Published in final edited form as:

J Nat Prod. 2016 April 22; 79(4): 784–791. doi:10.1021/acs.jnatprod.5b00810.

Cyclopenta[*b*]benzofuran and Secodammarane Derivatives from the Stems of *Aglaia stellatopilosa*

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Abstract

Four new 2,3-secodammarane triterpenoids, stellatonins A–D (**3–6**), together with a new 3,4-secodammarane triterpenoid, stellatonin E (**7**), and the known silvestrol (**1**), 5^{'''}-episilvestrol (**2**), and β -sitosterol, were isolated from a methanol extract of the stems of *Aglaia stellatopilosa* through bioassay-guided fractionation. The structures of the new compounds were elucidated using spectroscopic and chemical methods. The compounds were evaluated for their cytotoxic activity against three human cancer cell lines and for their antimicrobial activity using a microtiter plate assay against a panel of Gram-positive and Gram-negative bacteria and fungi.

Graphical Abstract

The genus *Aglaia* of the plant family Meliaceae is represented by approximately 120 known species distributed in the tropical rainforests of the Indo-Australasian region, ranging from India and Sri Lanka, to Australia (Queensland, Northern Territory, and Western Australia), east to Samoa in Polynesia, north to the Mariana Islands (Saipan, Roti, and Guam), and the Caroline Islands (Palau and Ponape) in Micronesia.^{1,2} *Aglaia* species are known for being the sources of three closely related structural groups of oxygen-containing heterocyclic secondary metabolites, namely, the cyclopenta[*b*]benzofurans (flavaglines), the

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ASSOCIATED CONTENT

Supporting Information

NMR spectra of compounds **3–7**, and ¹H NMR spectra of the (*R*)- and (*S*)-MTPA esters of **3** are available free-of-charge via the Internet at <http://pubs.acs.org/JNP>.

cyclopenta[*b*]benzopyrans (thapcapsins), and the benzo[*b*]oxepines (thapoxepines).^{3–7} Several cyclopenta[*b*]benzofurans have attracted considerable interest for their bioactive principles, particularly those with potential pesticidal and anticancer effects, inclusive of the two lead compounds, rocaglamide and the dioxanyl-ring containing silvestrol (**1**).^{3–7} Other types of secondary metabolites reported from *Aglaia* species include amides,^{8,9} flavonoids and other phenolic compounds,¹⁰ sesquiterpenes,¹⁰ and triterpenoids, particularly of the baccharane,^{10,11} cycloartane,^{12,13} and dammarane^{14,15} types. Some of these miscellaneous compounds have been found to possess interesting biological activities, including the antileukemic diamide, (–)-odorinol⁸ and the antiviral secodammarane triterpenoid, dammarenolic acid.¹⁶

Silvestrol (**1**) and its 5'''-epimer, episilvestrol (**2**), were characterized structurally and in terms of their absolute configurations initially from the twigs of an Indonesian plant, *Aglaia foveolata* Pannell and were both found to exhibit potent inhibitory activity for several human cancer cell lines.¹⁰ Further phytochemical work on the stem bark of *A. foveolata* led to the purification of two minor stereoisomers of silvestrol (**1**),¹⁷ while an extract of the leaves of this species afforded several cyclopenta[*bc*]benzopyran derivatives.¹¹ To date, no cyclopenta[*b*]benzofurans that do not contain a dioxanyl ring have been isolated from prior phytochemical work on *A. foveolata*.^{10,11,14,17}

In initial in vivo work on silvestrol (**1**), it was found to be active when administered both intraperitoneally (ip) and intravenously (iv) in the P388 murine leukemia model.¹⁰ Subsequently, silvestrol (**1**) has exhibited activity in other in vitro and in vivo test systems, including those germane to various B-cell malignancies,^{18–22} as well as breast and prostate cancer,²³ and hepatocellular carcinoma.²⁴ Mechanistically, silvestrol (**1**) is an inhibitor of protein translation, and affects the composition of the RNA helicase eukaryotic initiating factor (eIF) 4F complex.²³ An affinity purification procedure showed eIF4AI/II to be the molecular target of a biotinylated derivative of episilvestrol (**2**).²⁵ Silvestrol (**1**) has undergone preclinical toxicological evaluation through the NExT program of the U.S. National Cancer Institute.

Aglaia stellatopilosa Pannell, a species endemic to Central Borneo, Sarawak, Malaysia is found from sea level to an altitude of 1200 m above sea level. It is a dioecious tree or shrub with small fragrant flowers that can grow up to 8 m in height with a stem diameter of up to 10 cm. Its twigs are densely covered with brown stellate hairs, while its bark is usually greyish green.^{2,26} Phytochemically, no detailed report on this species has been published. Eight compounds were isolated in the present study from the stems of *A. stellatopilosa*, including the known silvestrol (**1**), episilvestrol (**2**), and β -sitosterol, and five new secodammarane derivatives (**3–7**). The isolated compounds were tested against several cancer cell lines (HT-29, MCF-7 and NCI-H460) as well as a small panel of bacterial and fungal strains, to evaluate their potential biological activities, and the results obtained are described herein.

