Bioactive Dihydro-β-agarofuran Sesquiterpenoids from the Australian Rainforest Plant *Maytenus bilocularis*

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ABSTRACT: Chemical investigations of the CH_2Cl_2 extract obtained from the leaves of the Australian rainforest tree *Maytenus bilocularis* afforded three new dihydro-*β*-agarofurans, bilocularins A−C (**1**−**3**), and six known congeners that included celastrine A (**4**), 1*α*,6*β*,8*α*-triacetoxy-9*α*-benzoyloxydihydro-*β*-agarofuran (**5**), 1*α*,6*β*-diacetoxy-9*α*-benzoyloxy-8*α*-hydroxydihydro-*β*-agarofuran (**6**), Ejap-10 (**11**), 1*α*,6*β*-diacetoxy-9*β*-benzoyloxydihydro-*β*-agarofuran (**12**), and Ejap-2 (**13**). The major compound **1** was used in semi-synthetic studies to afford four ester derivatives (**7**–**10**). The chemical structures of **1**−**3** were elucidated following analysis of 1D/2D NMR and MS data. The absolute configurations of bilocularins A (**1**) and B (**2**) were determined by single-crystal X-ray diffraction analysis. All compounds were evaluated for cytotoxic activity against the human prostate cancer cell line LNCaP; none of the compounds were active. However, several compounds showed similar potency as the drug efflux pump inhibitor verapamil in reversing the drug resistance of the human leukemia CEM/VCR R cell line. In addition, similar to verapamil, compound **5** was found to inhibit leucine uptake in LNCaP cells (IC_{50} = 15.5 μM), which was more potent than the leucine analogue 2-aminobicyclo[2,2,1]heptane-2-carbocyclic acid (BCH). This is the first report of secondary metabolites from *Maytenus bilocularis.*

The Celastraceae family is widely found in tropical and subtropical regions of the world. The potential of Celastraceae plants as producers of secondary metabolites that possess a broad range of bioactivity has attracted much interest as shown by the large number of natural products isolated from this family over the last 30 years.[1,2] Terpenoids, including monoterpenoid, sesquiterpenoid, diterpenoid, and triterpenoid are the most common bioactive constituents found in this family. However, the typical metabolites of the Celastraceae that have attracted the most interest are a class of poly-oxygenated tricyclic sesquiterpenoids known as dihydro-*β*-agarofurans.[1,2] They are known to exhibit a broad range of biological effects, such as cytotoxic,[3] anti-HIV,[4] multidrug resistance reversal,[5] neuroprotective,[6] and chemopreventive[7] activities.

The genus *Maytenus*, which consists of approximately 225 plant species, is one of the largest genera in the Celastraceae family.[8] *Maytenus* species have widely been used in folk medicine as remedies for treatment of several diseases, such as gastric ulcers, arthritis, and inflammatory disease.[8,9] The interest in *Maytenus* increased significantly following the discovery of the highly cytotoxic compound, maytansine, from an African plant, *Maytenus ovatus*.[8,10] *Maytenus bilocularis*, locally known as "orangebark", is an endemic Australian tree growing up to 10 m tall in the rainforests of New South Wales and Queensland.[11,12] Although many recent studies have reported the discovery of new bioactive constituents possessing various activities from *Maytenus*,[13-15] *M. bilocularis* has not been phytochemically investigated. Owing to an ongoing interest in the chemistry of Australian Celastraceae species,[3,16] we therefore selected *M. bilocularis* for chemical investigation.

Herein the isolation and structure elucidation of three new and six known dihydro-*β*-agarofuran sesquiterpenoids from a CH_2Cl_2 extract of the leaves of *M. bilocularis* are reported. All compounds were tested for their cytotoxicity toward the LNCaP human prostate cancer cell

line. Furthermore, the drug resistance reversal ability of a subset of compounds in the vinca alkaloid-resistant human leukemia CEM/VCR R cell line, and their effects on neutral amino acid uptake are also reported.

RESULTS AND DISCUSSION

The CH_2Cl_2 extract of the leaves of *M. bilocularis* was subjected to silica gel flash column chromatography and further purified by RP-HPLC to yield three new dihydro-*β*-agarofurans, bilocularins A−C (**1**−**3**), and six known congeners that included celastrine A (**4**), 1*α*,6*β*,8*α*-triacetoxy-9*α*-benzoyloxydihydro-*β*-agarofuran (**5**), 1*α*,6*β*-diacetoxy-9*α*-benzoyloxy-8*α*-hydroxydihydro-*β*-agarofuran (**6**), Ejap-10 (**11**), 1*α*,6*β*-diacetoxy-9*β*-benzoyloxydihydro-*β*-agarofuran (**12**), and Ejap-2 (**13**) (Figure 1).

Bilocularin A (**1**) was obtained as stable colourless crystals and was assigned the molecular formula $C_{28}H_{36}O_{10}$ following analysis of the (+)-HRESIMS sodium adduct ion at *m/z* 555.2197 $[M+Na]^+$ (calcd for $C_{28}H_{36}O_{10}Na$, 555.2201). The ^{1}H NMR spectrum of **1** revealed the signals corresponding to six methyl groups at δ_H 1.00, 1.42, 1.46, 1.50, 2.10, and 2.29, four oxygenated methine protons (δ_H 4.42, 5.40, 5.55, and 6.56), a pair of coupled oxymethylene doublets (δ_H 4.60, 4.92, J = 12.9 Hz), and one benzoyl group (5H, δ_H 7.46, 7.57, 8.06). The ^{13}C NMR and edited HSQC spectra suggested the presence of 28 carbon atoms, consisting of six methyls, three methylenes, eleven methines, and eight non-protonated carbons. The ^{13}C NMR resonances at δ_C 165.1, 169.8, 170.1, and 170.5 were indicative of four ester groups in **1**. Two oxygenated tertiary carbon signals were observed at δ_C 81.2 and δ_C 90.7, which were characteristic of the ethereal carbons of a dihydro-*β*-agarofuran.[3,17] Fragments of H-1/H_2-2/H_2-3/H-4/H_3-14 and H-6/H-7/H-8/H-9 were readily established from the ^{1}H-^{1}H COSY data of **1** (Figure 2). The remaining

structure of **1** was deduced following analysis of HMBC data. HMBC correlations from both H-1 and H-4 to C-10, as well as from H-4 and H_3-14 to C-5 constructed a partial structure of cyclohexane ring A substituted by a methyl group at C-4 (Figure 2). The HMBC spectrum of **1** also showed correlations from H-6 to C-5 and C-10, from H-7 to C-5, as well as from H-9 to C-1 and C-10, revealing the presence of another six-membered ring B, which was fused to the ring A (Figure 2). HMBC correlations from H-6 and H-7 to C-5 and C-11, along with the ^{13}C NMR resonances of C-5 (δ_C 90.7) and C-11 (δ_C 81.2), suggested that C-5 and C-11 were oxygenated tertiary carbons linked through an ether bond.[3,17] A *gem*-dimethyl group was located on the oxygenated tertiary carbon C-11 based on HMBC correlations from H_3-12 and H_3-13 to C-7 and C-11; these correlations also confirmed that the *gem*-dimethyl group and C-7 were connected through the non-protonated carbon C-11. Further HMBC correlations from H_2-15 to C-1, C-5, C-9, and C-10 located CH_2-15 at C-10. The presence of three acetoxy groups was indicated by HMBC correlations from the methyl protons at δ_H 1.46, 2.10, and 2.29 to carbonyl resonances at δ_C 170.1, 169.8, and 170.5, respectively. These considerations indicated that **1** was a dihydro-β-agarofuran sesquiterpenoid containing one benzoate and three acetoxy groups. The benzoate moiety was attached at C-9 based on HMBC correlations from both aromatic protons at δ_H 8.06 and δ_H 5.55 (H-9) to an ester carbonyl group at δ_C 165.1. Similarly, the three acetoxy groups were located at C-1, C-6, and C-15 on the basis of HMBC correlations from H-1, H-6, and H_2-15 to carbonyl carbons at δ_C 170.1, 169.8, and 170.5, respectively. Finally, a hydroxy group was positioned at C-8 by considering the deshielded ^{1}H and ^{13}C NMR resonances of CH-8 (δ_H 4.42 and δ_C 69.0). Based on these data, the 2D structure of **1** was established.

