*Acta Botanica Sinica*
植 物 学 报 2004, **46** (9): 1122 - 1127 http://www.chineseplantscience.com

# Antibacterial Activities of Neolignans Isolated from the Seed Endotheliums of *Trewia nudiflora*

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**Abstract**: Five neolignans including four new ones were obtained from the seed endotheliums of *Trewia nudiflora* L. Their structures were determined to be 9'-methyl americanol A (**1**), 9'-methyl isoamericanol A (**2**), 9'-ethyl americanol A (**3**), 9'-butyl americanol A (**4**), and americanin (**5**). Two acetylated products 3,4-diacetyl americanin (**5a**) and 3,4,9-triacetyl americanin (**5b**) had been prepared from compound **5**. All of these compounds were investigated on antibacterial assays when carbenicillin sodium, streptomycin sulfate and rifampicin were used as positive controls. Compounds **4** and **5** exhibited antibacterial activities against gram-positive bacterium *Staphylococcus aureus* and gram-negative bacterium *Mycobacterium tuberculosis* at the minimum inhibitory concentrations (MIC) of 50 μg/mL and 100 μg/mL, respectively, but **5a** and **5b** did not exhibit antibacterial activity at 200 μg/disk.
**Key words**: *Trewia nudiflora* ; seed endothelium; neolignan; antibacterial activity

Antimicrobial resistance is emerging in virtually all nosocomial and community pathogen-antimicrobial combinations, which highlights the need to stimulate further research into strategies aimed at preserving the effectiveness of currently available antibacterial agents and finding new classes of antibacterial agents. Natural products produced by higher plants evolved from selection for acquisition of improved defense against microbial attacks (Dixon, 2001), therefore, they are less likely to incur resistance.

The genus *Trewia* (Euphorbiaceae) includes only one species, which spread in India, Malaysia and China. Some maytansinoids isolated from the *Trewia nudiflora* seeds are tumor inhibitors (Powell *et al*., 1981; 1982). There were no reports about the isolation of antibacterial components from the seeds of *T. nudiflora*, particularly, from the seed endotheliums. We report herein the isolation, structure identification and the antibacterial activity against gram-positive bacterium *Staphylococcus aureus* and gram-negative bacterium *Mycobacterium tuberculosis* of seven neolignans including two acetyl derivatives.

## 1 Results and Discussion

Compound **1**, the HRESIMS determined the molecular formula to be $C_{19}H_{20}O_6$ (*m/z* 367.116 7 $[M + Na]^+$, calcd. 367.115 7). The IR (KBr) spectra of **1** revealed the presence of the hydroxyl (3 430 $cm^{-1}$), ether (1 274 $cm^{-1}$), double bond (1 615 $cm^{-1}$) and aromatic (1 586, 1 507 $cm^{-1}$) functionalities, which was supported by the UV absorbance at $\lambda_{max}$ 269.80 nm. The $^1$H-NMR spectrum indicated the presence of a *trans*-double bound (δ 6.49 (d, *J* = 15.9 Hz), δ 6.13 (dd, *J* = 6.2, 15.8 Hz)), two 1,3,4-trisubtituted benzene rings (δ 6.85 (s), δ 6.81 (d, *J* = 8.3 Hz), δ 6.75 (d, *J* = 8.5 Hz) and (δ 6.94 (s), δ 6.91 (d, *J* = 8.5 Hz), δ 6.89 (d, *J* = 8.4 Hz)), and the structure unit involving 1,4-dioxane ring (δ 3.96 (m, 1H), 4.78 (d, *J* = 8.0 Hz)), indicating that **1** was a neolignan. The HMBC experiments showed the $^1$H-$^{13}$C long-range correlations between the methoxyl protons at δ 3.34 (H-10') and the carbon at δ 74.1 (C-9'), and between the methylene protons at δ 4.01 (H-9') and the carbons at δ 58.0 (OMe-9'), 124.7 (C-8') and 133.6 (C-7'), between the methine protons at δ 6.85 (H-2) and the carbons at δ 120.4 (C-6) and 146.9 (C-4), between the methine protons at δ 6.81 (H-5) and the carbons at δ 129.4 (C-1) and 146.4 (C-3), between the methine protons at δ 6.94 (H-2') and the carbons at δ 121.3 (C-6') and 145.1 (C-4'), so determining the structure of the caffeyl alcohol moiety (Fig.1). The structure of the right nine-carbon moiety was determined by the HMBC correlations between the methylene protons at δ 3.65 (H-9) and the carbon at δ 77.5 (C-7), and between the methine proton at δ 4.78 (H-7) and the carbons at δ 62.0 (C-9), 79.8 (C-8), 129.4 (C-1) and 115.7 (C-2). The linkage of the two moieties was determined by the weak three-bond $^1$H-$^{13}$C long-range correlations between the methine proton at δ 4.78 (H-7) and the

