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PENTENYL COUMARINS FROM THE ROOTS AND STEMS OF YUNNAN LOCAL SUN CURED TOBACCO AND THEIR BIOACTIVITY

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Abstract – Three new (**1**-**3**), together with two known (**4** and **5**) pentenyl coumarins were isolated from the roots and stems of Yunnan local sun cured tobacco. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1**~**5** were tested for their anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity. The results revealed that compound **2** showed strong inhibition with inhibition zone diameter (IZD) of 18.6 ±2.6 mm, and compounds **1**, **3**, **4**, and **5** showed good inhibition with IZD of 14.2±2.2, 14.8±2.3, 12.4 ±2.2, and 15.1±1.8 mm, respectively. Compounds **1**-**5** were also tested for the antioxidant activity, and they showed notable antioxidant activity with IC_{50} values of 4.92, 4.18, 4.43, 4.65, and 4.07 μg/mL, respectively.

Nicotiana tabacum (Solanaceae) is an important economic crop because its leaves are used as raw material in tobacco industry.[1,2] In addition, people also used its aerial plant as insecticide, sedative, diaphoretic, anesthetic and emetic agents in Chinese folk medicines.[2] In previous phytochemical investigations on Nicotiana plants, more than 4000 compounds had been reported.[3,4] The main constituents identified were sesquiterpenoids,[5-7] flavonoids,[8-10] alkaloids,[11-13] furans,[14-16] coumarins,[17,18] and the like.

The roots and stems of *N. tabacum* are the main by-product in tobacco planting, and the multipurpose utilization of its roots and stems is an interesting topic, and arouses more and more attentions.[6,9,19] Coumarins also known as benzopyran-2-ones, is an important kind of secondary metabolites found in many high plants, and is probably produced by plants as a defense chemical to discourage predation.

There is a growing interest in coumarins due to their diversity in structural complexity and possess promising therapeutic perspectives with low toxicity.[20,21] In order to increase pharmacodynamic value of *N. tabacum*, our group has investigated the chemical constituents of a roots and stems of Yunnan local sun cured tobacco (Leye tobacco, a variety of *N, tabacum* widely cultivated in Huize prefecture, Yunnan province). As a result, three new (**1**-**3**), together with two known (**4** and **5**) pentenyl coumarins, were isolated. The structures of the isolated components were characterized by comprehensive spectroscopic analyses and by comparison with published data. Compounds **1**-**5** were also tested for their anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) and antioxidant activity.