The relative configuration of **1** was assigned following analysis of ^{1}H-^{1}H coupling constants and the ROESY data (Figure 3). Large coupling constants between H-1 and H-2 ($J_{1,2}$ = 12.0, 4.6

Hz) revealed the *β*-orientation of H-1. ROESY correlations between H-1 and H-9 and between H-1 and H-3*β* showed that these protons were cofacial. The coupling constant of 4.2 Hz between H-7 and H-8 suggested that H-7 and H-8 were *α*- and *β*-oriented, respectively. A small coupling constant between H-6 and H-7 ($J_{6,7}$ =1.1 Hz) indicated a dihedral angle close to 90º between these protons, and thus supported an *α*-orientation of H-6.[18] Notably, in all dihydro-*β*-agarofurans possessing an ester group at C-6, H-6 is *α*-oriented.[18,19] Finally, the *α*-orientation of CH_3-14 and CH_2-15 was determined on the basis of ROESY correlations between H-6 and H_3-14, H-6 and H_2-15, as well as between H-2*α* and H_2-15.

A single-crystal X-ray diffraction experiment using CuK*α* radiation[20] was performed on compound **1** (Figure 4), which unambiguously established the structure and absolute configuration of bilocularin A as (1*S*,4*R*,5*S*,6*R*,7*R*,8*R*,9*S*,10*S*)-1,6,15-triacetoxy-9-benzoyloxy-8-hydroxydihydro-*β*-agarofuran.

Compound **2**, obtained as colourless crystals, had the molecular formula $C_{28}H_{34}O_{10}$ assigned following analysis of the (+)-HRESIMS and NMR data. The ^{1}H NMR spectrum of **2** was similar to that of **1**, except for the absence of a methine proton signal in **2**, which corresponded to H-8 in **1**. Analysis of the NMR and MS data of **2** and comparison with **1** revealed that both compounds shared the same dihydro-*β*-agarofuran scaffold. However, the secondary hydroxy group in **1** was oxidized to a carbonyl group (C-8) in **2**. This was determined by HMBC correlations from H-6 (δ_H 6.42), H-7 (δ_H 3.05), and H-9 (δ_H 5.87) to a carbonyl carbon at δ_C 198.6. The ROESY spectrum of **2** exhibited correlations between H-1 and H-9, H-6 and H_3-14, as well as between H-6 and H_2-15, implying the relative configuration of **2** to be the same as compound **1**. The structure of **2** was confirmed by a single-crystal X-ray diffraction experiment using CuK*α* radiation (Figure 4), which also permitted the definition of its absolute configuration. Thus, the

structure of **2** (bilocularin B), was assigned as (1*S*,4*R*,5*S*,6*R*,7*R*,9*S*,10*S*)-1,6,15-triacetoxy-9-benzoyloxy-8-oxodihydro-*β*-agarofuran. It is worth mentioning that oxidation of bilocularin A (**1**) using Dess-Martin periodinane[21] afforded bilocularin B (**2**) with 93% yield.

The minor metabolite, compound **3**, was isolated as a colourless gum and was assigned the molecular formula $C_{28}H_{36}O_{10}$ by HRESIMS. These data indicated that **3** was a constitutional isomer of **1**. While the ^{1}H and ^{13}C NMR spectra of **3** showed a high degree of similarity with **1**, several key differences were readily identified. For instance, the H-6 resonance was shielded from δ_H 6.56 in **1** to δ_H 5.25 in **3**, while the H-8 signal was deshielded from δ_H 4.42 in **1** to δ_H 5.63 in **3**. COSY correlation between H-6 and an exchangeable OH-6 proton (δ_H 1.69), as well as HMBC correlations from both H-8 and the methyl protons at δ_H 2.00 to an ester carbonyl carbon at δ_C 169.8, indicated that in compound **3**, H-6 was attached to a carbon bearing a hydroxy group, while H-8 was part of an acetoxymethine group. The relative configuration of **3** was deemed to be identical to that of **1** following ROESY data analysis. On the basis of biosynthetic considerations and comparison of chiroptical and NMR data for this series, the absolute configuration of bilocularin C (**3**) was assigned as (1*S*,4*R*,5*S*,6*R*,7*S*,8*R*,9*S*,10*S*)-1,8,15-triacetoxy-9-benzoyloxy-6-hydroxydihydro-*β*-agarofuran.

The known compounds were characterized as celastrine A (**4**),[22] 1*α*,6*β*,8*α*-triacetoxy-9*α*-benzoyloxydihydro-*β*-agarofuran (**5**),[23,24] 1*α*,6*β*-diacetoxy-9*α*-benzoyloxy-8*α*-hydroxydihydro-*β*-agarofuran (**6**),[25] Ejap-10 (**11**),[26] 1*α*,6*β*-diacetoxy-9*β*-benzoyloxydihydro-*β*-agarofuran (**12**),[27] and Ejap-2 (**13**)[26] by comparison of their spectroscopic data with the literature. In this report, we have also included $[\alpha]_D$ values for compounds **4** and **12** along with ^{13}C NMR and $[\alpha]_D$ data for compounds **11** and **13**, which were not reported in the original papers. In addition, the electronic circular dichroism (ECD) data of compounds **1**–**13** are also reported. The ^{1}H NMR data of **11**

were consistent with the reported values,[28] however a discrepancy in the $[\alpha]_D$ sign was observed. Our experimental data showed a specific rotation of –47 (*c* 0.1, EtOH), while the literature reported an $[\alpha]_D$ value of +25 (*c* 0.1, EtOH).[28] This observation suggested that either the $[\alpha]_D$ of **11** was misassigned or that it was the enantiomer of **11**. Attempts to obtain authentic material for re-analysis were not successful.

A number of ester analogues were semi-synthesized from the major compound **1** in order to facilitate preliminary structure-activity relationship studies for this type of compound. Compound **1** was reacted with pivaloyl chloride, methoxyacetyl chloride, benzoyl chloride, and *p*-bromobenzoyl chloride in dry pyridine to generate **7**, **8**, **9**, and **10**, respectively. Their structures were confirmed by 1D/2D NMR and HRESIMS data. Benzoylation of **1** yielded the benzoylated derivative **9** (celaspene A).[29] The ECD spectrum of **9** showed a negative Cotton effect at 238 nm ($\Delta\varepsilon$ –26.0) and a positive Cotton effect at 223 nm ($\Delta\varepsilon$ +13.3) due to exciton coupling of the two benzoate chromophores at C-8α and C-9α, which were in accordance with the reported data.[29] Accordingly, the structure and absolute configuration of **9** were assigned as (1*S*,4*R*,5*S*,6*R*,7*R*,8*R*,9*S*,10*S*)-1,6,15-triacetoxy-8,9-dibenzoyloxydihydro-β-agarofuran. The absolute configuration of **9** established by the ECD studies was identical to that of **1** assigned through X-ray crystallographic analysis.

Owing to our continuing interest in the discovery and development of new prostate cancer active agents from nature,[30-33] compounds **1**–**13** were initially evaluated for their cytotoxicity towards the LNCaP human prostate cancer cell line (lymph node metastasis, androgen-sensitive) using a cell viability assay which is based on metabolic activity (alamarBlue). None of the compounds showed >30 % cell viability inhibition towards LNCaP cells at 10 μM after 72 h (see Table S31 Supporting Information for full biological results). Compounds **5**, **7**, and **9** exhibited

the higher activity with 14.6, 25.6, and 21.2% inhibition at 10 μM, respectively. In order to further investigate the biological effects of dihydro-β-agarofurans toward cancer cells, the major compounds **1** and **2**, and the most active compounds **5**, **7**, and **9** were subjected to further biological testing.

One of the main challenges in cancer treatment is the development of the multidrug resistance (MDR) phenotype due, in part, to the overexpression of multidrug transporters, which act as efflux pumps to reduce the intracellular concentration of chemotherapeutic agents.[34,35] P-glycoprotein (P-gp, *ABCB1*), MDR protein 1 (*MRP1*/*ABCC1*), and breast cancer resistance protein (*BCRP/ABCG2*) are the best characterized multidrug efflux pumps. Overexpression of P-gp has been detected in many cancer types in humans and therefore is an attractive target for cancer co-treatment therapy.[36] Recently, dihydro-β-agarofurans have been reported as promising MDR reversal agents by binding specifically to P-gp.[37-39] Owing to these findings, the drug resistance reversal ability of a subset of dihydro-β-agarofurans (compounds **1**, **2**, **5**, **7**, and **9**) was studied.