RESULTS AND DISCUSSION

Bioassay-guided fractionation of a MeOH extract of *A. stellatopilosa* stems using silica gel column chromatography and HPLC led to the isolation of the potentially cytotoxic cyclopenta[*b*]benzofuran derivatives, silvestrol (**1**, 17.5 mg) and episilvestrol (**2**, 10.2 mg), as purified from a silvestrol-enriched fraction that eluted with 100% EtOAc. These two compounds were present also in other highly cytotoxic fractions that eluted with CHCl₃-EtOAc (30:70) and EtOAc-MeOH (70:30). As determined quantitatively by HPLC, the combined active fractions were estimated to contain a total of 0.040% (w/w) of compound **1** and 0.053% (w/w) of compound **2**. In contrast, the percentage yields of silvestrol (**1**) as isolated from the fruits and stems of *A. foveolata* were 0.0085 and 0.01% (w/w), respectively.¹⁰ Also differing from the results of the present study, episilvestrol (**2**) was found to be only a minor constituent of *A. foveolata*, and was absent from the fruits but occurred in a low yield of 0.00064% (w/w) in the twigs.¹⁰ No further rocaglate derivatives were detected from any of the other fractions obtained from *A. stellatopilosa* stems. However, further fractionation using a combination of chromatographic methods afforded β -sitosterol and five new secodammarane derivatives (**3–7**).

Compound **3** was obtained as a white, needle-like solid. The IR spectrum indicated the presence of hydroxy group (3436 cm⁻¹), ester carbonyl (1734 cm⁻¹), and amide carbonyl (1693 and 1659 cm⁻¹) functionalities.²⁷ The HRESIMS showed an [M + Na]⁺ molecular ion peak at *m/z* 572.3953, corresponding to a molecular formula of C₃₂H₅₅NO₆Na (calcd 572.3927), which indicated six indices of hydrogen deficiency and the presence of a nitrogen atom. Three indices of hydrogen deficiency were accounted for by the carbonyl carbon (δ_C 179.5), two sp² carbons (δ_C 128.8 and δ_C 118.8), and one amide carbonyl carbon (δ_C 157.7). The structure of **3** could be proposed as comprising three rings (B/C/D) of a modified triterpenoid skeleton, in order to meet the remaining three indices of hydrogen deficiency. In the high-field region of the ¹H NMR spectrum, the signals observed were represented by seven tertiary methyl groups at δ_H 0.87 (3H, s, H-30), 0.98 (3H, s, H-18), 1.07 (3H, s, H₃-19), 1.11 (3H, s, H₃-29), 1.17 (3H, s, H₃-26), 1.18 (3H, s, H₃-28) and 1.22 (3H, s, H₃-27) and one methyl group attached to an oxygenated secondary carbon at δ_H 1.14 (3H, s, H₃-21). In addition, a methoxy group signal at δ_H 3.54 (3H, s, -OCH₃) and one oxygenated methine signal at δ_H 3.38 (1H, d, *J* = 10.5 Hz, H-24) were also observed. The

low-field region of the ^1H NMR spectrum exhibited signals for two olefinic protons at δ_{H} 4.88 (1H, d, $J = 14.4$ Hz, H-1) and 6.49 (1H, dd, $J = 10.8, 14.4$ Hz, H-2), as well as the signals of an NH unit at δ_{H} 7.03 (1H, d, $J = 10.8$ Hz, NH) and a formyl proton at δ_{H} 8.05 (1H, s, CHO), which was ascribed to a formamide group. The ^{13}C and DEPT NMR data showed 32 carbon signals, constituted by nine methyls, eight methylenes, six methines (one olefinic), four sp^3 quaternary carbons, one oxygenated methine (δ_{C} 79.0, C-24), two oxygenated tertiary carbons (δ_{C} 75.7, C-20 and δ_{C} 73.4, C-25), one ester carbonyl group (δ_{C} 179.5, C-3), and one formamide group (δ_{C} 157.7, NHCHO). These NMR data suggested that **3** is a triterpenoid with a skeleton similar to that of known secodammarane derivatives that have been isolated from other species in the genus *Aglaia*.^{15,16,28} Comparison of the NMR data with values for related dammarane triterpenoids revealed that major changes occurred at ring A in **3**. In the HMBC spectrum, H₃-28, H₃-29, and the protons of the methoxy group at δ_{H} 3.54 were all found to show strong correlations with the carbonyl group at δ_{C} 180.8 (C-3), which suggested that the cleavage of ring A occurred between C-2 and C-3, and that the resulting C-3 carboxylic acid group was esterified. Furthermore, key HMBC correlations between H₃-19 and the olefinic carbon at δ_{C} 128.8 (C-1), as well as the formyl proton at δ_{H} 8.05 (NHCHO) and another olefinic carbon at δ_{C} 118.8 (C-2), suggested that the formamide group is attached to C-2. An *E* configuration was assigned to the 1,2 double bond based on the large vicinal proton coupling constant ($J_{1,2} = 14.4$ Hz). During the ^1H NMR analysis, the presence of a minor isomer (**3a**) of **3** was observed. The ratio of **3** and **3a**, based on the integration of the formyl proton signals, was approximately 3:1 in CDCl_3 . This isomerization arose from the restricted C-N bond rotation of the monosubstituted formamide group. As mentioned above, the ^1H NMR signal of the formyl proton of **3** appeared at δ_{H} 8.05 as a singlet, indicating an undetectable small coupling constant between the formyl and NH protons, and suggested a *cis* conformation of the formamide group in compound **3**, which was confirmed by an observed NOE effect between the formyl and NH protons. Owing to compound quantity constraints no full NMR assignments were obtained for the minor isomer (**3a**). However, the corresponding signals of the formyl and NH protons could still be recognized at δ_{H} 8.23 (d, $J = 11.4$ Hz, CHO) and 7.10 (partly overlapped, NH), respectively, in the less crowded low-field region of the ^1H NMR spectrum. The large coupling constant of $J \approx 11.4$ Hz between these two protons indicated that the *trans* conformation is adopted by the formamide group in this minor isomer (**3a**). Therefore, compound **3**, the *cis*-isomer predominates in the equilibrium mixtures arising from the hindered C-N bond rotation, which is consistent with a previous report.²⁹ Furthermore, HMBC correlations from H-21 to C-20 and C-22, H-26 and H-27 to C-24 and C-25, as well as H-24 to C-22 and C-27 allowed the three hydroxy groups to be located at C-20, C-24, and C-25 of the side chain, respectively (Figure 1).