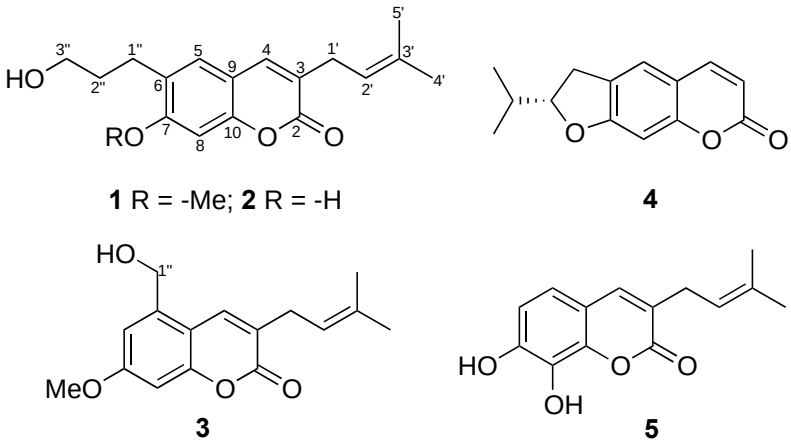

Figure 1. Pentenyl coumarins from the roots and stems of sun cured tobacco

The air-dried and powdered roots and stems of sun cured tobacco (5.8 kg) was extracted with 70% aqueous Me_2CO (3×12 L) under reflux for three times (4 h each) and concentrated under reduced pressure to yield a crude extract, which was suspended in water and partitioned with EtOAc. The EtOAc extract (362 g) was subjected repeatedly to column chromatography on silica gel, Sephadex LH-20, RP-18 and preparative HPLC to afford three new pentenyl coumarins, 7-methoxy-6-(3-hydroxypropyl)-3-prenyl-coumarin (**1**), 6-(3-hydroxypropyl)-7-hydroxy-3-prenylcoumarin (**2**), and 7-methoxy-5-hydroxymethyl-3-prenylcoumarin (**3**), together with two known pentenyl coumarins (**4** and **5**). The structures of the compounds **1**-**5** were as shown in Figure 1, and the ^{1}H and ^{13}C NMR data of the compounds **1**-**3** were listed in Table 1. The known compounds, compared with literature data, were identified as: (*S*)-marmesin (**4**)[22] and indicus H (**5**).[23]

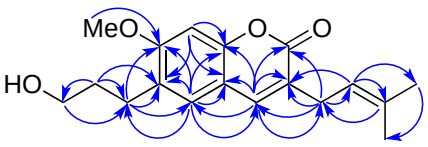

Figure 2. Key HMBC (↷) correlations of **1**

Compound **1** was isolated as a pale-yellow gum. It showed a molecular ion $[M+Na]^+$ at *m/z* 325.1410 in its HRESI-MS, corresponding to the molecular formula $C_{18}H_{22}O_4$ (calculated for 325.1416 $[M+Na]^+$), which indicated eight degrees of unsaturation. Its UV spectrum showed the maximum absorption at 220, 273, 336 nm. Strong absorption bands accounting for hydroxy

(3385 cm^{-1}), carbonyl (1706 cm^{-1}), and aromatic groups (1620, 1558, and 1472 cm^{-1}) could also be observed in its IR spectrum. The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) displayed signals for all 18 carbons and 22 protons, including a 1,2,4,5-tetrasubsititued-benzene (C-5~C-10, H-5, and H-8), an α,β-unsaturated ester carbonyl (-CH=C-C(O)O-, C-2~C-4, H-4), a prenyl group (-CH_2-CH=C(Me)$_2$; C-1'~C-5', H_2-1', H-2', H_3-4' and H_3-5'), a 3-hydroxypropyl group (C-1"~C-3", H_2-1"~H_2-3"), and a methoxy (δ_C 55.8, δ_H 3.85). To support the eight degrees of unsaturation in the molecule, and two oxidized quaternary carbon on benzene ring, the benzene ring and the α,β-unsaturated ester carbonyl should be formed a 3,6,7-trisubstituted coumarin system.[24] This deduction could also be supported by the HMBC correlations (Figure 2) from H-4 to C-2, C-3, C-5, C-9, and C-10, from H-5 to C-4, C-9, and C-10, and from H-8 to C-9 and C-10. In addition, the existence of prenyl group was supported by HMBC correlations from H_2-1′ to C-3′, from H-2′ to C-1′, C-3′, and C-4′, from H_3-4′ to C-3′ and C-2′, and from H_3-5′ to C-3′ and C-2′, and the existence of 3-hydroxypropyl group was supported by HMBC correlations from H_2-1″ to C-2″, from H-2″ to C-1″ and C-3″, and from H-3″ to C-1″.

Table 1. ^{1}H NMR and ^{13}C NMR Data of compounds **1-3 (**in $CDCl_3$, 500 and 125 MHz**)**

