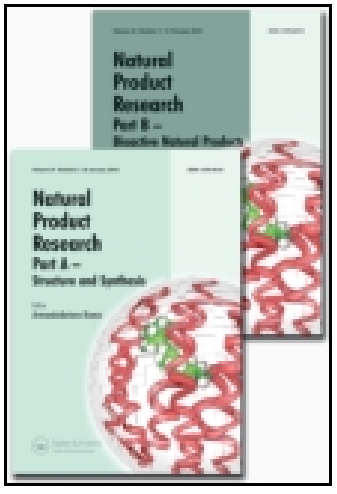

Natural Product Research: Formerly Natural Product Letters
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

A new picrotoxane sesquiterpene from the berries of Baccaurea ramiflora with antifungal activity against Colletotrichum gloeosporioides

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Published online: 13 Jan 2015.

To cite this article: Zheng-Hong Pan, De-Sheng Ning, Si-Si Huang, Yun-Fei Wu, Tao Ding & Lei Luo (2015) A new picrotoxane sesquiterpene from the berries of Baccaurea ramiflora with antifungal activity against Colletotrichum gloeosporioides, Natural Product Research: Formerly Natural Product Letters, 29:14, 1323-1327, DOI: 10.1080/14786419.2014.999335

To link to this article: http://dx.doi.org/10.1080/14786419.2014.999335

Natural Product Research, 2015
Vol. 29, No. 14, 1323–1327, http://dx.doi.org/10.1080/14786419.2014.999335

A new picrotoxane sesquiterpene from the berries of *Baccaurea ramiflora* with antifungal activity against *Colletotrichum gloeosporioides*

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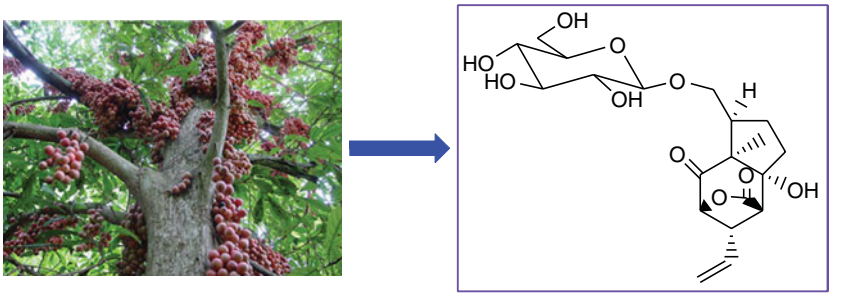
(*Received 12 October 2014; final version received 11 December 2014*)



Three picrotoxane sesquiterpenes including one new glycoside and two known constituents, sapidolide A (**2**) and picrotoximaesin (**3**), were isolated from the berries of *Baccaurea ramiflora*. The structure of the new sesquiterpene glycoside, ramifloside (**1**), was elucidated as 2-one-6α-hydroxy-13-nor-11-picrotoxen-3(15β)-olide 10-*O*-β-D-glucopyranoside on the basis of extensive spectroscopic analysis. Compounds **1–3** exhibited antifungal activity against *Colletotrichum gloeosporioides* with MICs of 12.5, 12.5 and 50 μg/mL.

Keywords: *Baccaurea ramiflora*; picrotoxane; sesquiterpene glycoside; antifungal activity; ramifloside

1. Introduction

Baccaurea ramiflora Lour., a tall evergreen tree in the family Euphorbiaceae, is widely distributed in the Karst region of southwest China (Li 1994). The whole plant has been used as a folk medicine in China to treat rheumatoid arthritis, cellulitis, apostema, injury from fall, etc. (Lin et al. 2003). Previous chemical investigations of *B. ramiflora* have showed the presence of phenolic, sesquiterpenoid and a series of volatile components (Yang et al. 2007, 2010; Xu, Guan, et al. 2007; Xu, Lin, et al. 2007). As a part of our search for naturally occurring bioactive metabolites from Karst plants, we investigated the chemical constituents of the berries of *B. ramiflora*, which led to the isolation of a new nor-picrotoxane sesquiterpene glycoside, named ramifloside (**1**), along with two picrotoxane sesquiterpenes: sapidolide A (**2**) and picrotoximaesin (**3**) (Bordoloi et al. 1996; Tane et al. 1996) (Figure 1). To the best of our