The absolute configuration of C-24 in compound **3** was determined using the Mosher ester method. Treatment of **3** with (*R*)- and (*S*)-MTPA chloride gave the C-24 (*S*)- and (*R*)-MTPA ester derivative, respectively. Analysis of the ^1H NMR chemical shift differences ($\delta_{\text{S-R}}$) between the (*S*)- and (*R*)-MTPA ester derivatives led to the assignment of the *R*-configuration at C-24 (Figure 2). The *R* configuration of C-20 was established by comparing the ^{13}C NMR chemical shifts of the diagnostic carbons C-17, C-21, and C-22 with those of reported dammarane derivatives with a C-20-hydroxy substituent.³⁰ In addition, key NOE

enhancements were observed for H-1 with H-5 and H-9, H₃-18 with H-13, as well as H₃-30 with H-9 and H-17, and revealed that the relative configuration of the skeleton of compound **3** is consistent with those of known dammarane derivatives. Therefore, the structure of compound **3** (stellatonin A), was identified as a mixture of methyl 20*R*,24*R*,25-trihydroxy-2,3-secodammar-2-[*cis*-formamide]-1-(*E*)-en-3-oate (**3**) and its minor isomer, methyl 20*R*,24*R*,25-trihydroxy-2,3-secodammara-2-[*trans*-formamide]-1-(*E*)-en-3-oate (**3a**).

Compound **4** was obtained as a white, amorphous solid. The IR spectrum indicated the presence of hydroxy (3333 cm⁻¹) and carbonyl (1713, 1707 cm⁻¹) groups. The HRESIMS showed an [M + Na]⁺ molecular ion peak at *m/z* 545.3809, which corresponded to a molecular formula of C₃₁H₅₄O₆Na (calcd 545.3818), indicating five indices of hydrogen deficiency. Two carbonyl groups (δ_C 203.3, C-2 and δ_C 180.3, C-3) accounted for two of the indices of hydrogen deficiency. This suggested that the structure of **4** also includes three intact rings (B/C/D) of a modified triterpenoid structure. The ¹³C NMR chemical shifts for rings B-D, including the C-17 side chain, were closely similar to those of compound **3**, with variations between these isolates occurring in the seco A-ring. Instead of signals of a formamide feature in compound **3**, a formyl group was recognized, based on a characteristic proton signal at δ_H 9.81 (1H, d, *J* = 3.5 Hz) in the ¹H NMR spectrum and a corresponding carbonyl signal at δ_C 203.3 in the ¹³C NMR spectrum of **4**. This formyl proton showed a COSY correlation with one of the C-1 protons at δ_H 2.31 (1H, dd, *J* = 14.4, 3.8 Hz, H-1a), and was thus assigned to C-2. The deduction was confirmed by key HMBC correlations from H-1 and H₃-19 to C-2, and from H-2 to C-1. Except for ring A, the remaining structural features of **4** were readily comparable with those of **3** based on a 1D and 2D NMR data analysis and by comparing with the published data of known analogues.³¹ Thus, the structure of compound **4** (stellatonin B) was assigned as methyl 20*R*,24*R*,25-trihydroxy-2,3-secodammar-2-al-3-oate.

Compound **5** was obtained as a white amorphous solid. The molecular formula of C₃₁H₅₄O₇ was deduced from the sodiated molecular ion peak [M+Na]⁺ in the HRESIMS at *m/z* 561.3760 (calcd 561.3767), consistent with the addition of one oxygen atom as compared to **4**. Compound **5** exhibited ¹H and ¹³C NMR data that were closely similar with those of compound **4**, with the major differences involving the C-2 functional group. The formyl group at C-2 in **4** was found to substituted by a hydroxycarbonyl group in **5**, based on the typical carbonyl signal that appeared at δ_C 175.3 in the ¹³C NMR spectrum. Due to this change, an upfield shift of nearly 8 ppm for C-1 in the ¹³C NMR spectrum was noted when compared with the same signal in compound **4**. The C-2 position of the carboxylic acid group was confirmed by HMBC correlations from H-1 to C-2, C-5, and C-9, as well as HMBC correlation from methyl group of C-18 to C-2. Further analysis of the 2D NMR data of **5** revealed the presence of comparable HMBC effects and NOE correlations to compound **4**. Accordingly, the structure of compound **5** (stellatonin C) was defined as 20*R*,24*R*,25-trihydroxy-2,3-secodammara-2,3-dioic acid-3-methyl ester.