Received 20 Nov. 2003 Accepted 24 Jun. 2004
Supported by the National Natural Science Foundation of China (30070007) and Natural Science Foundation of Yunnan Province (99B0017G).
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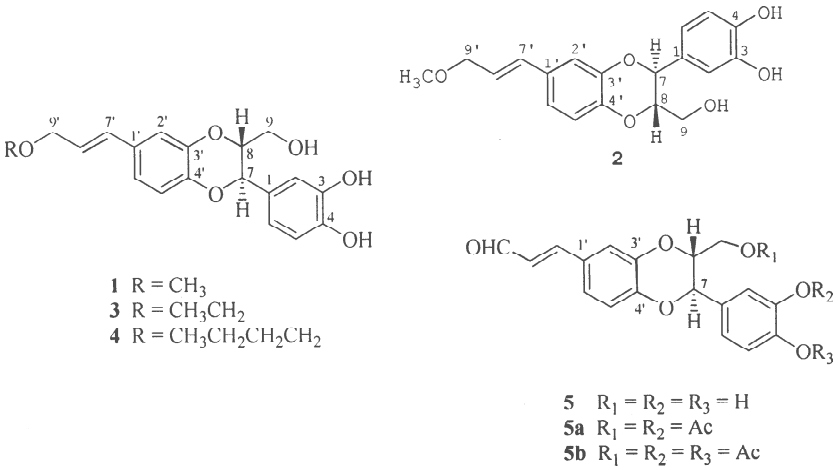


**Fig.1.** The structures of compounds **1 - 5**.

carbon at δ 145.1 (C-4'). The ROESY experiment showed $^1$H-$^1$H correlations between the protons at δ 4.78 (H-7) and at δ 3.46 (H-9b) and 3.65 (H-9a), indicating the *trans*-form of C-7/C-8 in the dioxane ring. Therefore, compound **1** was determined to be 9'-methoxy-7'-en-3',8:4',7-diepoxyneolignan-3,4,9-triol, namely 9'-methyl americanol A (Fukuyama *et al*., 1992).

Compound **2**, the HRESIMS determined the molecular formula to be $C_{19}H_{20}O_6$ (*m/z* 367.115 2 [M + Na]$^+$, calcd. 367.115 7). The $^1$H- and $^{13}$C-NMR spectra of **2** showed great similarities to those of compound **1**. The ROESY experiment showed $^1$H-$^1$H correlations between the protons at δ 4.83 (H-7) and at δ 3.49 (H-9b), indicating the *trans*-form of C-7/C-8 in the dioxane ring. Therefore, compound **2** was determined to be 9'-methyl-7'-en-3',7:4',8-diepoxyneolignan-3,4,9-triol, namely 9'-methyl isoamericanol A (Fukuyama *et al*., 1992).

Compound **3**, the HRESIMS determined the molecular formula to be $C_{20}H_{22}O_6$ (*m/z* 381.131 2 [M + Na]$^+$, calcd. 381.131 4). The data of $^1$H- and $^{13}$C-NMR spectra of **3** showed great similarities to those of compound **1** except for the replacement of methoxyl group with ethoxyl at C-9'. The HMBC experiments showed weak four-bond $^1$H-$^{13}$C long-range correlations between the proton at δ 3.49 (H-9b) and the carbon at δ 144.5 (C-3'), and the ROESY experiment showed $^1$H-$^1$H correlations between the protons at δ 4.77 (H-7) and at δ 3.71 (H-9a), indicating the *trans*-form of C-7/C-8 in the dioxane ring. Therefore, compound **3** was determined to be 9'-ethoxyl-7'-en-3',8:4',7-diepoxyneolignan-3,4,9-triol, namely 9'-ethyl americanol A (Fukuyama *et al*., 1992).