The IC_{50} value of the vinca alkaloid microtubule inhibitor, vinblastine was measured in the absence or presence of compounds **1**, **2**, **5**, **7**, and **9** (all at 5 μM) in the vinca alkaloid-resistant cell line, CEM/VCR R, which is a subline of the human T-cell acute lymphoblastic leukemia cell line, CEM.[40,41] The MDR cell line CEM/VCR R was demonstrated to overexpress P-gp mRNA and protein, exhibiting multidrug resistance to vincristine, vinblastine, adriamycin, daunorubicin, actinomycin D, teniposide, and tetracycline.[40,42] The tetracycline resistance was completely reversed with the P-gp inhibitors cyclosporine A and verapamil.[42] The latter is a calcium channel blocker that is routinely used as an inhibitor of multidrug resistance,[5] as it has been shown to bind to the efflux transporter P-gp.[43] Our data demonstrate that CEM/VCR R cells were 477.2-

fold less sensitive to vinblastine than the parental CEM cells (IC_{50} 1336.2 nM vs. 2.8 nM, Table 3). Compounds **1**, **2**, **5**, **7**, and **9** did not show any cytotoxicity toward CEM or CEM/VCR R cells on their own (data not shown) and slightly sensitized the CEM cells to vinblastine (Table 3). However, when CEM/VCR R cells were treated with compounds **1**, **2**, **5**, **7**, and **9** in combination with vinblastine, they strongly sensitized cells as indicated by the reduced IC_{50} values relative to that of vinblastine treatment alone (Table 3). Notably, at equimolar concentrations (5 μM), compounds **7** and **9** showed similar potency based on their reversal indices (RI) as the P-gp inhibitor verapamil, while the remaining compounds scored a lower RI (Table 3). Thus, compounds **1**, **2**, **5**, **7**, and **9** are active in inhibiting the MDR phenotype of CEM/VCR R cells, suggesting that they inhibit P-gp. Comparison of the MDR reversal activity for **1**, **2**, **7**, and **9** showed that compounds with an ester group at C-8 (**7** and **9**) were more potent than those with a hydroxy (**1**) or carbonyl (**2**) group at this position. These data indicated that ester substitution at C-8 improved MDR reversal activity for this series of dihydro-β-agarofurans.

Since verapamil has been shown to reduce amino acid transport by blocking calcium flux,[44] the leucine uptake in LNCaP cells was next examined. Verapamil significantly reduced leucine uptake to 82.7% of control at 10 μM, however was not as effective as 2-aminobicyclo[2,2,1]heptane-2-carbocyclic acid (BCH, 40.2% of control), an L-type amino acid transporter (LAT) family inhibitor (Figure 5A). In LNCaP cells, the essential amino acid leucine is delivered predominantly by LAT3 and regulates protein synthesis through mTORC1 signaling, thereby controlling cancer cell growth.[45-47] To determine the effects of our compounds on leucine uptake, a dose response analysis for each compound was performed. Compound **5** inhibited leucine uptake with an IC_{50} value of 15.5 μM (Figure 5B). Compounds **1** (IC_{50} = 124.5 μM) and

2 (IC_{50} = 151.2 μM) also inhibited leucine uptake at higher concentrations, while **7** and **9** did not show any effects on leucine transport (Figure 5B). These results showed that when C-15 is not substituted with an oxygen functionality (ie. **5** vs. **1**/**2**), leucine transport inhibition is more significant. Furthermore, ester substitution at C-8 (**7**/**9** vs. **1**/**2**) resulted in loss of activity. Apart from the LAT family inhibitor BCH, several other natural compounds (monoterpene glycoside isolated from *Pittosporum venulosum*) have been shown to inhibit LAT3-mediated leucine transport.[48,49] Whilst the dihydro-β-agarofurans structure class has had several different biological activities reported to date,[1,2] this is the first report of dihydro-β-agarofurans inhibiting leucine uptake.

In conclusion, three new and six known dihydro-β-agarofurans were obtained from *M. bilocularis*. Four semi-synthetic ester analogues were prepared from the major dihydro-β-agarofuran. Several compounds exhibited similar potency to the reference drug verapamil in reversing the drug resistance of the CEM/VCR R cells. In addition, one compound was found to inhibit leucine uptake in LNCaP cells and was more potent than the leucine analogue BCH. These preliminary findings warrant future mechanistic studies on how these compounds inhibit P-gp and LAT activity.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured using a Cole-Parmer melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO P-1020 polarimeter. UV spectra were obtained using a JASCO V-650 UV/vis spectrophotometer. CD spectra were recorded on a JASCO J-715 spectropolarimeter. IR data were acquired using an attached Universal Attenuated Total Reflectance (UATR) Two module on a PerkinElmer

spectrophotometer. NMR spectra were recorded on a Bruker AVANCE III HD 500 MHz NMR spectrometer at 25 °C. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks for $CDCl_3$ at δ_H 7.26 and δ_C 77.16, respectively. LRESIMS data were recorded on a Waters ZQ ESI mass spectrometer. HRESIMS data were acquired on a 12 T SolariX XR FT-ICR-MS. X-ray diffraction data were collected on an Oxford-Diffraction Gemini S Ultra CCD diffractometer utilizing CrysAlis software.[50] Alltech Davisil 30−40 μm 60 Å silica gel packed into an open glass column (25 × 90 mm) was used for flash column chromatography. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F_{254} pre-coated aluminum plates and was observed using UV light. Activon phenyl bonded silica was used for pre-adsorption work before HPLC separations, and the resulting material was packed into an Alltech stainless steel guard cartridge (10 × 30 mm). Phenomenex polypropylene solid-phase extraction (SPE) cartridges were used for purifications. A Waters 600 pump fitted with a Waters 996 photodiode array detector and Gilson 717-plus autosampler was used for semi-preparative HPLC separations. A Phenomenex Luna 5 μm phenyl-hexyl column (250 × 10 mm) was used for semi-preparative HPLC separations. A Fritsch Universal Cutting Mill Pulverisette 19 was used to grind the air-dried plant material. An Edwards Instrument Company Bio-line orbital shaker was used for plant extraction. All solvents used for chromatography, optical rotation, ECD, UV and MS were Lab-Scan HPLC grade. H_2O was Millipore Milli-Q PF filtered. All compounds were analyzed for purity by ^{1}H NMR spectroscopy and shown to be >95%, unless otherwise stated. All chemical reagents were purchased from Sigma-Aldrich.

Plant Material. Leaves of *M. bilocularis* (F. Muell.) Loes (Celastraceae) were collected from The Gap, Queensland, Australia in May 2015. A voucher specimen (RAD076) has been deposited at the Eskitis Institute, Griffith University, Brisbane, Australia.

Extraction and Isolation. The air-dried and ground leaves of *M. bilocularis* (10 g) were extracted with CH_2Cl_2 (2 × 250 mL) at room temperature under constant shaking for 2 h. The organic solvent was filtered and evaporated under reduced pressure to give 720 mg of CH_2Cl_2 extract that was subsequently chromatographed on a silica gel flash column (25 × 90 mm) using a stepwise gradient solvent system of *n*-hexane–EtOAc (100% *n*-hexane to 100% EtOAc), affording 100 fractions (100 × 10 mL). Each fraction was analyzed by TLC (*n*-hexane/EtOAc) and those displaying the same TLC profile were combined to afford 16 fractions (F1−F16); all these fractions were analyzed by ^{1}H NMR spectroscopy. Fractions F3, F9, and F11 were pure and identified as 1*α*,6*β*-diacetoxy-9*β*-benzoyloxydihydro-*β*-agarofuran (**12**, 8.4 mg, 0.084% dry wt), bilocularin B (**2**, 27.3 mg, 0.273% dry wt), and bilocularin A (**1**, 96.9 mg, 0.969% dry wt), respectively. Fraction F5 (48.2 mg) was pre-adsorbed to phenyl-bonded silica (~1 g), packed into a guard cartridge, and attached to a semi-preparative phenyl-hexyl HPLC column. Isocratic conditions of 60% MeOH (0.1% TFA)–40% H_2O (0.1% TFA) was initially employed for 5 min, followed by a linear gradient to 100% MeOH (0.1% TFA) over 45 min, then isocratic 100% MeOH (0.1% TFA) for 10 min, all at a flow rate of 4 mL/min. Sixty fractions (60 × 1 min) were collected from the start of the HPLC run and two pure compounds, 1*α*,6*β*,8*α*-triacetoxy-9*α*-benzoyloxydihydro-*β*-agarofuran (**5**, 32.1 mg, t_R 32–33 min, 0.321% dry wt) and Ejap-2 (**13**, 8.5 mg, t_R 36–37 min, 0.085% dry wt), were obtained. 1*α*,6*β*-Diacetoxy-9*α*-benzoyloxy-8*α*-hydroxydihydro-*β*-agarofuran (**6**, 10.1 mg, t_R 28–29 min, 0.101% dry wt) and celastrine A (**4**, 24.1 mg, t_R 33–34 min, 0.241% dry wt) were isolated from fraction F10 (42 mg) using the same HPLC conditions as those used for the purification of **5** and **13**. Fraction F14 (15.9 mg) was purified using a silica SPE cartridge (10 × 40 mm), which was flushed with 35% EtOAc–65% *n*-hexane (10 mL) and 40% EtOAc–60% *n*-hexane (10 mL). The fraction eluting with 40%

EtOAc–60% *n*-hexane afforded Ejap-10 (**11**, 13.4 mg, 0.134% dry wt). Fraction F16 (21.3 mg) was pre-adsorbed to phenyl-bonded silica (~1 g) and packed into a guard cartridge, which was then connected to a semi-preparative phenyl-hexyl HPLC column. Isocratic conditions of 50% MeOH (0.1% TFA)–50% H_2O (0.1% TFA) were held for 5 min, followed by a linear gradient to 100% MeOH (0.1% TFA) over 45 min, then isocratic 100% MeOH (0.1% TFA) for 10 min, all at a flow rate of 4 mL/min. Sixty fractions (60 × 1 min) were collected from the start of the HPLC run. Fractions 33–34 from this purification step were pooled and further purified on a silica SPE cartridge (10 × 40 mm) using a 5% stepwise gradient solvent system from 15% EtOAc–85% *n*-hexane to 40% EtOAc–60% *n*-hexane (10 mL elutions) to give bilocularin C (**3**, 0.7 mg, 0.007% dry wt).

