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Dihydro-β-agarofurans from the roots of the Australian endemic rainforest tree *Maytenus bilocularis* act as leucine transport inhibitors

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ABSTRACT

Phytochemical studies of the roots of the Australian plant, *Maytenus bilocularis*, resulted in the identification of six previously undescribed dihydro-β-agarofuran sesquiterpenoids, bilocularins D–I, along with three known natural products, namely 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran, pristimerin, and celastrol. The structures of all compounds were characterized via analysis of 1D/2D NMR and MS data. The absolute configuration of bilocularin D was defined by X-ray crystallography analysis. Bilocularins D and G, 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran, and celastrol inhibited leucine transport in the human prostate cancer cell line LNCaP with IC_{50} values ranging from 2.5–27.9 μM, which were more potent than the L-type amino acid transporter (LAT) family inhibitor 2-aminobicyclo[2,2,1]-heptane-2-carboxylic acid (BCH). Bilocularins D–F are the first examples of dihydro-β-agarofurans bearing a hydroxyacetate group.

1. Introduction

L-Type amino acid transporters (LATs) facilitate the transport of neutral amino acids, such as leucine, isoleucine, and valine through cell membranes (Grkovic et al., 2015). LAT family members are crucially important for cell survival since they mediate the uptake of essential amino acids, which are necessary for energy and protein production. The overexpression of LATs, such as LAT1 (SLC7A5) and LAT3 (SLC43A1) has been observed in various human cancers, including prostate cancer (Wang et al., 2011; Wang et al., 2013b). Furthermore, the inhibition of LAT1 and LAT3 has been reported to restrict leucine uptake, thereby regulating the activity of mammalian target of rapamycin complex 1 (mTORC1) (Wang et al., 2011; Wang et al., 2013b). Leucine is an essential amino acid needed for mTORC1 activation (Kimball et al., 1999). Since protein translation can only commence at a sufficient intra-cellular level of leucine, the inhibition of leucine transport into the cell presents an opportunity to inhibit the growth of cancer cells (Wang et al., 2011), and the LAT family transporters may therefore prove to be a novel drug target for future cancer therapies (Wang and Holst, 2015).

Recently, we have reported the chemical investigation of the leaves of the Australian rainforest plant, *Maytenus bilocularis* (F. Muell.) Loes (Celastraceae), that resulted in the discovery of the previously undescribed natural products, bilocularins A–C (**1**–**3**) (Wibowo et al., 2016a). Bilocularins A (**1**) and B (**2**) were the first reported dihydro-β-agarofurans, which inhibited leucine uptake in prostate cancer cells (Wibowo et al., 2016a). Moreover, several other dihydro-β-agarofuran natural products isolated from two other Australian Celastraceae plants, *Denhamia pittosporoides* and *Celastrus subspicata*, were subsequently shown by our group to possess leucine uptake inhibitory activity in the human prostate cancer cell line, LNCaP (Wibowo et al., 2016b, 2017). Driven by our

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previous discovery of dihydro-β-agarofurans that inhibited leucine uptake from the leaves of *M. bilocularis*, we extended our studies to the roots of *M. bilocularis*, which so far had not been phytochemically characterized. This paper reports the structures of six previously undescribed compounds, namely bilocularins D–I (**4**–**9**) together with three known natural products, 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran (**10**) (Tu, 1990), pristimerin (**11**) (Alvarenga et al., 1999), and celastrol (**12**) (Ngassapa et al., 1994). Furthermore, a subset of the discovered compounds was characterized with respect to inhibition of leucine uptake in the human prostate cancer cell line, LNCaP.

2. Results and discussion

The CH_2Cl_2 extract obtained from the roots of *Maytenus bilocularis* was separated by various chromatographic techniques to afford six previously undescribed dihydro-β-agarofuran sesquiterpenoids, bilocularins D–I (**4**–**9**), and three known natural products (Fig. 1).

Bilocularin D (**4**) was isolated as stable white crystals. The molecular formula $C_{37}H_{42}O_{13}$ was determined by (+)-HRESIMS data (*m/z* 717.2515 $[M+Na]^+$, calcd for 717.2518). The ^1H NMR spectrum of **4** exhibited signals for five methyl singlets (δ_H 1.56, 1.57 (6H), 2.11, and 2.14), four sets of methylene protons (δ_H 2.03/2.22, 2.22/2.62, 3.89/3.92, and 4.88/4.96), 17 methine protons (δ_H 2.32, 5.30, 5.76, 5.54, 6.17, 6.31, 7.38 (3H), 7.53 (4H), 7.62, 7.67, and 8.18 (2H)), and two hydroxy groups (δ_H 2.18 and 2.96); the latter two signals were confirmed following HSQC experiment. The ^{13}C NMR and HSQC spectra revealed 37 carbon resonances including five methyls, four methylenes, 17 methines, and 11 non-protonated carbons. The presence of five ester groups in **4** was suggested by the ^{13}C NMR signals at δ_C 165.6, 166.8, 170.0, 170.2, and 172.4. These data indicated that compound **4** belonged to the dihydro-β-agarofuran structure class (Wibowo et al., 2016a; Wibowo et al., 2016b, 2017), which was confirmed by detailed analysis of COSY and HMBC spectra (Fig. 2). Analysis of the HMBC spectrum indicated the presence of two acetate, a benzoate, a *trans*-cinnamate, and a hydroxyacetate moieties in **4**. The two acetates were located at C-2 and C-6 based on HMBC correlations from H-2 (δ_H 5.54) and H-6 (δ_H 6.17) to the ester carbonyls at δ_C 170.0 and 170.2, respectively. The benzoate group was attached to C-15 based on HMBC correlations from H_2-15 (δ_H 4.88/4.96) to the carbonyl carbon at δ_C 166.8. Similarly, HMBC correlations from the olefinic proton (δ_H 7.67) and H-9 (δ_H 5.30) to a carbon signal at δ_H 165.5 located the *trans*-cinnamate functionality at C-9. The presence of the hydroxyacetate group was confirmed by HMBC correlations from the exchangeable proton (δ_H 2.18) and the oxymethylene (δ_H 3.89/3.92) to a carbonyl resonance at δ_C 172.4. Furthermore, an HMBC correlation from H-1 (δ_H 5.76) to the carbon signal at δ_C 172.4 positioned the hydroxyacetate at C-1. Finally, a hydroxy group was attached at C-4 by considering the deshielded ^{13}C NMR resonance of C-4 (δ_C 70.0) and the HMBC correlation from the hydroxy proton signal at δ_H 2.96 to C-4.