Compound **4**, the HRESIMS determined the molecular formula to be $C_{22}H_{26}O_6$ (*m/z* 409.161 4 [M + Na]$^+$, calcd. 409.162 7). The $^1$H- and $^{13}$C-NMR spectra of **4** showed great similarities to those of compound **1** except for the replacement of methoxyl group with butoxyl at C-9'. The HMBC experiments showed $^1$H-$^{13}$C long-range correlations between the proton at δ 4.79 (H-7) and the carbon at δ 145.3 (C-4'), and weak four-bond $^1$H-$^{13}$C long-range correlations between the proton at δ 3.48 (H-9b) and the carbon at δ 144.9 (C-3'). Therefore, compound **4** was determined to be 9'-butoxyl-7'-en-3',8:4',7-diepoxyneolignan-3,4,9-triol, namely 9'-butyl americanol A (Fukuyama *et al*., 1992).

Compound **5** was readily determined to be 9'-aldehyde-7'-en-3', 7:4' 8-diepoxyneolignan-3,4,9-triol (american) (Woo and Kang, 1978) based on the NMR assignments, and the HMBC correlations between the proton at δ 4.85 (H-7) and the carbon at δ 145.7 (C-3') and the proton at δ 3.51 (H-9b) and the carbon at δ 148.1 (C-4'). The ROESY experiment showed $^1$H-$^1$H correlations between the protons at δ 4.85 (H-7) and at δ 3.51 (H-9b), indicating the *trans*-form of C-7/C-8 in the dioxane ring.

Compounds **1 - 5** isolated from the seed endotheliums and the two acetylated products of **5** were tested against *S. aureus* and *M. tuberculosis*. The disk diffusion method used in the preliminary screening showed that compounds **4** and **5** were active against the tested bacteria, but the acetylated products of compound **5** were inactive at the amount 200 μg/disk (Table 1). Except for compounds **4** and **5**, other natural neolignans did not exhibit any antibacterial activities at the amount 100 μg/disk. The minimum inhibitory concentrations (MIC) of compounds **4** and **5** against *S. aureus* and *M. tuberculosis* were 50 and 100 μg/mL, respectively. The MIC of the positive control streptomycin sulfate was 2 μg/mL.

**Table 1** The antibacterial activities of compounds **1 - 5**, **5a** and **5b**, and positive controls (the diameters of inhibition zones in mm)

| Tested organisms | Compounds (100 μg/disk) | | | | | Acetylated products (200 μg/disk) | | Positive controls | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **5a** | **5b** | Carbenicillin sodium (10 μg/disk) | Streptomycin sulfate (10 μg/disk) | Rifampicin (0.2 μg/disk) |
| *Mycobacterium tuberculosis* | - | - | - | 8.5 | 7.5 | - | - | 15.0 | 11.0 | 15.0 |
| *Staphylococcus aureus* | - | - | - | 8.5 | 7.5 | - | - | 12.0 | 10.0 | 15.0 |

-, no activities.

**Table 2** $^{1}$H-NMR data of compounds **1 - 5**

| Position | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| 2 | 6.85, s | 6.85, s | 6.80, s | 6.85, s | 6.87, d, 1.9 |
| 5 | 6.81, d, 8.3 | 6.81, d, 8.3 | 6.72, d, 8.5 | 6.80, d, 8.2 | 6.82, d, 8.2 |
| 6 | 6.75, d, 8.5 | 6.77, d, 8.5 | 6.74, d, 8.5 | 6.76, d, 8.5 | 6.80, dd, 1.9, 8.3 |
| 7 | 4.78, d, 8.0 | 4.83, d, 8.1 | 4.77, d, 8.2 | 4.79, d, 8.1 | 4.85, d, 8.1 |
| 8 | 3.96, m | 4.02, m | 3.92, br, s | 3.99, m | 4.09, ddd, 2.5, 4.5, 7.8 |
| 9 | 3.65, d, 11.4 | 3.71, d, 11.4 | 3.71, d, 11.4 | 3.67, d, 11.4 | 3.72, dd, 2.4, 12.4 |
| | 3.46, dd, 3.7, 12.2 | 3.49, d, 12.2 | 3.49, dd, 3.7, 12.2 | 3.48, m | 3.51, dd, 4.4, 12.4 |
| 3' | 6.94, s | 7.05, s | 6.93, s | 6.94, s | 7.24, d, 2.0 |
| 5' | 6.91, d, 8.5 | 6.99, d, 8.5 | 6.83, d, 8.3 | 6.91, d, 8.5 | 7.03, d, 8.3 |
| 6' | 6.89, d, 8.4 | 6.90, d, 8.4 | 6.84, d, 8.4 | 6.89, d, 8.4 | 7.23, dd, 2.0, 8.4 |
| 7' | 6.49, d, 15.9 | 6.53, d, 15.9 | 6.46, d, 16.0 | 6.50, d, 15.7 | 7.57, d, 15.8 |
| 8' | 6.13, dd, 6.2,15.8 | 6.16, dd, 6.2, 15.7 | 6.10, dd, 7.8, 14.0 | 6.16, dd, 6.2,15.8 | 6.65, dd, 7.8, 15.8 |
| 9' | 4.01, d, 6.1 | 4.04, m | 4.07, d, 6.2 | 4.08, ddd, 6.1, 6.2, 6.2 | 9.58, d, 7.7 |
| | | | 4.02, d, 6.2 | | |
| RO-9' | $CH_3O$- | $CH_3O$- | $CH_3CH_2O$- | $CH_3CH_2CH_2CH_2O$- | |
| | 3.34, s, 3H | 3.34, s, 3H | 3.48, q, 6.4, 2H | 3.48, m, 2H | |
| | | | 1.23, t, 6.3, 3H | 1.57, m, 2H | |
| | | | | 1.40, q, 7.7, 2H | |
| | | | | 0.93, t, 7.3, 3H | |