Bilocularin A (**1**): stable colourless crystals (EtOH); mp 209–211 °C; $[\alpha]_D^{24}$–32 (*c* 0.1, MeOH); ECD λ_{ext} (MeOH) 230 ($\Delta\varepsilon$ +0.3), 245 ($\Delta\varepsilon$ –1.1) nm; UV (MeOH) λ_{max} (log ε) 231 (4.16), 276 (3.05) nm; IR (UATR) ν_{max} 3492, 1738, 1371, 1237, 1095, 1059, 716 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 8.06 (2H, m, OBz-9), 7.57 (1H, m, OBz-9), 7.46 (2H, m, OBz-9), 2.29 (3H, s, OAc-15), 2.10 (3H, s, OAc-6), 1.46 (3H, s, OAc-1), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 170.5 (C, OAc-15), 170.1 (C, OAc-1), 169.8 (C, OAc-6), 165.1 (C, OBz-9), 133.7 (CH, OBz-9), 129.8 (2CH, OBz-9), 129.6 (C, OBz-9), 128.8 (2CH, OBz-9), 21.5 (CH_3, OAc-15), 21.3 (CH_3, OAc-6), 20.9 (CH_3, OAc-1), for other signals, see Table 2; (+)-LRESIMS *m/z* 555 (100) $[M+Na]^+$; (+)-HRESIMS *m/z* 555.2197 $[M+Na]^+$ (calcd for $C_{28}H_{36}O_{10}Na$, 555.2201).

Bilocularin B (**2**): stable colourless crystals (EtOH); mp 272–274 °C; $[\alpha]_D^{24}$+2 (*c* 0.1, MeOH); ECD λ_{ext} (MeOH) 247 ($\Delta\varepsilon$ –0.6), 292 ($\Delta\varepsilon$ +0.9) nm; UV (MeOH) λ_{max} (log ε) 231 (3.95), 274 (2.86) nm; IR (UATR) ν_{max} 2935, 1741, 1640, 1374, 1228, 1039, 869, 704 cm^{-1}; ^1H NMR

($CDCl_3$, 500 MHz) δ_H 7.99 (2H, m, OBz-9), 7.57 (1H, m, OBz-9), 7.44 (2H, m, OBz-9), 2.13 (3H, s, OAc-6), 2.06 (3H, s, OAc-15), 1.53 (3H, s, OAc-1), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 170.6 (C, OAc-15), 170.0 (C, OAc-1), 169.2 (C, OAc-6), 165.3 (C, OBz-9), 133.6 (CH, OBz-9), 129.9 (2CH, OBz-9), 129.2 (C, OBz-9), 128.7 (2CH, OBz-9), 21.2 (CH_3, OAc-6), 20.84[a] (CH_3, OAc-15), 20.80[a] (CH_3, OAc-1), for other signals, see Table 2; (+)-LRESIMS *m/z* 531 (100) $[M+H]^+$; (+)-HRESIMS *m/z* 553.2040 $[M+Na]^+$ (calcd for $C_{28}H_{34}O_{10}Na$, 553.2044). [a]Interchangeable signals.

Bilocularin C (**3**): stable colourless gum; $[\alpha]_D^{24}$–65 (*c* 0.04, MeOH); ECD λ_{ext} (MeOH) 212 ($\Delta\varepsilon$ –1.2), 244 ($\Delta\varepsilon$ –1.2) nm; UV (MeOH) λ_{max} (log ε) 231 (4.28), 273 (3.18) nm; IR (UATR) ν_{max} 3484, 2945, 1733, 1370, 1232, 1278, 1105, 1038, 715 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.01 (2H, m, OBz-9), 7.56 (1H, m, OBz-9), 7.44 (2H, m, OBz-9), 2.19 (3H, s, OAc-15), 2.00 (3H, s, OAc-8), 1.47 (3H, s, OAc-1), for other signals, see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 170.4 (C, OAc-15), 170.2 (C, OAc-1), 169.8 (C, OAc-8), 164.9 (C, OBz-9), 133.4 (CH, OBz-9), 129.70 (2CH, OBz-9), 129.67 (C, OBz-9), 128.7 (2CH, OBz-9), 21.8 (CH_3, OAc-15), 21.2 (CH_3, OAc-8), 20.9 (CH_3, OAc-1), for other signals, see Table 2; (+)-LRESIMS *m/z* 555 (100) $[M+Na]^+$; (+)-HRESIMS *m/z* 555.2193 $[M+Na]^+$ (calcd for $C_{28}H_{36}O_{10}Na$, 555.2201).

Celastrine A (**4**): white amorphous powder; $[\alpha]_D^{24}$–15 (*c* 0.1, MeOH); ECD λ_{ext} (MeOH) 228 (+0.7), 245 (–0.5) nm; lit. ECD λ_{ext} (solvent not reported) 226 ($\Delta\varepsilon$ +3.8) nm;[22] (+)-LRESIMS *m/z* 597 (100) $[M+Na]^+$.

1α,6β,8α-Triacetoxy-9α-benzoyloxydihydro-β-agarofuran (**5**): white amorphous powder; $[\alpha]_D^{24}$–93 (*c* 0.1, MeOH); lit. $[\alpha]_D^{25}$–51.1 (*c* 0.28, MeOH);[24] ECD λ_{ext} (MeOH) 225 ($\Delta\varepsilon$ +1.2), 247 ($\Delta\varepsilon$ –1.8) nm; (+)-LRESIMS *m/z* 539 (100) $[M+Na]^+$.

1α,6β-Diacetoxy-9α-benzoyloxy-8α-hydroxydihydro-β-agarofuran (**6**): white amorphous powder; $[\alpha]_D^{25}$–29 (*c* 0.5, $CHCl_3$); lit. $[\alpha]_D^{23}$–78.7 (*c* 0.525, $CHCl_3$);[25] ECD λ_{ext} (MeOH) 240 ($\Delta\varepsilon$ –2.6) nm; (+)-LRESIMS *m/z* 497 (100) $[M+Na]^+$.

Ejap-10 (**11**): white amorphous powder; $[\alpha]_D^{25}$–47 (*c* 0.1, EtOH); lit. $[\alpha]_D^{20}$+25 (*c* 0.1, EtOH);[28] ECD λ_{ext} (MeOH) 228 ($\Delta\varepsilon$ –6.0), 281 ($\Delta\varepsilon$ +0.6) nm; The ^{1}H NMR data were in agreement with the literature data;[26,28] ^{13}C NMR δ_C 170.9 (C, OAc-15), 169.94[a] (C, OAc-1), 169.89[a] (C, OAc-6), 165.7 (C, OBz-9), 133.8 (CH, OBz-9), 130.2 (2CH, OBz-9), 128.9 (C, OBz-9), 128.6 (2CH, OBz-9), 90.3 (C, C-5), 81.4 (C, C-11), 77.8 (CH, C-9), 75.4 (CH, C-8), 74.7 (CH, C-6), 73.8 (CH, C-1), 65.2 (CH_2, C-15), 55.6 (CH, C-7), 52.6 (C, C-10), 33.2 (CH, C-4), 30.7 (CH_3, C-13), 26.4 (CH_2, C-3), 25.9 (CH_3, C-12), 22.4 (CH_2, C-2), 21.5 (CH_3, OAc-6), 21.3 (CH_3, OAc-15), 20.9 (CH_3, OAc-1), 15.9 (CH_3, C-14); (+)-LRESIMS *m/z* 555 (100) $[M+Na]^+$. [a]Interchangeable signals.

1α,6β-Diacetoxy-9β-benzoyloxydihydro-β-agarofuran (**12**): white amorphous powder; $[\alpha]_D^{25}$+3 (*c* 0.1, MeOH); ECD λ_{ext} (MeOH) 233 ($\Delta\varepsilon$ –1.5), 281 ($\Delta\varepsilon$ +0.2) nm; (+)-LRESIMS *m/z* 481 (100) $[M+Na]^+$.

