMR data of **1** and **2** are listed in Table 1. The known compounds, compared with the literature, were identified as nicotianasesterpene A (**3**) [16], procurcumenol (**4**) [22], 4-isopropyl-7-methoxy-6-methylnaphthalene-1-carboxylate (**5**) [15], tobterpene B (**6**) [23], and 1-β-hydroxy-α-cyperone (**7**) [24].

Tabanoid A (**1**) was isolated as a yellow gum. Its molecular formula was determined to be C$_{16}$H$_{20}$O$_2$ with seven degrees of unsaturation based on HR-ESI-MS at *m/z* 267.1361 [M + Na]$^+$ (calcd for C$_{16}$H$_{20}$O$_2$Na, 267.1368). The ^{1}H NMR spectrum of **1** displayed two aromatic protons [δ 7.17 (s), H-6 and 6.80 (s), H-9], two olefinic protons [δ 6.53 (s), H-3 and 6.21 (d, J = 9.8 Hz), H-10], one methyl singlet [δ 2.18 (s), H$_3$-15], one oxidated methylene [δ 4.54 (s), H$_2$-14], one methane [δ 3.09 (m), H-11], and a methyl doublet integrating for six hydrogens [δ 1.23, (d, J = 6.8 Hz), H$_6$-12, 13]. The ^{1}H–^{1}H COSY cross correlations (Fig. 1) of H$_3$-12, 13/H-11/H-10 indicated that the existence of a methylpropylidene moiety (=CH-CH(CH$_3$)$_2$) [16]. In addition, the ^{13}C and DEPT NMR data also revealed that compound **1** had the presence of 16 carbon signals, including one 2,4,7,8,10-pentasubstituted benzofulvene ring (C-1–C-9), one oxidated methylene (C-14), one isopropyl group (C-11–C-13), and the isopropyl was linked to aromatic methine on benzofulvene (C-10) to form a methylpropylidene moiety, and one methoxy group (δ$_C$ 56.0). In addition, the existence of a benzofulvene ring was supported by the HMBC correlations (Fig. 1) from H-3 to C-1, C-2, C-4, C-5, from H-6 to C-1, C-4, C-5, and from H-9 to C-2, C-5, the existence of methylpropylidene moiety was supported the HMBC correlations from H-10 to C-11, C-12, 13, from H-11 to C-10, C-12, 13, from H$_6$-12, 13 to C-10, and the ^{1}H–^{1}H COSY correlations (Fig. 1) of H-10/H-11/H$_6$-12, 13.
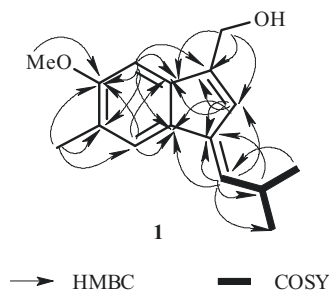
Fig. 1. Key HMBC and ^{1}H–^{1}H COSY
correlations of **1**.

Since the benzofulvene skeleton was determined, the remainder signals, a methylpropylidene moiety, a hydroxymethyl group, a methyl group, and a methoxy group can be considered as the substituents on the benzofulvene ring, and their positions can also be determined by further analysis of its HMBC correlations. The methylpropylidene moiety located at C-2 was supported by the HMBC correlations from H-10 to C-1, C-2, and C-3, from H-11 to C-2. The hydroxymethyl group located at C-4 was supported by the HMBC corrections from H_2-14 to C-3, C-4, C-5, and from H-3 to C-14. The methyl group located at C-8 was supported by the HMBC corrections from H_3-15 to C-7, C-8, and C-9, and from H-9 to C-15. Finally, The HMBC correlation from the methoxy proton signal (δ 3.79) to C-7 suggested the methoxy group is located at C-7. In addition, the *E*-configuration of the double bond between C-2 and C-10 was determined by the comparison of NMR data of **1** with the known compound, nicotianasesterpene A [16], and the ROESY correlation observed between H-11 and H-3. Thus, in this way, the structure of **1** was established.

Compound **2** was also obtained as a yellow gum, and assigned the molecular formula $C_{16}H_{20}O$, according to the ion peak in HR-ESI-MS at *m/z* 251.1408 [M + Na]$^{+}$, which indicated 7 degrees of unsaturation. Its UV, IR, ^{1}H and ^{13}C NMR spectral data depicted that compound **2** has a similar structure to **1**. The chemical shift differences resulted from the disappearance of a hydroxymethyl resonance and the appearance of a methyl resonance (C-14 and H_3-14) in **2**. These changes indicated that the hydroxymethyl at C-4 in **1** was converted into a methyl group in **2**. The HMBC correlation from H_3-14 to C-3, C-4, and C-5, from H-3 to C-14, also supported the methyl group being located at C-4. In addition, the positions of the methoxy group and another methyl group can also be determined by further analysis of its HMBC correlations. The structure of **2** was therefore defined, and given the trivial name of tabanoid B.

Compounds **1** and **2** were screened for anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity according to arbitrary criterion [25] with the diameter of inhibition zone (IZD) as follows: very weak inhibition (with IZD of 6–8 mm), weak inhibition (with IZD of 8–12 mm), good inhibition (with IZD of 12–16 mm), and strong inhibition (with IZD of > 16 mm) activities, respectively. The IZD of the positive control (vancomycin) was 32 mm and the IZD of the negative control was considered as zero. The results revealed that compounds **1** and **2** showed good inhibition with IZD of 15.2 ± 2.5, and 12.8 ± 2.2 mm, respectively.