$^{1}$H-NMR, and HMBC, $^{1}$H-$^{1}$H COSY and ROESY spectra were obtained at 500 MHz and recorded in $CD_3OD$ at room temperature, respectively. Coupling constants are presented in Hz. Unless otherwise indicated, all proton signals integrate to 1H.

**Table 3** $^{13}$C-NMR data of compounds **1 - 5**

| Position | **1** | **2** | **3** | **4** | **5** |
|---|---|---|---|---|---|
| 1 | 129.4 | 129.4 | 128.0 | 129.6 | 129.1 |
| 2 | 115.7 | 115.6 | 114.2 | 115.5 | 115.6 |
| 3 | 146.4 | 146.7 | 146.7 | 146.6 | 146.7 |
| 4 | 146.9 | 147.2 | 147.2 | 147.1 | 147.3 |
| 5 | 116.3 | 116.4 | 115.3 | 116.4 | 118.6 |
| 6 | 120.4 | 120.4 | 119.6 | 120.4 | 120.4 |
| 7 | 77.5 | 77.7 | 77.7 | 77.7 | 77.6 |
| 8 | 79.8 | 80.0 | 80.0 | 80.1 | 80.5 |
| 9 | 62.0 | 62.1 | 62.1 | 62.1 | 61.9 |
| 1' | 131.6 | 131.8 | 131.8 | 131.9 | 129.1 |
| 2' | 115.8 | 115.8 | 114.8 | 115.8 | 118.1 |
| 3' | 144.7 | 144.9 | 144.5 | 144.9 | 145.7 |
| 4' | 145.1 | 145.5 | 144.9 | 145.3 | 148.1 |
| 5' | 117.9 | 117.3 | 116.8 | 118.0 | 116.4 |
| 6' | 121.3 | 121.0 | 120.0 | 120.9 | 124.0 |
| 7' | 133.6 | 133.6 | 132.3 | 133.3 | 155.2 |
| 8' | 124.7 | 125.0 | 123.9 | 125.3 | 127.8 |
| 9' | 74.1 | 74.2 | 73.1 | 72.5 | 196.0 |
| RO-9' | 58.0 | 58.0 | 65.6 | 71.5 | |
| | | | 15.0 | 32.9 | |
| | | | | 20.3 | |
| | | | | 14.2 | |

$^{13}$C-NMR spectra were obtained at 125 MHz and recorded in $CD_3OD$ at room temperature.

Previous studies showed that neolignans had various bioactivities including antitumour, antimitotic, antiviral activity and specifically inhabiting certain enzymes (Macrae and Towers, 1984), and Zacchino *et al.* (1999) reported that 8-O-4'-neolignans had antifungal activity. But the antibacterial activity of diepoxyneolignan was not reported before our work. The four new neolignans **1 - 4** produced remarkably different inhibitory zones in paper disc diffusion assay (Table 1), indicating that the acyl substitution at C-9' affect the antibacterial activities. Compound **5** gave decent activities against *S. aureus* and *M. tuberculosis* (MIC 100 μg/mL), while its acetylated products **5a** and **5b** were inactive at 200 μg/disc, indicating that the free hydroxyl groups contributed to the antibacterial activities in our experiments as well.