Ejap-2 (**13**): white amorphous powder; $[\alpha]_D^{25}$+35 (*c* 0.08, MeOH); ECD λ_{ext} (MeOH) 231 ($\Delta\varepsilon$ –2.1), 279 ($\Delta\varepsilon$ +0.4) nm; The ^{1}H NMR data were in agreement with reported data;[26] ^{13}C NMR δ_C 170.8 (C, OAc-15), 170.2 (C, OAc-6), 169.8 (C, OAc-1), 165.5 (C, OBz-9), 133.5 (CH, OBz-9), 130.2 (2CH, OBz-9), 129.5 (C, OBz-9), 128.5 (2CH, OBz-9), 89.7 (C, C-5), 82.6 (C, C-11), 78.3 (CH, C-6), 73.6 (CH, C-1), 70.2 (CH, C-9), 65.5 (CH_2, C-15), 53.2 (C, C-10), 48.9 (CH, C-7), 34.8 (CH_2, C-8), 33.5 (CH, C-4), 30.5 (CH_3, C-13), 26.5 (CH_2, C-3), 26.1 (CH_3, C-12), 22.3 (CH_2, C-2), 21.5 (CH_3, OAc-6), 21.3 (C, OAc-15), 20.9 (CH_3, OAc-1), 16.7 (CH_3, C-14); (+)-LRESIMS *m/z* 539 (100) $[M+Na]^+$.

Oxidation of Bilocularin A (1). Compound **1** (10 mg, 0.0188 mmol) was dissolved in 250 μL of CH_2Cl_2 before Dess-Martin periodinane[21] (21.5 mg, 0.0507 mmol) was added. The solution was stirred at room temperature for 1.5 h. The mixture was purified using a silica SPE cartridge (10 × 40 mm) flushed with 10 mL of CH_2Cl_2 to give bilocularin B (**2**, 9.3 mg, 93%).

Acetylation of Bilocularin A (1). Compound **1** (7.5 mg, 0.0141 mmol) was dissolved in dry pyridine (6 drops) and Ac_2O (500 μL, 5.3 mmol) was added dropwise. The mixture was stirred overnight at room temperature. The reaction mixture was dried and subjected to HPLC using a semi-preparative phenyl-hexyl column at a flow rate of 4 mL/min and isocratic conditions of 50% MeOH (0.1% TFA)–50% H_2O (0.1% TFA) for 5 min, followed by a linear gradient to 100% MeOH (0.1% TFA) over 45 min, then isocratic 100% MeOH (0.1% TFA) for 10 min. Sixty fractions (60 × 1 min) were collected from the start of the HPLC run. Fractions 41–42 yielded the previously known natural product, celastrine A (**4**, 2.0 mg, 25%).[22]

Pivaloylation of Bilocularin A (1). Compound **1** (11.7 mg, 0.0220 mmol) was dissolved in dry pyridine (2 drops) before pivaloyl chloride (200 μL, 1.6 mmol) was added. The solution was stirred for 16 h at room temperature. The reaction mixture was dried and purified by silica SPE cartridge (10 × 40 mm) using a 5% stepwise gradient from *n*-hexane to 20% EtOAc–80% *n*-hexane (10 mL elutions). The fraction that eluted with 20% EtOAc–80% *n*-hexane contained the pivaloylated compound **7** (3.5 mg, 25%).

8-Pivaloyloxybilocularin A (**7**): white amorphous powder; $[\alpha]_D^{24}$–41 (*c* 0.1, MeOH); ECD λ_{ext} (MeOH) 240 ($\Delta\varepsilon$ +0.5), 273 ($\Delta\varepsilon$ –0.2) nm; UV (MeOH) λ_{max} (log ε) 232 (4.06), 274 (2.93) nm; IR (UATR) ν_{max} 2973, 1736, 1369, 1271, 1229, 1141, 1096, 714 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 8.03 (2H, m, OBz-9), 7.55 (1H, m, OBz-9), 7.43 (2H, m, OBz-9), 6.36 (1H, d, *J* = 0.8 Hz, H-6), 5.68 (1H, d, *J* = 5.7 Hz, H-9), 5.60 (1H, dd, *J* = 5.7, 3.9 Hz, H-8), 5.40 (1H, dd, *J* =

11.9, 4.8 Hz, H-1), 4.96 (1H, d, *J* = 13.3 Hz, H-15a), 4.71 (1H, d, *J* = 13.3 Hz, H-15b), 2.42 (1H, br d, *J* = 3.9 Hz, H-7), 2.23 (1H, m, H-4), 2.21 (1H, m, H-3*β*), 2.20 (3H, s, OAc-15), 2.11 (3H, s, OAc-6), 1.83 (1H, m, H-2*α*), 1.73 (1H, m, H-2*β*), 1.59 (3H, s, H-12), 1.46 (1H, m, H-3*α*), 1.44 (3H, s, OAc-1), 1.42 (3H, s, H-13), 1.18 (9H, s, OPiv-8), 1.00 (3H, d, *J* = 1.0 Hz, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 177.6 (C, OPiv-8), 171.0 (C, OAc-15), 170.1 (C, OAc-1), 169.8 (C, OAc-6), 164.9 (C, OBz-9), 133.5 (CH, OBz-9), 129.8 (2CH, OBz-9), 129.4 (C, OBz-9), 128.6 (2CH, OBz-9), 90.9 (C, C-5), 81.5 (C, C-11), 79.6 (CH, C-1), 74.9 (CH, C-6), 73.9 (CH, C-9), 70.3 (CH, C-8), 61.5 (CH_2, C-15), 53.0 (CH, C-7), 50.8 (C, C-10), 39.0 (C, OPiv-8), 33.7 (CH, C-4), 30.5 (CH_3, C-13), 27.2 ($3CH_3$, OPiv-8), 26.6 (CH_2, C-3), 24.5 (CH_3, C-12), 23.4 (CH_2, C-2), 21.7 (CH_3, OAc-15), 21.4 (CH_3, OAc-6), 20.9 (CH_3, OAc-1), 15.2 (CH_3, C-14); (+)-LRESIMS *m/z* 639 (100) $[M+Na]^+$; (+)-HRESIMS *m/z* 639.2773 $[M+Na]^+$ (calcd for $C_{33}H_{44}O_{11}Na$, 639.2776).

Methoxyacetylation of Bilocularin A (1). Compound **1** (12.1 mg, 0.0227 mmol) was dissolved in dry pyridine (2 drops) before 200 μL (2.2 mmol) of methoxyacetyl chloride was added. The solution was stirred overnight at room temperature. The reaction mixture was dried and purified using the same protocol described for the purification of compound **4** to give compound **8** (6.2 mg, 45%, t_R 36–37 min).

8-Methoxyacetoxybilocularin A (**8**): white amorphous powder; $[\alpha]_D^{24}$–20 (*c* 0.3, MeOH); ECD λ_{ext} (MeOH) 230 ($\Delta\varepsilon$ +0.5), 250 ($\Delta\varepsilon$ –0.4) nm; UV (MeOH) λ_{max} (log ε) 232 (4.02), 273 (2.92) nm; IR (UATR) ν_{max} 2980, 1740, 1371, 1276, 1232, 1127, 715 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H 8.02 (2H, m, OBz-9), 7.55 (1H, m, OBz-9), 7.44 (2H, m, OBz-9), 6.61 (1H, d, *J* = 0.5 Hz, H-6), 5.68 (2H, overlapping signals, H-8, H-9), 5.40 (1H, dd, *J* = 11.8, 5.2 Hz , H-1), 4.97 (1H, d, *J* = 13.2 Hz, H-15a), 4.53 (1H, d, *J* = 13.2 Hz, H-15b), 4.07 (1H, d, *J* = 16.3 Hz,

COC*H*$_2$OCH$_3$), 3.97 (1H, d, *J* = 16.3 Hz, COC*H*$_2$OCH$_3$), 3.24 (3H, s, COCH$_2$OC*H*$_3$), 2.45 (1H, br s, H-7), 2.28 (3H, s, OAc-15), 2.25 (1H, m, H-4), 2.22 (1H, m, H-3*β*), 2.11 (3H, s, OAc-6), 1.76 (1H, m, H-2*β*), 1.69 (1H, m, H-2*α*), 1.59 (3H, s, H-12), 1.47 (1H, m, H-3*α*), 1.47 (3H, s, OAc-1), 1.43 (3H, s, H-13), 1.01 (3H, d, *J* = 7.5 Hz, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 170.8 (C, OAc-15), 170.1 (C, OAc-1), 169.9 (C, OAc-6), 169.7 (C, *C*OCH$_2$OCH$_3$), 164.9 (C, OBz-9), 133.6 (CH, OBz-9), 129.8 (2CH, OBz-9), 129.4 (C, OBz-9), 128.8 (2CH, OBz-9), 90.8 (C, C-5), 81.2 (C, C-11), 79.5 (CH, C-1), 74.7 (CH, C-6), 72.8 (CH, C-9), 70.9 (CH, C-8), 69.6 (CH$_2$, CO*C*H$_2$OCH$_3$), 60.5 (CH$_2$, C-15), 59.3 (CH$_3$, COCH$_2$O*C*H$_3$), 53.0 (CH, C-7), 50.9 (C, C-10), 33.4 (CH, C-4), 30.5 (CH$_3$, C-13), 26.4 (CH$_2$, C-3), 24.7 (CH$_3$, C-12), 23.1 (CH$_2$, C-2), 21.5 (CH$_3$, OAc-15), 21.4 (CH$_3$, OAc-6), 20.9 (CH$_3$, OAc-1), 15.3 (CH$_3$, C-14); (+)-LRESIMS *m/z* 627 (100) [M+Na]$^+$; (+)-HRESIMS *m/z* 627.2410 [M+Na]$^+$ (calcd for $C_{31}H_{40}O_{12}Na$, 627.2412).