Compound **6** was obtained as a white, needle-like solid. The HRESIMS showed an [M+Na]⁺ molecular ion peak at *m/z* 554.3826 (calcd 554.3821), corresponding to a molecular formula of C₃₂H₅₃NO₅Na, consistent with seven indices of hydrogen deficiency. From the ¹H and ¹³C NMR data of **6**, the structures of the seco A-ring moiety and the ring B/C/D

subunits were assigned as being closely comparable to those of **3**, with major differences observed at the side chain attached to ring D due to the presence of a tetrahydrofuran-2-yl group. In the HMBC spectrum, correlations between H₃-21/C-17, C-20, and C-22, H₃-26 and H₃-27, C-24, and C-25, supported the proposed side chain (Figure 1). When comparing the ¹³C NMR data of **6** with those of other reported secodammaranes, diagnostic resonances inclusive of those of C-20-C-22 and C-24 showed similarities with analogous data for methyl isofoveolate B and shoreic acid.²⁸ Thus, a 20*S*, 24*R* configuration of the tetrahydrofuran-2-yl moiety was proposed. As for compound **3**, **6** also was found to contain a trace amount of a *trans*-configured isomer of the formamide unit based on NMR spectroscopic data analysis. Hence, the structure of this compound (stellatonin D) was elucidated as a mixture of methyl 20*S*,24*R*-epoxy-25-hydroxy-2,3-secodammar-2-[(*cis*-formamide)-1-(*E*)-en-3-oate (**6**) and its minor isomer, methyl 20*S*,24*R*-epoxy-25-hydroxy-2,3-secodammara-2-[*trans*-formamide]-1-(*E*)-en-3-oate (**6a**).

Compound **7** was obtained as a white, amorphous solid. The HRESIMS data showed an [M + Na]⁺ molecular ion at *m/z* 529.3856, and corresponded to a molecular formula of C₃₁H₅₄O₅Na (calcd 529.3869), indicating the compound to have five indices of hydrogen deficiency. Comparison of the NMR data of this compound with those of compounds **4** and **5**, showed the absence of a tertiary methyl group in **7**, and instead, a terminal vinyl group was recognized based on the ¹³C NMR resonances at δ_C 147.7 (C) and δ_C 113.6 (CH₂) and the corresponding proton signals at δ_H 4.84 and 4.65 (each 1H, brs, H-28). In the HMBC spectrum, a relatively low-field methyl group at δ_H 1.73 (3H, s, H₃-29) showed correlations with the two olefinic carbons and the C-5 methine carbon (δ_C 50.8). In addition, HMBC correlations from the two vinyl protons to C-5 and C-29 were also observed. Thus, the presence of a ^{4,28} double bond was confirmed, which implied that instead of the 2,3-secodammarane skeleton for compounds **3–6**, compound **7** is based on a 3,4-secodammarane ring system.²⁸ In the HMBC spectrum, the protons of the methoxy group at δ_H 3.67 and H-2 showed strong correlations with the carbonyl group at δ_C 174.8 (C-3), which suggested that methylation occurred at the C-3 carboxylic acid group. Several known 3,4-secodammarane derivatives isolated from *Aglaia* species have been found to contain a tetrahydrofuran-2-yl moiety in the D ring.³² However, compound **7** possessed a trihydroxylated side chain, similar as in compounds **3**, **4** and **5** (Figure 1). Accordingly, the structure of compound **7** (stellatonin E) was defined as methyl 20*R*,24*R*,25-trihydroxy-3,4-secodammara-4(28)-en-3-oate.

Compounds **1** and **2** were both found to possess significant cytotoxic activity against the HT-29,¹⁷ MCF-7, and NCI-H460 human cancer cell lines. Compounds **1** and **2** also showed growth inhibition activity of >90% against two yeasts, *Saccharomyces cerevisiae* and *Rhodoturula glutinis*, at a concentration of 200 μM. Compound **7** exhibited the highest activity against the Gram-positive bacteria that were evaluated, namely, *Bacillus subtilis* and *Staphylococcus aureus*, with 90% inhibition at concentrations of 20 and 80 μM, respectively (Table 2).

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were obtained using Stuart SMP3 melting point apparatus, and are uncorrected. Optical rotations were measured on a Perkin-Elmer 343 automatic polarimeter. IR spectra (in CHCl_3) were recorded on a Thermo Scientific Nicolet 6700 FT-IR and Perkin Elmer Frontier FTIR spectrometer. UV spectra (in MeOH) were recorded on a Varian Cary 50 UV-visible spectrophotometer. NMR spectra were recorded on a Bruker AVIIIHD-700 or 600 NMR spectrometer with cryoprobe, and also with AVIII-400, AVIII-500, or DRX-400 NMR spectrometers. Mass spectra were run on Bruker MicroTOF-QII and Waters Q-TOF Micro mass spectrometers. Column chromatography was performed using silica gel (60–200 mesh; J.T. Baker, Center Valley, PA, USA; 230–400 mesh; Sorbent Technologies, Atlanta, GA, USA) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Analytical TLC was conducted on pre-coated 250 μm thickness silica gel plates (UV₂₅₄, aluminum backed; Merck, Darmstadt, Germany). Analytical HPLC was conducted on a 250 $\times$ 4.6 mm i.d Phenomenex Luna C₁₈ (5 μm) column or 150 $\times$ 4.6 mm i.d. Agilent Eclipse XDB C₁₈ (5 μm) column using a Hitachi Elite LACHrom or an Agilent 1100 Series instrument. Semi-preparative HPLC was conducted on a 250 $\times$ 9.4 mm i.d. Agilent Eclipse XDB C₁₈ (5 μm) column using an Agilent 1200 series semi-preparative HPLC instrument.

Plant Material

The stems of *Aglaia stellatopilosa* were collected in Ba'kelalan (03° 57' N; 115° 36' E; 1173 m altitude), Sarawak, Malaysia in June, 2008 by Tu Chu Lee, and were identified by Jovita Elderson, with the taxonomic identification confirmed by C.M.P. A voucher specimen (SABC4440) has been deposited in the Traditional Knowledge Herbarium, Sarawak Biodiversity Centre, Kuching, Sarawak, Malaysia.