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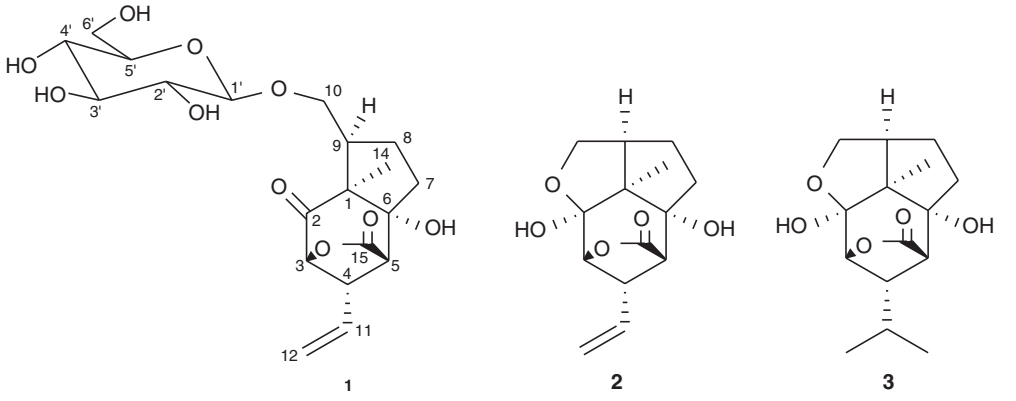

Figure 1. Structure of compounds **1**–**3**.

knowledge, the new compound was the first nor-picrotoxane glycoside from plant origin, and the two known constituents were reported for the first time from this plant. This article describes the isolation, structural elucidation and antifungal activity of these picrotoxane sesquiterpenes.

2. Results and discussion

Compound **1** was obtained as a white amorphous solid. The molecular formula of **1** was established to be $C_{20}H_{28}O_{10}$ by HR-ESI-MS at *m/z* 451.1576 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_{10}Na$, 451.1575), indicating seven degrees of unsaturation. Its IR spectrum implied the presence of hydroxyl (3424 cm^{-1}) and carbonyl (1794 and 1711 cm^{-1}) groups. The ^{13}C NMR and DEPT spectra exhibited 20 carbon signals due to 1 methyl, 5 methylene (including 2 oxygenated and 1 olefinic), 10 methine (including 6 oxygenated and 1 olefinic), 2 quaternary carbon (including 1 oxygenated) and 2 carbonyl resonances. The ^1H NMR spectrum revealed the presence of a terminal double bond at δ_H 5.16 (2H, dd, $J = 17.5$, 10.5 Hz) and δ_H 6.07 (ddd, $J = 17.5$, 10.5, 10.5 Hz), and a tertiary methyl singlet at δ_H 1.29. The six characteristic ^{13}C NMR signals at δ_C 104.3, 77.5, 77.5, 74.9, 71.1 and 62.3 suggested the presence of a glucose moiety. Gas chromatography (GC) analysis of hydrolyzate of **1** established the D-configuration of the sugar moiety. Careful analysis of NMR data of **1** revealed that the remaining signals were very similar to those of sapidolide A (**2**), except for the presence of the above sugar signals and an extra ketonic signal (δ_C 209.1) in **1** and the absence of C-2 signal (δ_C 103.0) in **2**. The HMBC correlations of a ketonic carbon at δ_C 209.1 with H-3 (δ_H 4.53), H-4 (δ_H 3.65), H-9 (δ_H 2.22) and H-14 (δ_H 1.29) indicated that the ketonic group was attached to C-2 (see Supplementary material, Figure S1). The glucose moiety located at C-10 was confirmed by the HMBC correlation between H-1′ (δ_H 4.13) and C-10 (δ_C 70.8) and has a β-anomeric orientation determined by the large coupling constant ($J = 9.0$ Hz) of the anomeric proton H-1′. In order to better determine the stereochemistry of **1**, the NMR spectra of **1** recorded in DMSO-d_6 solvent were determined (see Supplementary material, Figures S8–S11). The relative configuration of the aglycone of **1** was characterised on the basis of the NOESY spectrum, in which NOE correlations were found between H-11 and H-3, H-5, Me-14, OH-6; H-9 and Me-14, OH-6; and H-3 and Me-14. Furthermore, the NMR data of **1** were in very close accord with those of sapidolide A (**2**), which was also isolated from the same plant, and its stereochemistry was confirmed by X-ray diffraction experiment (Bordoloi et al. 1996). Therefore, the structure of compound **1** was elucidated as 2-one-6α-hydroxy-13-nor-11-picrotoxen-3(15β)-olide 10-*O*-β-D-glucopyranoside, named as ramifloside.