Benzoylation of Bilocularin A (1). Compound **1** (5.2 mg, 0.0098 mmol) was dissolved in dry pyridine (6 drops) before benzoyl chloride (300 μL, 2.6 mmol) and a crystal of 4-dimethylaminopyridine (DMAP) were added. The solution was stirred at room temperature for 72 h. The reaction mixture was dried and purified in the same manner as **4** to afford the known compound celaspene A (**9**, 2.0 mg, 32%, t_R 46–47 min). The spectroscopic data of **9** were in agreement with literature data.[29]

Celaspene A (**9**): $[\alpha]_D^{24}$–13 (*c* 0.06, CH_2Cl_2); literature value $[\alpha]_D^{21}$–21.4 (*c* 0.19, CH_2Cl_2);[29] ECD λ_{ext} (MeCN) 223 ($\Delta\varepsilon$ +13.3), 238 ($\Delta\varepsilon$ –26.0) nm; lit. ECD λ_{ext} (MeCN) 222 ($\Delta\varepsilon$ +9.4), 239 ($\Delta\varepsilon$ –13.9) nm;[29] UV (MeCN) λ_{max} (log ε) 242 (3.14), 256 (sh, 2.90), 271 (sh, 2.87) nm; (+)-LRESIMS *m/z* 659 (100) [M+Na]$^+$.

***p*-Bromobenzoylation of Bilocularin A (1).** Compound **1** (8.2 mg, 0.0154 mmol) was reacted with *p*-bromobenzoyl chloride under the same conditions as those for the formation of **9**. The

reaction mixture was purified using the same procedure described for **4** to afford 2.6 mg (24% yield) of compound **10** (t_R 52–53 min).

8-p-Bromobenzoyloxybilocularin A (**10**): white amorphous powder; $[\alpha]_D^{25}$–30 (*c* 0.03, MeOH); ECD λ_{ext} (MeOH) 228 ($\Delta\varepsilon$ +17.3), 246 ($\Delta\varepsilon$ –40.7) nm; UV (MeOH) λ_{max} (log ε) 237 (4.12) nm; IR (UATR) ν_{max} 2930, 1738, 1370, 1275, 1263, 1230, 1096, 712 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ_H 7.92 (2H, m, OBz-9), 7.86 (2H, d, *J* = 8.5 Hz, OBzBr-8), 7.56 (2H, d, *J* = 8.5 Hz, OBzBr-8), 7.53 (1H, m, OBz-9), 7.38 (2H, m, OBz-9), 6.74 (1H, s, H-6), 5.78 (1H, d, *J* = 5.9 Hz, H-9), 5.75 (1H, dd, *J* = 5.9, 3.4 Hz, H-8), 5.45 (1H, dd, *J* = 11.5, 5.0 Hz, H-1), 4.79 (1H, d, *J* = 13.3 Hz, H-15a), 4.72 (1H, d, *J* = 13.3 Hz, H-15b), 2.61 (1H, d, *J* = 3.4 Hz, H-7), 2.27 (1H, m, H-4), 2.25 (1H, m, H-3β), 2.13 (3H, s, OAc-6), 2.02 (3H, s, OAc-15), 1.75 (2H, m, H-2), 1.63 (3H, s, H-12), 1.49 (3H, s, OAc-1), 1.48 (1H, m, H-3α), 1.46 (3H, s, H-13), 1.00 (3H, d, *J* = 7.6 Hz, H-14); ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C 170.9 (C, OAc-15), 170.1 (C, OAc-1), 170.0 (C, OAc-6), 165.5 (C, OBzBr-8), 164.9 (C, OBz-9), 133.5 (CH, OBz-9), 131.9 (2CH, OBzBr-8), 131.5 (2CH, OBzBr-8), 129.7 (2CH, OBz-9), 129.5 (C, OBz-9), 129.2 (C, OBzBr-8), 128.7 (2CH, OBz-9), 128.5 (C, OBzBr-8), 90.7 (C, C-5), 81.2 (C, C-11), 79.5 (CH, C-1), 74.9 (CH, C-6), 73.0 (CH, C-9), 71.8 (CH, C-8), 61.2 (CH_2, C-15), 53.4 (CH, C-7), 50.9 (C, C-10), 33.5 (CH, C-4), 30.5 (CH_3, C-13), 26.5 (CH_2, C-3), 24.8 (CH_3, C-12), 23.2 (CH_2, C-2), 21.6 (CH_3, OAc-15), 21.4 (CH_3, OAc-6), 20.9 (CH_3, OAc-1), 15.3 (CH_3, C-14); (+)-LRESIMS *m/z* 737 (100) $[^{79}Br{:}M+Na]^+$, 739 (100) $[^{81}Br{:}M+Na]^+$; (+)-HRESIMS *m/z* 737.1580 $[M+Na]^+$ (calcd for $C_{35}H_{39}{}^{79}BrO_{11}Na$, 737.1568).

X-ray Crystallography Analysis. Colourless crystals of bilocularin A (**1**) and bilocularin B (**2**) were obtained by crystallization from EtOH. Unique data sets for **1** and **2** were measured at 200 K on an Oxford-Diffraction GEMINI S Ultra CCD diffractometer with CuKα radiation

utilizing CrysAlis software.[50] The structures were solved by the direct methods package SIR97[51] and refined by full matrix least squares refinement on F^2 using the WinGX software package[52] incorporating SHELXL-2013.[53] Anisotropic thermal parameters were refined for non-hydrogen atoms; $(x, y, z, U_{iso})_H$ were included and constrained at estimated values. Conventional residuals at convergence are quoted; statistical weights were employed. The absolute configurations of **1** and **2** were determined by anomalous dispersion effects (**1** 1955 Bijvoet pairs, Flack parameter 0.02(6): **2** 1825 Bijvoet pairs, Flack parameter 0.05(6)).[20] ORTEP-3[52] and PLATON[54] software was utilized to prepare material for publication. Full .cif deposition resides with the Cambridge Crystallographic Data Centre (CCDC Nos. 1453302 and 1453303). Copies can be obtained free of charge on application at the following address: http://www.ccdc.cam.ac.uk/cgi-bin/catreq.cgi).

Crystal Data for Bilocularin A (**1**). $C_{28}H_{36}O_{10}$. M = 532.57, monoclinic, space group $P2_1$, a = 11.2388(2), b = 8.4266(1), c = 14.8577(3) Å, β = 100.786(2)°, U = 1382.24(4) Å^3, Z = 2, D_c = 1.28 g cm^{-3}, μ = 0.81 mm^{-1}, Crystal size: 0.43 × 0.25 × 0.19 mm. $T_{min/max}$ = 0.07, 1.00. 10995 reflections collected, 4245 unique (R_{int} = 0.025), R = 0.029 [4114 reflections with $I > 2\sigma(I)$], wRF^2 = 0.074 (all data).

Crystal Data for Bilocularin B (**2**). $C_{28}H_{34}O_{10}$. M = 530.55, orthorhombic, space group $P2_12_12_1$, a = 10.9490(2), b = 12.5734(2), c = 20.2474(3) Å, U = 2787.38(8) Å^3, Z = 4, D_c = 1.26 g cm^{-3}, μ = 0.80 mm^{-1}, Crystal size: 0.39 × 0.31 × 0.25 mm. $T_{min/max}$ = 0.93, 1.00. 12699 reflections collected, 4296 unique (R_{int} = 0.025), R = 0.040 [4112 reflections with $I > 2\sigma(I)$], wRF^2 = 0.108 (all data).