No.	Compound **1**		Compound **2**		Compound **3**	
	δ_C (m)	δ_H (m, *J*, Hz)	δ_C (m)	δ_H (m, *J*, Hz)		
2	163.0 s		162.9 s		163.1 s	
3	126.0 s		125.9 s		126.7 s	
4	138.7 d	7.39 s	138.9 d	7.43 s	139.9 d	7.39 s
5	127.4 d	7.10 s	128.4 d	7.05 s	136.7 s	
6	123.8 s		124.5 s		108.2 d	6.95 d (1.8)
7	158.2 s		155.5 s		159.9 s	
8	101.4 d	6.77 s	103.6 d	6.62 s	98.2 d	6.82 d (1.8)
9	114.5 s		115.0 s		122.5 s	
10	152.9 s		153.2 s		154.1 s	
1'	28.9 t	3.33 (d) 7.2	29.0 t	3.29 (d) 7.2	28.9 t	3.30 (d) 7.2
2'	121.0 d	5.25 (t) 7.2	121.2 d	5.29 (t) 7.2	121.6 d	5.29 (t) 7.2
3'	134.6 s		134.4 s		134.0 s	
4'	26.1 q	1.96 s	26.3 q	1.95 s	26.5 q	1.97 s
5'	21.8 q	1.63 s	21.6 q	1.62 s	21.2 q	1.65 s
1"	24.0 t	2.72 (d) 7.8	23.9 t	2.70 (d) 7.8	63.9 t	4.63 s
2"	36.4 t	1.85 m	35.9 t	1.83 m		
3"	63.6 t	3.62 (t) 6.6	63.7 t	3.65 (t) 6.6		
-OMe	55.8 q	3.85 s			55.7 q	3.82 s
Ar-OH				10.87 s		

Since the coumarin skeleton was determined, the positions of substituents (prenyl, 3-hydroxypropyl, and methoxy groups) also can determined by further analysis of its HMBC data (Figure 2). The HMBC correlation from H_2-1" to C-5, C-6, and C-7, from H_2-2" to C-5, from H-5 to C-1" showed the 3-hydroxypropyl group was located at C-6. The HMBC correlations from the H_2-1′ to C-2, C-3, and C-4,

from H-2′ to C-3, and from H-4 to C-1′ indicated the prenyl group was located at C-3. Finally, the HMBC correlations from the methoxy resonance (δ_H 3.85) to C-7 confirmed that the methoxy group was located at C-7. Thus, the structure of **1** was established, and gave the systematic name of 7-methoxy-6-(3-hydroxypropyl)-3-prenylcoumarin.

6-(3-Hydroxypropyl)-7-hydroxy-3-prenylcoumarin (**2**) was obtained as a pale yellow gum and showed a quasi-molecular ion at *m/z* 311.1263 $[M+Na]^+$ in the HRESIMS (calcd *m/z* 311.1259), corresponding to the molecular formula $C_{17}H_{20}O_4$. The ^1H and ^{13}C NMR spectra of **2** were highly similar to those of **1**, indicating that **2** shared the same overall structure with **1**. The chemical shift differences resulted from the highfield shift of C-7 from δ_C 158.2 ppm to δ_C 155.5 ppm, and the disappearance of a methoxy resonance and appearance of a phenolic hydroxy resonance (δ_H 10.87 s) in **2**. These changes indicated that a methoxy group at C-7 in **1** was converted into a phenolic hydroxy group in **2**. The HMBC correlation from the phenolic hydroxy proton (δ_H 10.87) to C-6, C-7 and C-8 also indicated that the phenolic hydroxy group located at C-7. In addition, the positions of the prenyl group and hydroxypropyl group can also be determined by further analysis of its HMBC correlations. The structure of **2** was therefore defined.

Compound **3**, a yellow gum, giving a $[M+Na]^+$ peak at *m/z* 297.1110 in its HRESIMS spectrum, was assigned with a molecular formula of $C_{16}H_{18}O_4$, with one $-CH_2CH_2-$ unit less than that of **1**. The NMR data (Table 1) of **3** were also similar to those of **1** in C-1~C-10 and C-1′~C-5′, except for the disappearance of a hydroxypropyl group and appearance of a hydroxymethyl, and the substituents position variation in **3**. By further analysis of its HMBC correlations, the hydroxymethyl group located at C-5 was supported by the HMBC correlations from H_2-1" to C-5, C-6, and C-9, from H-6 to C-1", the prenyl group located at C-3 was supported by the HMBC correlations from the H_2-1′ to C-2, C-3, and C-4, from H-2′ to C-3, and from H-4 to C-1′, the methoxy group located at C-7 was supported by the HMBC correlation of the methoxy resonance (δ_H 3.82) to C-7. Thus, the structure of 7-methoxy-5-hydroxymethyl- 3-prenylcoumarin (**3**) was established.