Plant lignans including neolignans isolated in our work are well established phytoalexins, and are widely distributed in various plant tissues. Our experiments indicated the diepoxyneolignan subtype of neolignans had evident antibacterial activity, and showed primary structure-activity relationships, revealing the need to carry out further researches into this type of structures and indicating that seed endotheliums may be unique resources of antimicrobial products.

## 2 Experimental

### 2.1 General experimental procedures

Optical rotations were measured on a JASCO DIP-370 Digital Polarimeter. The IR spectra were measured on a Perkin-Dlmer-577 spectrophotometer. UV spectra were obtained on a Shimadzu double-beam 210A spectrophotometer. The NMR spectra were recorded on Brucker DRX-500 spectrometers. EIMS, ESIMS and LC-MS analysis were carried out on VG AutoSpec-3000 and Finnigen LCQ Advantage spectrometer, respectively. TLC was performed on plates precoated with silica gel (Qingdao Marine Chemical Ltd., China). Reversed-phase $C_{18}$ silica gel for column chromatography was obtained from Merck. Sephadex LH-20 for column chromatography was purchased from Amersham Biosciences. Solvents were distilled before used.

### 2.2 Plant materials

The seeds of *Trewia nudiflora* L. were collected in Xishuangbanna, Yunnan Province of China, December 1998. A voucher specimen (No. K. M. Feng 20159) was deposited at Kunming Institute of Botany, The Chinese Academy of Sciences.

### 2.3 Extraction and isolation

The seed endotheliums of *T. nudiflora* (dry weight: 6.5 kg) were extracted three times with 70% ethanol. The ethanol extract was portioned between EtOAc and water. The EtOAc extract was extracted with petroleum ether and chloroform to produce two fractions PE and CH. Fraction CH (105 g) was subjected to column chromatography over silica gel (300 g, 200 - 300 mesh, Qingdao Marine Chemical Ltd.) eluted with chloroform containing increasing amount of methanol (10 L) to produce nine fractions (CH-1 to CH-9) based on TLC results. Fractions 1 - 7 were determined to mainly contain wax and lipids by TLC and LC-MS analysis, which were free of lignans and did not show antimicrobial activities in our experiments, therefore, were not subjected to further isolation. Fraction CH-8 (1.84 g) was further chromatographed on a silica gel (30 g) column and eluted with chloroform first (60 mL) and then petroleum ether containing increasing amount of acetone (3 L) to produce three fractions (CH-8-1 to CH-8-3). Fraction CH-8-1 (0.643 g) was subjected to column chromatography over Sephadex LH-20 (30 g) eluting with methanol (200 mL) to produce three factions (CH-8-1-1 to CH-8-1-3), and fraction CH-8-1-3 (0.263 g) was subjected to MPLC over reversed-phase $C_{18}$ silica gel (24 g) eluting with MeOH-$H_2O$ (3:2, V/V, 2 L) to afford compound **4** (175 mg). Fraction CH-8-2 (0.5 g) was subjected to column chromatography over Sephadex LH-20 (30 g) eluting with methanol (200 mL) to produce two fractions (fraction CH-8-2-1 and CH-8-2-2), and fraction CH-8-2-2 was subjected to column chromatography over reversed-phase $C_{18}$ silica gel (50 g) eluting with MeOH-$H_2O$ (1:1, V/V, 2.5 L) to afford compound **1** (128 mg). Fraction CH-9 (15.838 g) was further chromatographed on silica gel (80 g) column and eluted with chloroform containing increasing amount of acetone (5 L) to produce six fractions (CH-9-1 to CH-9-6). Fraction CH-9-5 (3.26 g) was subjected to MPLC over reversed-phase $C_{18}$ silica gel (130 g) and eluted with MeOH-$H_2O$ (2:3, V/V, 2.5 L) to afford compound **5** (800 mg). Fraction CH-9-2 (0.5 g) was chromatographed on Sephadex LH-20 column (30 g) eluting with methanol and further subjected to column chromatography over reversed-phase $C_{18}$ silica gel (50 g) eluting with MeOH-$H_2O$ (1:1, V/V) to afford compound **3** (20 mg). Fraction CH-9-6 (2.15 g) was chromatographed on Sephadex LH-20 column (30 g) eluting with methanol (300 mL) to produce four fractions (CH-9-6-1 to CH-9-6-4). Fraction CH-9-6-2 (0.45 g) was chromatographed on reversed-phase $C_{18}$ silica gel column (50 g) and eluted with MeOH-$H_2O$ (1:1, V/V, 1.8 mL) containing increasing amount of methanol to produce compound **2** (5 mg).