AlamarBlue Assay. LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in phenol-red free RPMI-1640 medium (Thermo Fisher Scientific) supplemented with 5% FBS (Thermo Fisher Scientific) at 37 °C in an

atmosphere containing 5% CO_2 and maintained in log phase growth. Cell viability as a function of metabolic activity was measured by an alamarBlue endpoint assay (Thermo Fisher Scientific) as previously described.[16] Briefly, LNCaP (4000 cells per well) were seeded for 24 h into 96-well tissue culture plates (Corning, Corning, NY, USA) and treated with the indicated compounds. Metabolic activity was measured with alamarBlue according to the manufacturer's instructions (Thermo Fisher Scientific) after 72 h of treatment. Compounds were dissolved in DMSO and diluted in growth medium (final concentration 0.3%). Control cells were treated with the equivalent dose of DMSO (negative control) or vinblastine (50 nM; Sigma Aldrich) as a positive control. Each data point was measured in triplicate, and repeated in at least four independent experiments.

Drug Resistance Reversal Assay. Human T-cell acute lymphoblastic leukemia cells, CCRF-CEM (CEM), and drug-resistant subline CCRF-CEM/VCR R (CEM/VCR R)[40,41] were kind gifts from Professor Kavallaris (Children's Cancer Institute, Lowy Cancer Research Centre, UNSW). All cells were maintained in suspension in RPMI 1640 (10% FBS) as described previously. [40,55] Cells were seeded in 96-well plates (20000 cells per well) and co-treated at the same time with a dose titration of vinblastine and the indicated compounds at 5 μM for 72 h. Cell viability as a function of metabolic activity was measured by an alamarBlue endpoint assay according to the manufacturer's instructions. Calculations of half-maximal inhibitory concentration (IC_{50}) after 72 h of treatment were performed with GraphPad Prism (GraphPad Software). Each data point was performed in triplicate, and repeated in at least two independent experiments.

Leucine Transport Inhibition Assay. The [^{3}H]L-leucine uptake was performed as detailed previously.[45] Briefly, cells were cultured in 6-well plates in RPMI media. After collecting and counting, cells (3×10^4/well) were incubated with 0.3 μCi [^{3}H]L-leucine (200 nM; PerkinElmer)

in leucine-free RPMI media (Invitrogen) with 10% (v/v) dialyzed FBS in the presence of different concentrations of compounds for 15 min at 37 °C. Cells were collected, transferred to filter paper using a 96-well plate harvester (Wallac PerkinElmer), dried, exposed to scintillation fluid and counts measured using a liquid scintillation counter (PerkinElmer). GraphPad Prism was used to determine the IC_{50} of each compound. Each data point was determined in triplicate, and repeated in three independent experiments. An unpaired two-tailed Student's t-test was used to test for significant differences of data points.

ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra for bilocularins A-C (**1**–**3**); 1D NMR spectra for compounds **7**, **8**, **10**, **11**, and **13**; COSY, HMBC, and ROESY diagrams for compounds **2**–**3**; and alamarBlue assay data for LNCaP cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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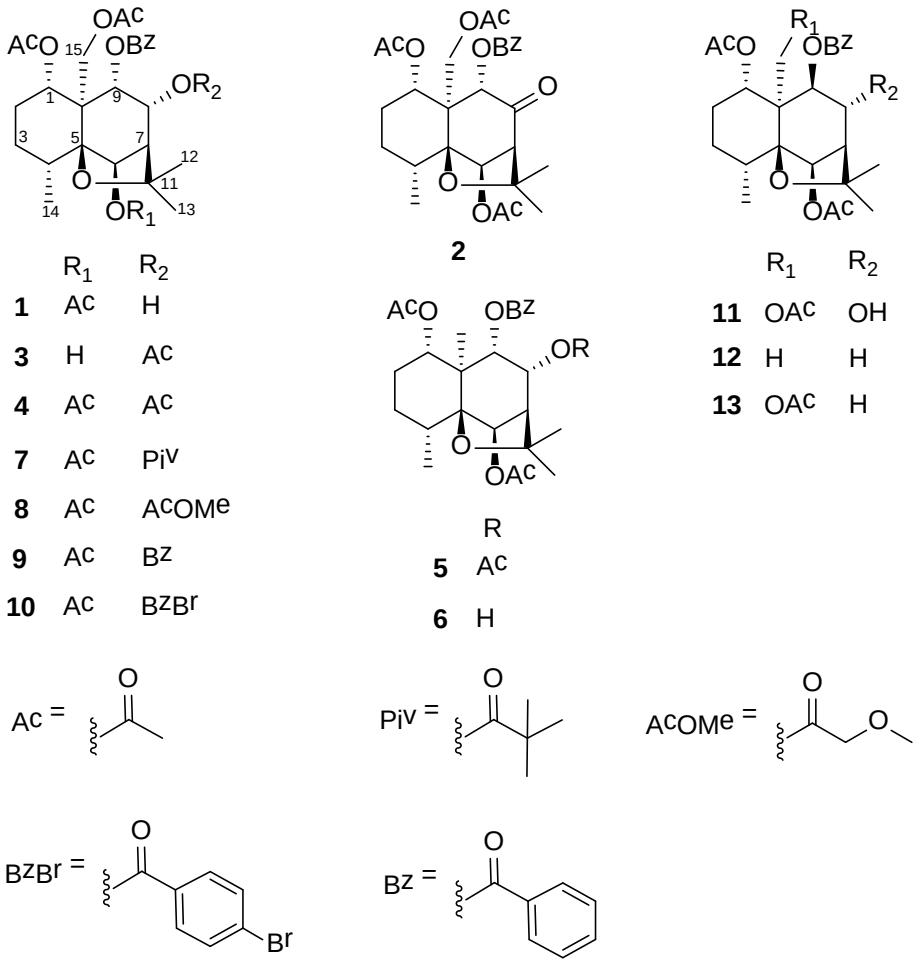


Figure 1. Chemical structures of natural products (**1**–**6** and **11**–**13**) and semi-synthetic analogues (**7**–**10**).

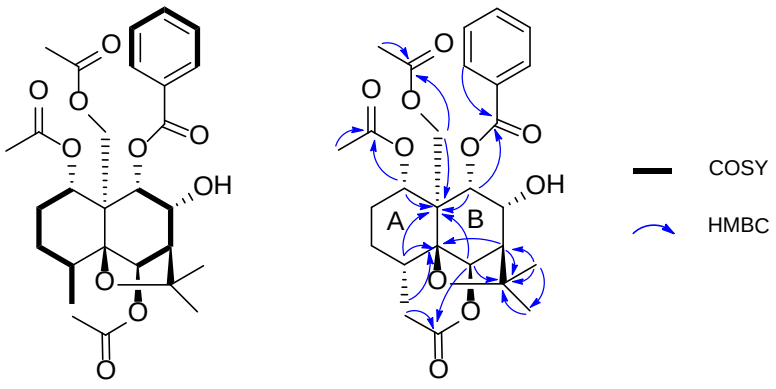


Figure 2. COSY and selected HMBC correlations of bilocularin A (**1**).

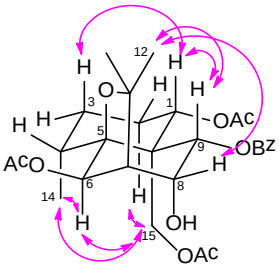


Figure 3. Key ROESY correlations of bilocularin A (**1**).