Because detailed ^{1}H and ^{13}C NMR spectral data of sapidolide A (**2**) were not reported in the literature (Bordoloi et al. 1996), we performed the complete assignments of 1D NMR spectra of **2** (see Supplementary material, Table S1). The other known compound was identified as picrotoximaesin (**3**) by analysis of the spectroscopic data and comparison with the literature values (Tane et al. 1996). Compounds **2** and **3** were isolated from *B. ramiflora* for the first time.

Since sapidolide A (**2**) was reported to exhibit antifungal activity (Bordoloi et al. 1996), the three isolates were tested for the antifungal activity against the phytopathogen *Colletotrichum gloeosporioides*, using carbendazim as a positive controls (MIC = 6.25 μg/mL). Compounds **1** and **2** exhibited significant antifungal activities against *C. gloeosporioides* with minimum inhibitory concentrations (MICs) of 12.5 and 12.5 μg/mL, while compound **3** exhibited weak antifungal activity with MIC of 50 μg/mL. The above results suggest that the terminal double bond in **1** and **2** plays an important role in the antifungal activity.

3. Experimental

3.1. *General experimental procedures*

Optical rotations were measured with a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). IR spectroscopy was measured in a Bio-Rad FTS-135 spectrometer (Bio-Rad, Richmond, CA, USA) with KBr pellets. MS were performed on an Agilent 6500 Q-TOF spectrometer (Agilent Technologies, Santa Clara, CA, USA). 1D NMR and 2D NMR spectra were recorded on DRX-500 spectrometer (Bruker Co., Ettlingen, Germany) with TMS as internal standard. Unless specified, chemical shifts (δ) are expressed in ppm with reference to the solvent signals. Column chromatography was performed either on silica gel (100–200 and 200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm; Merck, Darmstadt, Germany) or on Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsala, Sweden). Fractions were monitored by TLC, and spots were visualised by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. Solvents were distilled before use.

3.2. *Plant material*

The berries of *B. ramiflora* were collected from Longzhou County of Guangxi province, China, in August 2013. Voucher specimens (GXIB20130801) were deposited at the Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization, Guangxi Institute of Botany and were identified by one of the authors (Associate Professor Tao Ding).

3.3. *Fungal material*

The fungal strain *C. gloeosporioides* was purchased from the Agricultural Culture Collection of China, Beijing, China. It was deposited at the Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization, Guangxi Institute of Botany. This fungus was incubated in the potato dextrose (PD) medium.

3.4. *Extraction and isolation*

The air-dried and powdered berries of *B. ramiflora* (2.7 kg) were extracted with EtOH (3 × 10 L, each 24 h) at room temperature and filtered. The filtrate was evaporated to give a residue, which was suspended in H_2O (3 L) and then extracted with EtOAc (4 × 2 L). The EtOAc extract (580 g) was subjected to silica gel column chromatography with a step gradient system (petroleum ether–Me_2CO, 1:0 to 0:1) to obtain fractions 1–4. Fraction 2 was subjected to silica gel column chromatography (petroleum ether–Me_2CO, 4:1 to 0:1) and then further purified by Sephadex

LH-20 ($CHCl_3$–MeOH, 1:1) to yield **2** (500 mg) and **3** (60 mg). Fraction 3 was further subjected to silica gel column chromatography using petroleum ether–Me_2CO (4:1 to 0:1) as eluent to provide subfractions 3.1–3.3. Subfraction 3.2 was applied to an RP-18 gel column with a gradient elution (H_2O–MeOH, 40–80%), and further purified by silica gel column chromatography ($CHCl_3$–Me_2OH, 9:1) to obtain compound **1** (260 mg).