Since certain of the coumarin derivatives exhibit potential antibacterial and antioxidant activity.[25,26] Compounds **1**~**5** were evaluated for their antibacterial and antioxidant activity.

The anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity was evaluated for according to arbitrary criterion [27] with inhibition zone diameter (IZD) as follow: 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 was 32 mm and the negative control to zero. The results revealed that compound **2** showed strong inhibition with IZD of 18.6 ±2.6 mm, and compounds **1**, **3**, **4**, and **5** showed good inhibition with IZD of 14.2±2.2, 14.8±2.3, 12.4 ±2.2, and 15.1±1.8 mm, respectively.

The antioxidant activity was also tested by the detection of the oxidative products with the

2',7'-dichlorofluorescin diacetate (DCFH) method reported previously.[28] The results revealed that compounds **1**-**5** shows notable antioxidant activity with IC_{50} values of 4.92, 4.18, 4.43, 4.65 and 4.07 μg/mL, respectively.

RXPERIMENTAL

General Experimental Procedures. 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 internal standard. ESIMS and HRESIMS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. Semi-preparative 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, Qing-dao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40 - 63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 dextran gel (27-163 μm, Sigma-Aldrich, Inc, USA), or MCI gel CHP-50 revised phase resin (50 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H_2SO_4 in EtOH and heating.

Plant Material. The roots and stems of Yunnan local sun cured tobacco (Leye tobacco, a variety of *Nicotiana tabacum* L. widely cultivated in Huize prefecture, Yunnan province) were in Huize prefecture, Yunnan Province, P. R. China, in September 2017. The identification of the plant material was verified by Prof. Y. J. Chen (Yunnan University of Nationalities). A voucher specimen (Ynni-17-09-116) has been deposited in herbarium of Yunnan Minzu University, P. R. China.

Extraction and Isolation. The air-dried and powdered roots and stems of sun cured tobacco (5.8 kg) was extracted with 70% aqueous Me_2CO (3×12 L) under reflux for three times (4 h each), and concentrated under reduced pressure to yield a crude extract, which was suspended in water and partitioned with EtOAc. The EtOAc extract (362 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with $CHCl_3$/MeOH 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, 63.4 g) by silica gel column chromatography, eluted with $CHCl_3$/Me_2CO (9:1-2:1), yielded mixtures B1–B7. Subfraction B1 (9:1, 5.22 g) was subjected to silica gel column chromatography using petroleum ether/Me_2CO, and then semi-preparative HPLC (68% MeOH/H_2O, flow rate 20 mL/min) to give **1** (14.8 mg). Subfraction B2 (8:2, 6.36 g) was loaded on another silica gel column using petroleum ether-EtOAc elution, and then separated semi-preparative HPLC (62% MeOH-H_2O, flow rate 20 mL/min) to afford **2** (15.0 mg), **3** (12.4 mg), and **4** (11.8 mg). Subfraction B3 (7:3, 7.87 g) was separated on the other silica gel column eluted by petroleum ether- EtOAc, followed by

semi-preparative HPLC (54% MeOH-H_2O, flow rate 20 mL/min) to give **5** (20.6 mg).