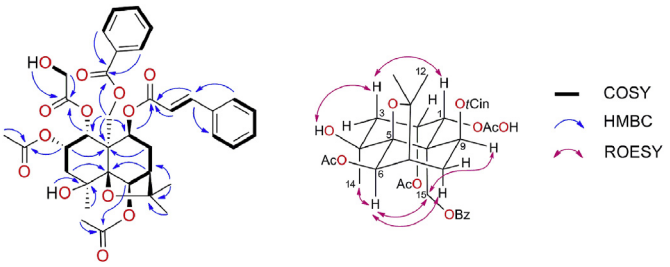

Fig. 2. Diagnostic 2D NMR correlations for bilocularin D (**4**).

The relative configuration of **4** was assigned following analysis of ^1H-^1H coupling constants and the ROESY spectrum (Fig. 2). The β-orientation of H-1 and H-2 was assigned based on the small coupling constant between the two protons ($J_{1,2} = 3.5$ Hz) (Gao et al., 2016; Núñez et al., 2016). The ROESY spectrum of **4** exhibited correlations between H-1 and H-3β, indicating that these two atoms were co-facial. Furthermore, ROESY cross-peaks between H-6 and H_2-15, as well as between H_3-14 and H-6 determined the α-orientation of H-6, CH_3-14, and CH_2-15. The structure of **4** was confirmed by X-ray crystallography studies (Fig. 3), which also defined its absolute configuration as (1*R*,2*S*,4*S*,5*S*,6*R*,7*R*,9*S*,10*R*)-2,6-diacetoxy-15-benzoyloxy-9-cinnamoyloxy-1-hydroxyacetoxy-4-hydroxydihydro-β-agarofuran.

Compound **5**, which was purified as a white amorphous powder, had a molecular formula of $C_{39}H_{44}O_{13}$, which was assigned

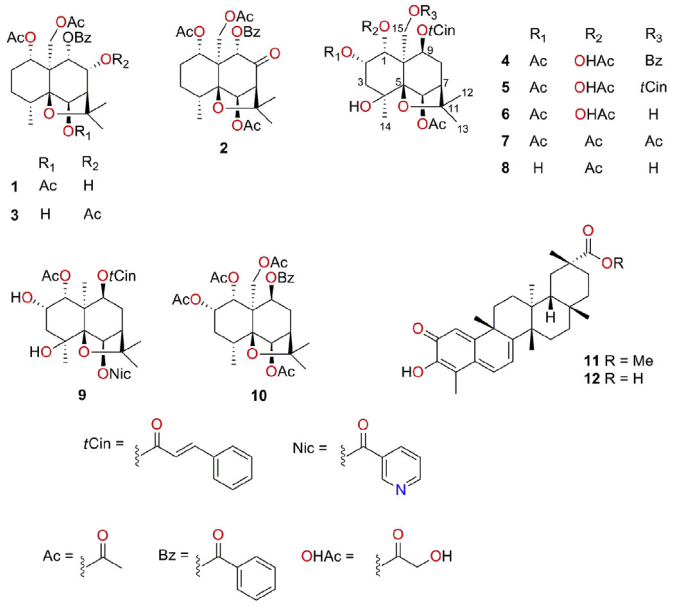

Fig. 1. Structures of bilocularins A–I (**1**–**9**), 1α,2α,6β,15-tetraacetoxy-9β-benzoyloxydihydro-β-agarofuran (**10**), pristimerin (**11**), and celastrol (**12**).

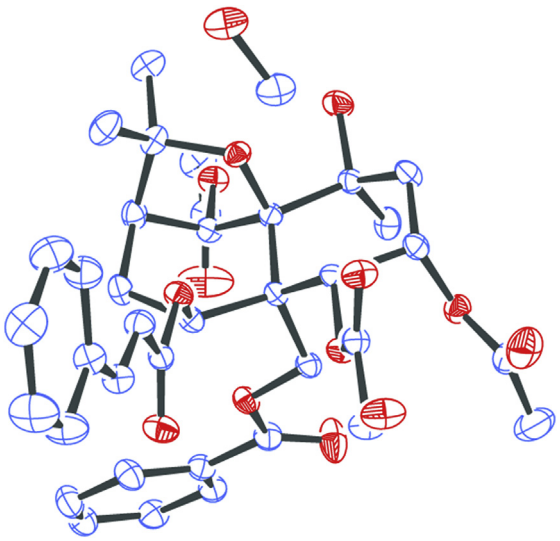

Fig. 3. ORTEP of bilocularin D (**4**), hydrogen atoms are omitted for clarity.