EXPERIMENTAL

General Methods. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning IR spectra. ^{1}H, ^{13}C, and 2D NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as the internal standard. ESI-MS and HR-ESI-MS analyses were measured on an Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. Semipreparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (2.12 mm × 25 cm) or Venusil MP C_{18} (2.0 mm × 25 cm) columns. Column chromatography was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 µm, Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, Inc, USA), or MCI gel (75–150 µm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC and visualized by spraying with 5% H_2SO_4 in ethanol and heating.

Plant Material. The tobacco (YNZY-20-06), a gene editing mutant with solanesol (a high content tetraterpene in tobacco) synthesis gene knockout, cultivated by Yunnan China Tobacco Industry Co. Ltd., was planted in the Greenhouse in

Kunming, Yunnan Province. The tobacco stems were picked when the leaves were at a mature stage, and the voucher specimen (YNNI-21-09) was deposited in the Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan Minzu University, P. R. China.

Extraction and Isolation. The air-dried tobacco stems (4.0 kg) were crushed to 30 mesh, and were extracted four times with 70% aqueous acetone (4 × 8 L) at room temperature and filtered. All the extract was combined and condensed under reduced pressure (≤ 60°C), which was partitioned between EtOAc and H_2O. The EtOAc part was discolored with MCI. The concentrated 80% ethanol elution partial from MCI column obtained a purified extract of 126 g. The purified extract was applied to silica gel (200–300 mesh) column chromatography, eluting with a $CHCl_3$–CH_3OH gradient system (10:0, 9:1, 8:2, 7:3, 6:4, 5:5), to give six fractions A–F. Further separation of fraction B (9:1, 25.8 g) by silica gel column chromatography, eluted with $CHCl_3$–$(CH_3)_2CO$ (9:1–2:1), yielded mixtures B1–B6. Subfraction B2 (8:2, 8.15 g) was subjected to silica gel column chromatography using petroleum ether–acetone and semipreparative HPLC (52–58% MeOH–H_2O, flow rate 12 mL/min) to give **1** (116.4 mg), **2** (18.2 mg), **3** (15.7 mg), and **7** (18.6 mg). Subfraction B3 (7:3, 6.27 g) was subjected to silica gel column chromatography using petroleum ether–acetone and semipreparative HPLC (48–52% MeOH–H_2O, flow rate 12 mL/min) to give **4** (114.5 mg), **5** (17.3 mg), and **6** (18.4 mg).

Anti-MRSA Agar Disc Diffusion Assay. The MRSA strain ZR11 was clinically isolated from infectious samples of critically ill patients in the Clinical Laboratory of the First People′s Hospital of Yunnan Province, and confirmed by standard cefoxitin disk diffusion test following CLSI standard procedures [25]. The anti-MRSA activity of the compounds was evaluated via the disc diffusion method. The ZR11strain was inoculated in Mueller Hinton Broth and incubated at 37°C for 24 h. The turbidity of bacterial suspension was adjusted to 0.5 McFarland standard, which equals 1.5×10^8 colony-forming units (CFU)/mL. Sterile filter paper discs (6 mm) were impregnated with 20 µL (50 µg) of each compound and placed on inoculated Mueller Hinton agar containing bacterial suspension which was adjusted to 0.5 McFarland standard. The commercially available discs containing 30 µg Vancomycin were used as the positive control, whereas discs without samples (5% DMSO) acted as the negative control. The inhibition zones including the diameter of the disc (mm) were measured and compared after incubation at 37°C for 24 h. The tests were carried out in triplicate for each sample.

5-Methoxy-6-methyl-1-(2-methylpropylidene)-1*H*-inden-3-yl)methanol (1), $C_{16}H_{20}O_2$, pale-yellow gum, obtained as a yellow gum. UV (MeOH, λ_{max}, nm) (log ε): 210 (3.84), 262 (3.29), 304 (3.08). IR (KBr, v_{max}, cm^{-1}): 3426, 3075, 2928, 1625, 1552, 1467, 1349, 1160, 1042, 867, 792. ^{1}H and ^{13}C NMR (500 and 125 MHz, $CDCl_3$) data, see Table 1. ESI-MS *m/z* 267 [M + Na]$^+$; HR-ESI-MS *m/z* 267.1361 [M + Na]$^+$ (calcd for $C_{16}H_{20}NaO_2$, 267.1368).

5-Methoxy-3,6-dimethyl-1-(2-methylpropylidene)-1*H*-indene (2), $C_{16}H_{20}O$, pale-yellow gum, obtained as a yellow gum. UV (MeOH, λ_{max}, nm) (log ε): 210 (3.84), 262 (3.29), 304 (3.08). IR (KBr, v_{max}, cm^{-1}): 3070, 2936, 1618, 1548, 1462, 1357, 1158, 1049, 850, 784. ^{1}H and ^{13}C NMR (500 and 125 MHz, $CDCl_3$) data, see Table 1. ESI-MS *m/z* 251 [M + Na]$^+$; HR-ESI-MS *m/z* 251.1408 [M + Na]$^+$ (calcd for $C_{16}H_{20}ONa$, 251.1412).

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