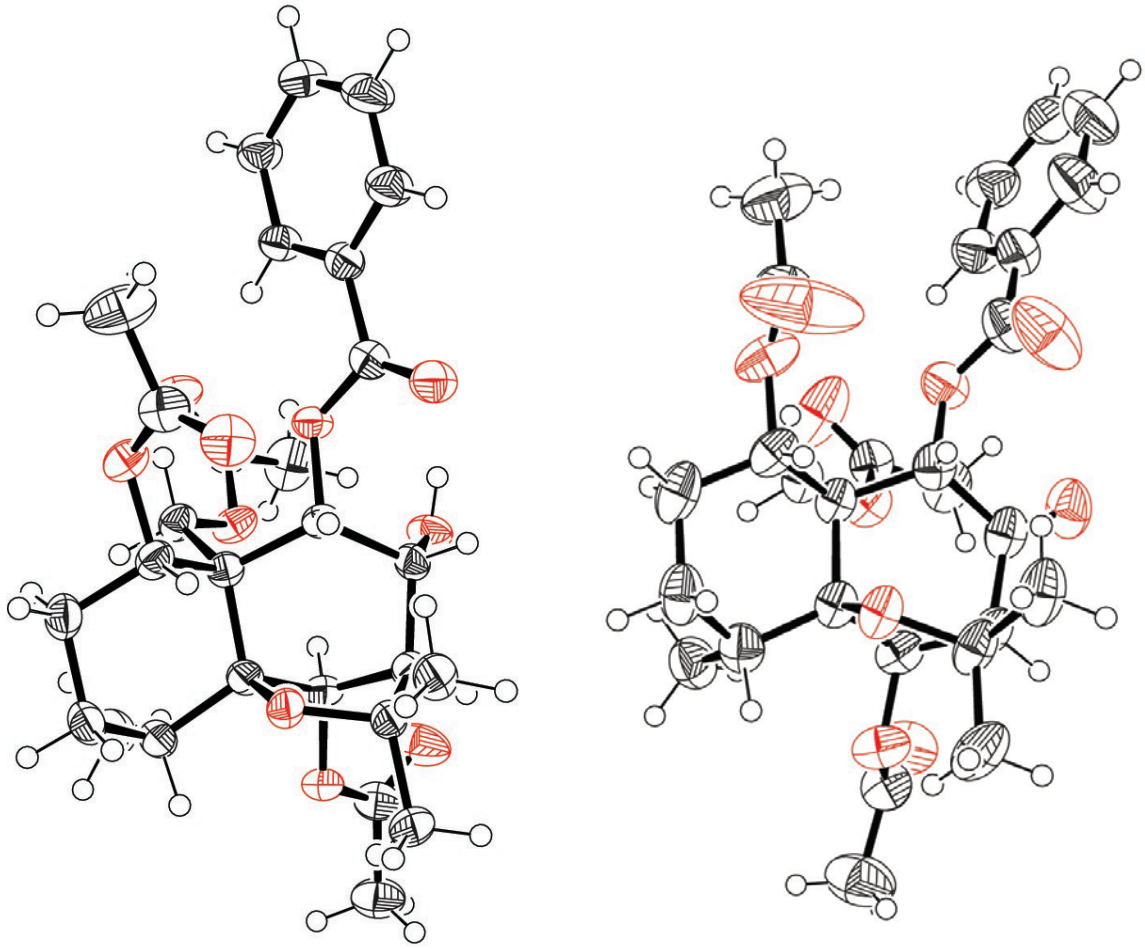


Figure 4. ORTEP drawings of bilocularin A (**1**) (left) and bilocularin B (**2**) (right).

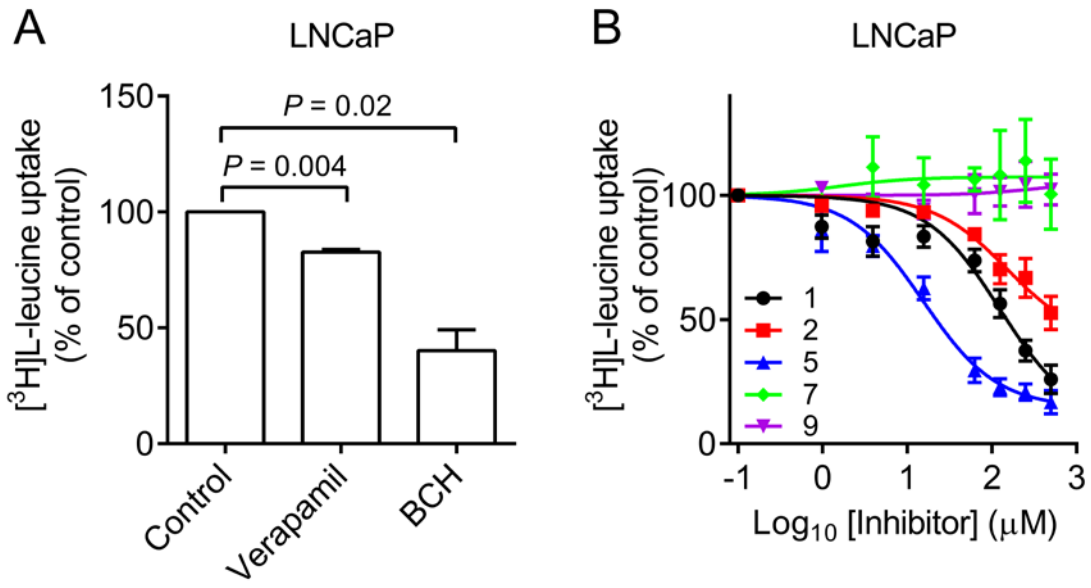


Figure 5. Characterization of compound effects on leucine uptake in LNCaP cells. (A) Leucine uptake assay in the presence of verapamil (10 μM) or BCH (10 mM) was performed in LNCaP cells (n=3, mean ± S.E.M). (B) Leucine uptake assays in the presence of different doses of compounds **1**, **2**, **5**, **7** or **9** was performed in LNCaP cells (n=3, mean ±S.E.M). An unpaired two-tailed Student's t-test was used to test for significant differences.

Table 1. **^{1}H NMR (500 MHz) Spectroscopic Data of Bilocularins A–C (1–3) in $CDCl_3$[a]**

position	1	2	3
	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)	δ_H, mult. (*J* in Hz)
1	5.40, dd (12.0, 4.6)	5.50, dd (12.1, 4.4)	5.39, dd (10.5, 6.1)
2*α*	1.61, m	1.58, m	1.74,[b] m
2*β*	1.71, m	1.77, m	1.74,[b] m
3*α*	1.45, m	1.55, m	1.48, m
3β	2.18, m	2.22, m	2.21, m
4	2.20, m	2.41, m	2.29, m
6	6.56, d (1.1)	6.42, d (1.0)	5.25, d (4.4)
7	2.40, br d (4.2)	3.05, d (1.0)	2.37, d (3.6)
8	4.42, dd (5.7, 4.2)	-	5.63, dd (5.8, 3.6)
9	5.55, d (5.7)	5.87, s	5.64, d (5.8)
12	1.50, s	1.53, s	1.56, s
13	1.42, s	1.51, s	1.52, s
14	1.00, d (7.4)	1.07, d (7.5)	1.21, d (7.5)
15	4.60, d (12.9)	4.37, d (12.5)	4.65, d (13.1)
	4.92, d (12.9)	5.03, d (12.5)	4.95, d (13.1)
OH-6	-	-	1.69, d (4.4)
OH-8	[c]	-	-

[a] NMR data corresponding to ester groups are provided in the Experimental Section.
[b] Overlapping signals.
[c] Not observed.

Table 2. ^{13}C NMR (125 MHz) Spectroscopic Data of Bilocularins A–C (1–3) in $CDCl_3$[a]

position	1	2	3
	δ_C, type	δ_C, type	δ_C, type
1	79.0, CH	77.2, CH	79.6, CH
2	23.2, CH_2	23.4, CH_2	23.4, CH_2
3	26.4, CH_2	26.4, CH_2	26.7, CH_2
4	33.2, CH	33.8, CH	33.2, CH
5	90.7, C	92.1, C	92.1, C
6	74.0, CH	74.6, CH	73.3,[b] CH
7	55.5, CH	65.3, CH	54.7, CH
8	69.0, CH	198.6, C	70.7, CH
9	75.6, CH	80.0, CH	73.2,[b] CH
10	51.6, C	51.3, C	50.7, C
11	81.2, C	83.1, C	81.5, C
12	24.5, CH_3	25.1, CH_3	24.9, CH_3
13	30.6, CH_3	30.1, CH_3	31.0, CH_3
14	15.6, CH_3	16.4, CH_3	16.3, CH_3
15	60.7, CH_2	60.5, CH_2	61.2, CH_2

[a] NMR data corresponding to ester groups are provided in the Experimental Section.
[b] Interchangeable signals.

Table 3. Drug Resistance Reversal Ability of Compounds 1, 2, 5, 7, and 9 (all at 5 μM) in a Drug Resistant Cell Line.

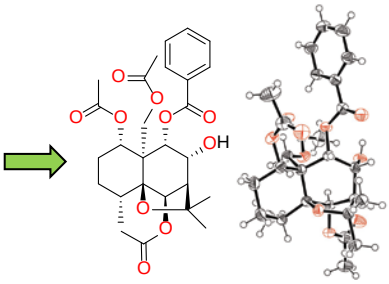
Treatment	CEM		CEM/VCR R	
	IC_{50} (nM)[a]	RI[b]	IC_{50} (nM)[a]	RI[b]
vinblastine	2.8 ± 1.1	-	1336.2 ± 302.1	-
+compound **1**	1.6 ± 1.0	1.7	36.8 ± 2.3	36.3
+compound **2**	1.5 ± 0.8	1.8	26.4 ± 0.0	50.7
+compound **5**	1.4 ± 0.4	2.0	21.9 ± 9.2	60.9
+compound **7**	1.9 ± 1.0	1.5	10.8 ± 3.1	123.3
+compound **9**	1.3 ± 0.6	2.1	10.2 ± 3.4	131.2
+verapamil	1.0 ± 0.0	2.9	20.7 ± 16.9	64.6

[a]IC_{50} values were determined using a viability assay (alamarBlue) in two independent experiments performed in triplicates (mean ± SD). Verapamil (5 μM) was used as positive control.

[b]RI: reversal index. The RI was calculated as the ratio of the IC_{50} (vinblastine) to the IC_{50} (vinblastine + tested compound at 5 μM).

Table of Content Graphic

Maytenus bilocularis



Bilocularin A (1)