Anti-MRSA agar disc diffusion assay. The MRSA strain ZR11 was clinical 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.[27] The anti-MRSA activity of the compounds was evaluated via the disc diffusion method. The ZR11 strain was inoculated in Müeller Hinton Broth and was incubated at 37 °C for 24h. The turbidity of bacterial suspension was adjusted to 0.5 McFarland standard which equals to 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 Müeller Hinton agar containing bacterial suspension which adjusted to 0.5 McFarland standard .The commercially available discs containing 30 μg Vancomycin were used as positive control whereas discs without samples (5% DMSO) acted as negative control. The inhibition zones including the diameter of the disc (mm) were measured and compared after incubation at 37 °C for 24h. The tests were carried out in triplicate for each sample.

Antioxidant assay. The antioxidant activity was tested by 2',7'-dichlorofluorescin diacetate (DCFH) method reported previously.[28] Myelomonocytic HL-60 cells (1×10^6 cells/mL, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37 °C in 5% CO_2: 95% air. 125 μL of the cell suspension was added to each well of a 96-well plate. After treatment with a different concentration of the test material for 30 min, the cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Then the cells were incubated for 15 min after the addition of 5 μg/mL DCFH-DA (Mole-cular Probes). DCFH-DA is a non-fluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyse DCFH-DA to 2',7'-dichlorofluorescin (DCFH), and the reactive oxygen species (ROS) generated within HL-60 cells oxidize DCFH to the fluorescent dye 2',7'-dichlorfluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA treated control incubations with and without the test materials. The levels of DCF were measured using a CytoFluor 2350, fluorescence measurement system (Millipore) with an excitation wavelength at 485 nm (bandwidth 20 nm) and emission at 530 nm (band width 25 nm).

7-Methoxy-6-(3-hydroxypropyl)-3-prenylcoumarin (**1**): $C_{18}H_{22}O_4$, obtained as yellow gum, and it has a faint pleasant fragrance similar to vanilla essence; UV (MeOH), λ_{max} (log ε) 220 (4.16), 273 (3.72), 336 (3.90) nm; IR (KBr) ν_{max} 3385, 2964, 2258, 1706, 1620, 1558, 1472, 1261, 1163, 1042, 854, 785 cm^{-1}; ^1H and ^{13}C NMR data ($CDC1_3$, 500 and 125 MHz) see Table1; positive ESIMS *m/z* 325 $[M+Na]^+$; positive HRESIMS *m/z* 325.1410 $[M+Na]^+$ (calcd for $C_{18}H_{22}NaO_4$, 325.1416).

6-(3-Hydroxypropyl)-7-hydroxy-3-prenylcoumarin (**2**): $C_{17}H_{20}O_4$, obtained as yellow gum, and it has a faint pleasant fragrance similar to vanilla essence; UV (MeOH), λ_{max} (log ε) 222 (4.15), 271 (3.69), 334

(3.87) nm; IR (KBr) ν_{max} 3408, 2960, 2264, 1708, 1615, 1542, 1465, 1257, 1156, 1039, 868, 753 cm^{-1}; ^{1}H and ^{13}C NMR data ($CDC1_3$, 500 and 125 MHz) see Table1; positive ESIMS *m/z* 311 $[M+Na]^+$; positive HRESIMS *m/z* 311.1263 $[M+Na]^+$ (calcd for $C_{17}H_{20}NaO_4$, 311.1259).

7-Methoxy-5-hydroxymethyl-3-prenylcoumarin (**3**): $C_{16}H_{18}O_4$, obtained as yellow gum, and it has a faint pleasant fragrance similar to vanilla essence; UV (MeOH), λ_{max} (log ε) 220 (4.22), 270 (3.89), 332 (3.90) nm; IR (KBr) ν_{max} 3378, 2957, 2260, 1704, 1612, 1550, 1457, 1264, 1149, 1062, 845, 792 cm^{-1}; ^{1}H and ^{13}C NMR data ($CDC1_3$, 500 and 125 MHz) see Table1; positive ESIMS *m/z* 297 $[M+Na]^+$; positive HRESIMS *m/z* 297.1110 $[M+Na]^+$ (calcd for $C_{16}H_{18}NaO_4$, 297.1103).

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