Extraction and Isolation

The dried ground stems of *A. stellatopilosa* (36.7 kg) were extracted three times with MeOH (140 L for the first stage and 75 L each for the second and third stages) at room temperature for 3 days, filtered and concentrated under reduced pressure to yield 2.13 kg of a thick, dark brown syrup. An aliquot (150 g) of the MeOH extract was fractionated by Si gel column chromatography using gradient mixtures from hexanes $\rightarrow$ CHCl_3 $\rightarrow$ EtOAc $\rightarrow$ MeOH as eluents to obtain 14 fractions (F1 $\rightarrow$ F14). Among these, F11 was found to be the most cytotoxic against NCI-H460 and MCF-7 cells, with GI₅₀ values of 0.032 $\mu\text{g/mL}$ and 0.025 $\mu\text{g/mL}$, respectively. Fraction F11 (613.7 mg) was subjected to semi-preparative HPLC (MeOH-H₂O, 55:45) to yield compounds **1** (17.5 mg) and **2** (10.2 mg). Fraction F8 (3.72 g) formed a white precipitate and was separated further using a column containing Sephadex LH-20, which was eluted with acetone, to afford nine sub-fractions (F8-1 $\rightarrow$ F8-9). Sub-fraction F8-4, which was found to be cytotoxic against HT-29 and MCF-7 cells with GI₅₀ values of 12.1 $\mu\text{g/mL}$ and 21.2 $\mu\text{g/mL}$, respectively, was subjected to successive fractionation using Sephadex LH-20 (MeOH), Si gel column chromatography (CH_2Cl_2 $\rightarrow$ EtOAc $\rightarrow$ MeOH), and HPLC ($\text{CH}_3\text{CN-H}_2\text{O}$ (30:70) $\rightarrow$ 100:0) to obtain **4** (172.6 mg) and **7** (53.8 mg). Compound **4** (10 mg) was also obtained from F8-5 as well as **5** (2.3 mg) using Si

gel column chromatography (CHCl₃-EtOAc (100:0) → 0:100), followed by semi-preparative HPLC (CH₃CN-H₂O (20:80) → 100:0) and analytical HPLC (CH₃CN-H₂O (50:50)).

A part of the MeOH extract (100 g) was partitioned sequentially with hexanes (6 × 600 mL) and EtOAc (6 × 600 mL). The EtOAc fraction was concentrated under reduced pressure to yield 51.7 g of an EtOAc-soluble extract. An aliquot (50 g) of the dried EtOAc fraction (GI₅₀ values for the NCI-H460 and MCF-7 cell lines, 1.1 and 1.2 µg/mL, respectively) was fractionated by Si gel column chromatography using a gradient mixtures from hexanes → (CH₃)₂CO → MeOH as eluents to afford eight fractions (AS01 → AS08). Fraction AS(2)04D was separated by passage over a Sephadex LH-20 column (acetone) followed by C₁₈ silica gel column chromatography (CH₃CN-H₂O (50:50) → 100:0) and semi-preparative HPLC (CH₃CN-H₂O (20:80) → 100:0) to obtain **3** (11.4 mg).

An additional 100 g of the crude MeOH extract was fractionated by Si gel column chromatography using gradient mixtures of hexanes and MeOH as eluents to obtain 70 fractions. Fraction F35 appeared as a milky suspension, and, when washed with hexanes and a small amount of CH₂Cl₂, yielded a white precipitate of β-sitosterol (11.0 mg). Additionally, fractions F39-F44, which each formed a precipitate, were pooled (6.83 g), and subjected to a second Si column chromatographic stage with a gradient mobile phase of CH₂Cl₂ and EtOAc mixtures, affording 50 subfractions. Of these, F36, on being washed with C₆H₁₄, yielded compound **6** (18.0 mg).

Stellatonin A (3)—White needle-like solid; mp 182–183 °C; $[\alpha]_D^{20} + 33$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 220 (4.06) nm; IR (film) $\nu_{\max}$ 3436, 2953, 2871, 1723, 1734, 1693, 1659, 1535, 1455, 1390, 1255, 1127, 1071, 960, 765, 578 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 572.3953 [M + Na]⁺ (calcd for C₃₂H₅₅NO₆Na, 572.3927).

Stellatonin B (4)—White amorphous powder; $[\alpha]_D^{20} + 30$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 220 (3.06), 260 (3.04) nm; IR (film) $\nu_{\max}$ 3333, 2951, 2657, 2359, 2342, 1713, 1707, 1458, 1391, 1377, 1258, 1146, 1126, 1074, 1013, 752, 667 cm⁻¹; ¹H and ¹³C NMR, see Table 1, HRESIMS m/z 545.3809 [M + Na]⁺ (calcd for C₃₁H₅₄O₆Na, 545.3818).

Stellatonin C (5)—White amorphous powder; $[\alpha]_D^{20} + 34$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 208 (2.96) nm; IR (film) $\nu_{\max}$ 3020, 2951, 2857, 2359, 2341, 1720, 1458, 1350, 1215, 748, 667 cm⁻¹; ¹H and ¹³C NMR, see Table 1; HRESIMS m/z 561.3760 [M + Na]⁺ (calcd for C₃₁H₅₄O₇Na, 561.3767).

Stellatonin D (6)—White needle-like solid; mp 202–203 °C; $[\alpha]_D^{20} + 10$ (c 0.2, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 230 (4.45) nm; IR (film) $\nu_{\max}$ 3375, 3258, 3175, 2966, 2878, 1729, 1718, 1675, 1660, 1522, 1389, 1377, 1284, 1254, 963, 768 cm⁻¹; ¹H NMR and ¹³C NMR, see Table 1; HRESIMS m/z 554.3826 [M + Na]⁺ (calcd for C₃₂H₅₃NO₅Na, 554.3821).