3.4.1. *Ramifloside (**1**)*

White amorphous solid; $[\alpha]_D^{12.4}$ − 8.8 ($c = 0.11$, MeOH); IR (KBr) ν_{max} cm^{-1}: 3424, 2922, 1794, 1711, 1635, 1382, 1315, 1075, 1019; HR-ESI-MS (positive mode) *m/z*: 451.1576 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_{10}Na$, 451.1575). ^1H NMR data (MeOH; 500 MHz), δ: 4.53 (1H, d, $J = 5.5$ Hz, H-3), 3.65 (1H, m, H-4), 2.87 (1H, d, $J = 4.5$ Hz, H-5), 2.33 (1H, dd, $J = 6.5$, 12.5 Hz, H-7a), 1.84 (1H, m, H-7b), 1.91 (1H, m, H-8a), 1.35 (1H, m, H-8b), 2.22 (1H, m, H-9), 3.88 (1H, dd, $J = 4.5$, 9.5 Hz, H-10a), 3.16 (1H, br d, $J = 9.5$ Hz, H-10b), 6.07 (1H, ddd, $J = 10.5$, 10.5, 17.5 Hz, H-11), 5.16 (2H, dd, $J = 10.5$, 17.5 Hz, H_2-12), 1.29 (3H, s, H_3-14), 4.13 (1H, d, $J = 9.0$ Hz, H-1′), 3.13 (1H, dd, $J = 7.5$, 9.0 Hz, H-2′), 3.32 (1H, m, H-3′), 3.28 (1H, m, H-4′), 3.24 (1H, m, H-5′), 3.82 (1H, dd, $J = 12.0$, 2.0 Hz, H-6′a), 3.68 (1H, m, H-6′b). ^{13}C NMR data (MeOH; 125 MHz), δ: 59.6 (C-1), 209.1 (C-2), 85.0 (C-3), 49.6 (C-4), 54.8 (C-5), 82.7 (C-6), 41.2 (C-7), 28.0 (C-8), 55.1 (C-9), 70.8 (C-10), 132.4 (C-11), 120.0 (C-12), 24.8 (C-14), 176.5 (C-15), 104.3 (C-1′), 74.9 (C-2′), 77.5 (C-3′), 71.1 (C-4′), 77.5 (C-5′), 62.3 (C-6′).

3.5. *Acid hydrolysis of compound 1 and determination of sugar component*

Compound **1** (1.0 mg) was hydrolysed with 1 M HCl–dioxane (1:1, 1 mL) at 80°C for 4 h. The reaction mixture was partitioned between EtOAc and H_2O three times. The aqueous layer was neutralised with 2 M $NaHCO_3$ and evaporated *in vacuo*. The residue was dissolved in pyridine (0.5 mL), to which L-cysteine methyl ester hydrochloride in pyridine (0.1 M, 0.5 mL) was added. After reacting at 60°C for 1 h, trimethylsilylimidazole (0.5 mL) was added to the reaction mixture and kept at 60°C for another 30 min. The mixture was partitioned between *n*-hexane and H_2O, and the *n*-hexane extract was analysed by GC–MS. By comparison of the retention time of the authentic sample, the monosaccharide of compound **1** was determined to be D-glucose ($t_R = 19.25$).

3.6. *Antifungal activity against* C. gloeosporioides

Antifungal assay was performed by the microdilution method in 96-well flat-microtitre plates using PD medium (Li et al. 2012). The compounds were made up to 2 mg/mL in DMSO. Carbendazim was used as positive control and the equal concentration of DMSO was used as a negative control. The fungus was incubated in the PD medium for 18 h at 28 ± 0.5°C at 150 rpm, and spores of different microorganism concentrations were diluted to approximately 1×10^6 CFU with PD medium. In flat-microtitre plates, tested compounds, fungal suspension and sterile water were added to make up final concentrations of the compounds in the range of 1.54–200 μg/mL. After incubation at 28 ± 0.5°C for 48 h, MIC was determined as the lowest concentrations that produce complete growth inhibition of the tested microorganisms. All experiments were repeated three times.

4. Conclusion

In conclusion, three picrotoxane sesquiterpenes were isolated from the berries of *B. ramiflora*, including one new glycoside named as ramifloside (**1**). Compounds **1**–**3** exhibited antifungal activity against *C. gloeosporioides* with MICs of 12.5, 12.5 and 50 μg/mL.

Supplementary material

Supplementary material relating to this article is available online, alongside Table S1 and Figures S1–S15.

Funding

This work was supported financially by the National Natural Science Foundation of China [number 21102023] and the funds of Guangxi Key Laboratory of Functional Phytochemicals Research and Utilization [number ZRJJ2012-8], [number ZRJJ2013-8].

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