### 2.4 Acetylation of compound 5

Compound **5** (300 mg) was dissolved in 20 mL acetic

anhydride and pyridine (1:1, V/V) and warmed at 60 °C for 10 min and stayed at ambient temperature over night. The mixture after being dried with flow nitrogen was subjected to column chromatography over silica gel (30 g) and eluted with chloroform containing increasing amount of acetone (1 L) to produce **5a** (200 mg) and **5b** (100 mg), respectively.

### 2.5 Identification

**Compound 1** White powder. $[\alpha]_D^{23}$ -6.67° (*c* 0.30, MeOH). UV $\lambda_{max}^{MeOH}$ nm: 204, 269, 356; EIMS *m/z* (rel. Int): 344 $[M]^+$ (68), 312 (8), 191 (26), 166 (93), 148 (92), 123 (100), 91 (48), 77 (23); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3 437, 1 615, 1 586, 1 506, 1 436, 1 274. See the $^1H$- and $^{13}C$-NMR data in Table 2 and Table 3, respectively.

**Compound 2** White powder. $[\alpha]_D^{23}$ -40.00° (*c* 0.05, MeOH)/-19.17° (*c* 0.30, MeOH). UV $\lambda_{max}^{MeOH}$ nm: 206, 270, 380; EIMS *m/z* (rel. Int): 344 $[M]^+$ (21), 312 (4), 191 (6), 166 (76), 148 (36), 123 (100), 91 (51), 77 (26); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3 432, 2 925, 1 630, 1 507, 1 442, 1 273. See the $^1H$- and $^{13}C$-NMR data in Table 2 and Table 3, respectively.

**Compound 3** Yellow powder. $[\alpha]_D^{23}$ -1.67° (*c* 0.60, MeOH). UV $\lambda_{max}^{MeOH}$ nm: 206, 270, 380; EIMS *m/z* (rel. Int): 358 $[M]^+$ (31), 344 $[M-CH_3]$ (54), 312 (8), 191 (22), 166 (100), 148 (90), 123 (95), 91 (52), 77 (26); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3 441, 1 612, 1 587, 1 508, 1 449, 1 276. See the $^1H$- and $^{13}C$-NMR data in Table 2 and Table 3, respectively.

**Compound 4** Yellow powder. $[\alpha]_D^{23}$ -7.86° (*c* 1.40, MeOH). UV $\lambda_{max}^{MeOH}$ nm: 206, 270, 380; EIMS *m/z* (rel. Int): 386 $[M]^+$ (60), 312 (15), 166 (100), 148 (85), 123 (80), 91 (36), 77 (14); IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3 423, 2 933, 1 612, 1 587, 1 507, 1 450, 1 274. See the $^1H$- and $^{13}C$-NMR data in Table 2 and Table 3, respectively.

**Compound 5** Yellow crystal. EIMS *m/z* (rel. Int): 228 $[M]^+$ (100), 310 (21), 166 (50), 148 (49), 123 (70), 91 (28), 77 (23). See the $^1H$- and $^{13}C$-NMR data in Table 2 and Table 3, respectively.

**Compound 5a** Yellow oil, $[\alpha]_D^{23}$ -8.6° (*c* 0.70, MeOH), ESIMS *m/z*: 455 $[M+1]^+$.

**Compound 5b** Yellow oil, $[\alpha]_D^{23}$ -10.9° (*c* 0.55, MeOH), ESIMS *m/z*: 413 $[M+1]^+$.

### 2.6 Antibacterial assay

The preliminary screening of the antibacterial activity was assessed against *S. aureus* and *M. tuberculosis* by using disk diffusion method (Jorgensen *et al*., 1999). Carbenicillin sodium (10 μg), streptomycin sulfate (10 μg) and rifampicin (0.2 μg) were used as the positive controls, respectively. The minimum inhibitory concentration (MIC) of compounds **4** and **5** was evaluated using agar dilution technique (Jorgensen *et al*., 1999) in Petri dishes (containing beef extract, peptone and agar). Different concentrations of the two compounds were dissolved in dimethyl sulfoxide (DMSO) to give serious twofold dilutions that were added to each dish, resulting in the concentrations of testing compounds ranging from 25 to 400 μg/mL, and streptomycin sulfate was used as positive control.

**Acknowledgements:** The authors are grateful to Mr. HE Yi-Neng and Ms. LIANG Hui-Ling in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, The Chinese Academy of Sciences, for measuring NMR and MS data, respectively.

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(Managing editor: WANG Wei)