Stellatonin E (7)—White amorphous powder; $[\alpha]_D^{20} + 37$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 215 (3.10) nm; IR (film) $\nu_{\max}$ 3406, 3347, 2951, 2870, 2361, 2342, 1726, 1458,

1375, 1258, 1171, 1157, 1126, 1070, 1018, 893, 752, 667 cm^{-1} ; ^1H and ^{13}C NMR, see Table 1; HRESIMS m/z 529.3856 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{31}\text{H}_{54}\text{O}_5\text{Na}$, 529.3869).

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of Compound 3

The (*R*)- and (*S*)-MTPA ester derivatives of compound **3** were prepared in a manner described previously.^{30, 31} In brief, 1.5 mg of this compound were added into two NMR tubes, and dried completely. Pyridine- d_5 (0.5 mL) was added to each tube. Then, (*S*)-(+)- α -methoxy- α -(trifluoromethyl)phenylacetyl (MTPA) chloride (10 μL) or (*R*)-MTPA chloride (10 μL), was injected into the NMR tubes separately under N_2 gas and quickly mixed with the dissolved sample. After the reaction was completed, the amide-iminol tautomerizations were still observed for both ester derivatives in pyridine- d_5 . Samples were chromatographed over an open C_{18} column using MeOH- H_2O mixtures (50:50 to 100% MeOH) for elution to remove the deuterated pyridine solvent and other impurities. The ^1H NMR chemical shifts of the purified (*R*)- and (*S*)-MTPA esters of **3** were recorded in MeOH- d_4 . HSQC and HMBC experiments were used to establish the ^1H NMR assignments, and only fully assigned signals were used for the δ_{S-R} calculations.

(24R)-MTPA Ester of Stellatonin A (3)— ^1H NMR data (400 MHz, MeOH- d_4) δ_{H} 7.899 (1H, s, H-1), 7.602 (1H, s, H-1), 4.968 (1H, d, $J = 6.9$ Hz, H-24), 3.510 (3H, s, H-OMe-3), 1.808 (2H, m, H-23), 1.675 (1H, m, H-11), 1.775 (1H, m, H-5), 1.607 (1H, m, H-7, H-13, H-16, H-17), 1.480 (2H, m, H-11), 1.479 (2H, m, H-15), 1.479 (1H, m, H-9, H-16), 1.408 (2H, m, H-6), 1.325 (1H, m, H-7), 1.325 (2H, m, H-7, H-22), 1.172 (3H, s, H-26), 1.129 (3H, s, H-27, H-28), 1.083 (3H, s, H-29), 1.048 (3H, s, H-19), 0.971 (3H, s, H-18), 0.942 (3H, s, H-21), 0.864 (3H, s, H-30).

(24S)-MTPA Ester of Stellatonin A (3)— ^1H NMR data (400 MHz, MeOH- d_4) δ_{H} 7.570 (1H, s, H-1), 7.389 (1H, s, H-1), 4.948 (1H, d, $J = 6.1$ Hz, H-24), 3.503 (3H, s, H-OMe-3), 1.649 (1H, m, H-17), 1.618 (1H, m, H-5), 1.618 (2H, m, H-23, H-16), 1.563 (1H, m, H-7), 1.538 (1H, m, H-13), 1.488 (1H, m, H-11), 1.383 (1H, m, H-9), 1.383 (2H, m, H-15, H-22), 1.289 (2H, m, H-6), 1.289 (1H, m, H-7), 1.243 (2H, m, H-12), 1.127 (3H, s, H-28), 1.110 (3H, s, H-26), 1.077 (3H, s, H-29), 1.071 (3H, s, H-27), 1.025 (3H, s, H-19, H-21), 0.942 (3H, s, H-18), 0.855 (3H, s, H-30).

Preparation of Silvestrol (1) and Episilvestrol (2) Standard and Sample Solutions

Silvestrol (**1**) and episilvestrol (**2**) were obtained from Cerylid Biosciences Ltd. (Richmond, Victoria, Australia), and had a purity of >95% according to HPLC analysis. Compounds **1** and **2** were dissolved in HPLC grade MeOH and sonicated to yield standard stock solutions of 1 mg/mL. The working standard solutions of 0.8, 4, 20 and 100 $\mu\text{g/mL}$ were prepared by serial dilution of the standard stock solutions, respectively. Similarly to the standard solutions, all highly active fractions containing **1** and **2** were dissolved in HPLC grade MeOH to obtain a concentration of 1 mg/mL. After proper mixing and filtering of the sample solution, it was considered ready for HPLC analysis.

HPLC Analysis of the Concentration Levels of Silvestrol (1) and Episilvestrol (2) in *A. stellatopilosa* Stems

Quantitative analysis was performed on a reversed-phase analytical HPLC system, Agilent 1100 Series using the following conditions: working standard concentration: 0.8 to 100 µg/mL; sample concentration: 1000 µg/mL; injection volume: 20 µL; mobile phase: gradient elution of CH₃CN/H₂O (0–10 min, from 10:90 to 100:0; 11–15 min, 100% CH₃CN); UV detection wavelength: 210 nm; flow rate: 1.5 mL/min and column temperature: 35 °C. A four-point calibration curve was established by plotting the peak heights (absorbance) against the corresponding concentrations of the standard solutions. The concentrations of **1** and **2** in the samples were then calculated using peak data and standard calibration curves.

Cytotoxicity Assays

The in vitro screening of all compounds isolated was evaluated through bioassay against three human cancer cell lines, namely, colon (HT-29), breast (MCF-7) and lung (NCI-H460), according to a published protocol.^{33,34} The HT-29, MCF-7 and NCI-H460 cells were obtained from American Type Culture Collection (ATCC) (catalog numbers of HTB-38, HTB-22 and HTB-177, respectively).

Antimicrobial Assays

Antimicrobial assays were performed using a microtiter plate-based assay by procedures recommended in the *ISO 20776-1:2006 Manual*, with some modifications.³⁵ Samples were dissolved in sufficient DMSO to a final concentration of 200 µM and two-fold dilutions were performed until a 25 µM concentration was obtained. Ten pathogenic microorganisms were tested: Gram-positive bacteria, namely, *Bacillus subtilis*, BS (strain NBRC #3134), *Micrococcus luteus*, ML (strain NBRC #12708), and *Staphylococcus aureus*, SA (strain NBRC #12732); Gram-negative bacteria: *Escherichia coli*, EC (strain NBRC #3301), *Porphyromonas gingivalis*, PG (strain CCUG #25893), and *Pseudomonas aeruginosa*, PA (strain NBRC #12689); yeasts: *Pityrosporum ovale*, PO (strain CCUG #24229), *Rhodotulula glutinis*, RG (strain NBRC #1125), and *Saccharomyces cerevisiae*, SC (strain ATCC # 9763); and a fungus: *Aspergillus niger*, AN (strain NBRC #4066). All tested microorganisms were obtained from culture collection centers [NITE Biological Resource Centre (NBRI), Culture Collection of the University of Göteborg (CCUG) or American Type Collection (ATCC)], and all experiments were conducted using a level 2 biosafety cabinet. Several antibiotics were used as reference control compounds (bacteria: kanamycin sulphate, streptomycin, ampicillin, penicillin G, and chloramphenicol; yeasts and the fungus: myconazole, nystatin, and thiabendazole). The yeasts and the fungal strain were assayed on Sabouraud dextrose broth (g/L): yeast extract, 15:0 and glucose, 20:0, and the bacteria on Luria Bertani broth (g/L): yeast extract, 5:0; tryptone, 10:0 and NaCl, 10:0. Test inocula were prepared from fresh bacterial cultures by serial dilution to yield 1×10⁵ cells/mL for bacterial strains and 1×10⁶ cells/mL for fungi and yeast strains. Each inoculum was added to all 96 wells and the plates were incubated at 28 °C for 24 h or at 28 °C for 48 h (*A. niger*). Optical densities (OD) were taken at 600 nm before (Initial OD) and after incubation (Final OD) using a VersaMax™ Turnable Microplate Reader (VWR, Visalia, CA, USA). The values for minimal inhibitory concentrations (MIC) were taken at the lowest concentration where

spectrophotometrically 90% growth reduction or inhibition was measured. The assays were performed with three replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Sarawak State Government and in part by grant P01CA125066 awarded to Dr. A.D. Kinghorn from the National Cancer Institute, NIH, Bethesda, MD. We thank the following colleagues from the Sarawak Biodiversity Centre: Mr. Tu Chu Lee for the collection of plant samples; Ms. Jovita Elderson for the botanical identification; Ms. Asma' Saiyidaitina Aisyah for performing some of the cytotoxicity assays, and Mr. Chia Hwa Chuan for the antimicrobial tests. We would also like to acknowledge the Centre for Research and Instrumentation (CRIM), National University of Malaysia (UKM) and Structural Biology and Biophysics, Malaysia Genome Institute (MGI) for the NMR spectra obtained for compounds **3** and **6**.

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Figure 1.
Selected key COSY (bold lines), HMBC (→) and NOESY (↔) correlations of **3**, **4**, **6**, and **7**.

Figure 2.
 δ_{S-R} values of MTPA esters of **3**.

Table 1

¹H and ¹³C NMR Chemical Shifts of Compounds 3–7.

position	^{3a,e}		^{4f}		^{5b}		^{6d,e}		^{7b}	
	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}
1	4.88,d (14.4)	128.8	2.31,dd (14.4,3.8) 2.11,d (18.5)	51.0	2.43,d (18.0) 2.30,d (18.0)	43.2	4.89,d (14.4)	128.8	1.62,brd (2.5)	34.6
2	6.49,dd (10.8,14.4)	118.8	9.81,d (3.5)	203.3	7.89, s	175.3 ^d	6.49,dd (10.8,14.4)	118.8	2.35,m; 2.16,m	28.6
3		179.5		180.3		181.9		179.4		174.8
4		46.3 ^c		45.7		47.7		46.4		147.7
5	1.64,d (7.7)	55.6	1.89,dd (9.5,2.6)	44.9	1.74,m	43.8	1.66, ^c m	55.6	1.97,dd (10.0,2.4)	50.8 ^c
6	1.58, ^c s; 1.51,d (9.8)	19.7	1.48, m	21.8	1.35,m	26.6	1.59, ^c m; 1.48, ^c m	19.8	1.80, ^c t (4.8); 1.37,m	24.8
7	1.58, ^c s; 1.31,m	34.7	1.60, ^c m; 1.32,m	34.7	1.63, ^c m; 1.28,m	35.7	1.59, ^c m; 1.30,m	34.8	1.57,m; 1.23, s	34.0
8		40.1		40.5		41.5		40.1		40.1
9	1.36, ^c m	46.8	2.29,dd (14.4,3.9)	51.1	2.56,d (10.0)	50.0	1.38, ^c m	47.0	1.49,brd (4.0)	41.0
10		46.3 ^c		42.3		42.9		44.4		39.2
11	1.36, ^c m; 1.27,s	22.9	1.60, ^c m	19.7	1.63, ^c m; 1.54,m	22.0	1.38, ^c m	23.0	1.40, ^c m; 1.26,m	22.2
12	1.31, ^c m; 1.22,s	29.9	1.70, ^c m; 1.50, m	25.0	1.76, ^c m; 1.63, ^c m	23.7	1.88, m; 1.79, ^c m	26.4	1.80, ^c t (4.8)	27.5
13	1.60, ^c s	43.1	1.57, ^c m	42.6	1.63, ^c m	43.5	1.59, ^c m	43.6	1.61, ^c m	42.7
14		50.8		50.9		52.0		50.6		50.8 ^c
15	1.44,m; 1.11, ^c s	31.2	1.40, m; 1.08,m	31.3	1.49,m; 1.08,m	32.4	1.45,m; 1.11, ^c s	31.5	1.45,m; 1.12,m	31.4
16	1.78,d (2.8); 1.51,d (9.8)	25.2	1.75, ^c m	27.3	1.85, ^c m	28.9	1.70,m; 1.13,s	27.4	1.61, ^c m; 1.40, ^c m	25.8
17	1.79,d (4.2)	49.9	1.75, ^c m	50.7	1.76, ^c m	51.2	1.79, ^c m	49.6	1.80, ^c t (4.8)	50.1
18	0.98,s	16.5	0.85,s	16.4	0.93,s	17.1	0.98,s	15.4	0.99,s	15.5
19	1.07,s	15.4	0.93,s	20.3	0.94,s	20.7	1.07,s	14.6	0.84,s	20.3
20		75.7		75.6		76.3		85.6		75.7
21	1.14,s	25.5	1.12,s	25.3	1.12,s	25.2	1.12, s	24.6	1.15,s	25.4
22	1.71,m; 1.58, ^c s	37.2	1.70, ^c m; 1.57, ^c m	37.3	1.85, ^c m; 1.43,m	39.5	1.66, ^c m; 1.63,m	36.1	1.71,m; 1.61, ^c m	37.1
23	1.60, ^c s; 1.38,d (5.6)	25.8	1.57, ^c m; 1.38, m	25.7	1.76, ^c m; 1.63, ^c m	26.0	1.79, ^c m; 1.48,m	25.9	1.40, ^c m	25.0
24	3.38,d (10.5)	79.0	3.36,d (9.6)	78.9	3.22,brdd (8.4, 1.6)	80.6	3.74,m	83.6	3.39,d (9.6)	79.0

position	3^{a,e}		4^c		5^b		6^{d,e}		7^b	
	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}	δ_{H} , mult. (J in Hz)	δ_{C}
25		73.4 ^d		73.4		74.0		71.7		73.3
26	1.17, s	23.6	1.21, ^c s	22.0	1.14, s	25.3	1.11, ^c s	23.6	1.17, s	23.4 ^c
27	1.22, s	26.8	1.15, s	23.4	1.17, s	25.6	1.20, ^c dd (12.0, 3.0)	27.6	1.23, s	26.8
28	1.18, s	29.1	1.24, s	29.9	1.24, ^c s	28.4	1.18, dd (12.0, 3.0)	29.1	4.84, brs; 4.65, brs	113.6
29	1.11, ^c s	22.3	1.21, ^c s	26.7	1.24, ^c s	24.3	1.38, ^c s	23.0	1.73, s	23.4 ^c
30	0.87, s	14.6	0.96, s	15.5	1.01, s	15.7	0.87, s	16.5	0.89, s	16.5
NH	7.03, d (10.8)						6.93, d (10.8)			
CHO	8.05, s	157.7					8.05, s	157.7		
OMe-3	3.54, s	51.8	3.57, s	51.9	3.63, s	52.5	3.54, s	51.7	3.67, s	51.8

^a₁H NMR spectra were measured at 700 MHz and ¹³C NMR spectra were measured at 175 MHz. The spectra of compound **3** were obtained in CDCl₃.

^b₁H NMR spectra were measured at 400 MHz and ¹³C NMR spectra were measured at 100 MHz. The spectra of compound **5** were obtained in methanol-*d*₄ and the spectra of **7** obtained in CDCl₃.

^c₁H NMR spectra were measured at 500 MHz and the ¹³C NMR spectra measured at 125 MHz. The spectra of **4** were obtained in CDCl₃.

^d₁H NMR spectra were measured at 600 MHz and the ¹³C NMR spectra were measured at 150 MHz. The spectra of compound **6** were obtained in CDCl₃. Assignments made were supported using 2D NMR spectra.

^eOverlapping signals

^dSignal inferred from ¹³C and HMBC spectra.

^eOnly the NMR data of the major isomer are fully assigned.

Table 2

Biological Activities of Compounds Isolated from *A. stellatopilosa*

compound	cancer cell line				antimicrobial activity ^c								
	HT-29 ^a	MCF-7 ^b	NCI-H460 ^b	SA	ML	BS	EC	PA	PG	SC	RG	PO	AN
1	0.0007 ¹⁷	0.00017	0.00023	-	-	-	-	-	-	++	++	+	-
2	0.001 ¹⁷	0.00038	0.00092	-	-	-	-	-	-	++	++	++	-
3	>10	>10	>10	-	-	-	-	-	+	-	-	+	-
4	>10	>10	>10	+	-	-	-	-	-	-	-	-	-
5	>10	>10	>10	-	-	-	-	-	-	-	-	-	-
6	>10	>10	>10	-	-	-	-	-	-	-	-	-	-
7	>10	>10	>10	++	-	++	-	-	+	-	-	-	+

^aResults are expressed as ED₅₀ values (μM).^bResults are expressed as GI₅₀ values (μM).^cResults are expressed as % inhibition; (++) = 90%; (+) = 60–89% and (-) = no inhibition. See the Experimental Section for the meaning of the abbreviations.