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Cytotoxic Homoisoflavones from the Bulbs of *Bellevalia eigii*

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Abstract

Eight new and 10 known compounds, were isolated from an organic extract of the bulbs of *Bellevalia eigii* as part of a search for anticancer leads from native plants of Jordan. Of these, the series of 16 homoisoflavonoids (**1–16**), comprise the seven new analogues 7-*O*-methyl-3'-hydroxy-3,9-dihdropunctatin (**3**), 6-hydroxy-7-*O*-methyl-3,9-dihdropunctatin (**6**), 7,4'-di-*O*-methyl-3'-hydroxy-3,9-dihdropunctatin (**9**), 7-*O*-methylpunctatin (**10**), 7-*O*-methyl-3'-hydroxypunctatin (**13**), 5-hydroxy-7,8-dimethoxychroman-4-one (**14**), and 7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihdropunctatin (**15**), as well as the known ferulic acid-derived acrylamide (**17**), and the new methylthioacrylate bellegimycin (**18**). The structures were elucidated using a set of spectroscopic and spectrometric techniques; the absolute configurations of compounds **1–9**, **15**, and **16** were determined using ECD spectroscopy, while a modified Mosher's ester method was used for compound **18**. Optical rotation data for the known compounds **1**, **2**, and **8** are reported here for the first time. The cytotoxic activities of all compounds were evaluated

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Notes

The authors declare no competing financial interest.

Supporting Information

UPLC chromatograms of compounds **1–18**, ECD spectra of known compounds, and ¹H and ¹³C NMR spectra for compounds **1–18**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

using the MDA-MB-435 (melanoma) and HT-29 (colon) cancer cell lines. Compounds **4** and **9** were the most potent on the latter cell line with IC₅₀ values of 1.0 and 1.1 μ M, respectively. Compounds **1-18** were assessed for antimicrobial activity using a collection of bacteria and fungi; compounds **4** and **12** showed promising activity against the bacterium *Mycobacterium smegmatis* with MIC values of 17 and 24 μ g/mL, respectively.

Homoisoflavonoids, a rare class of natural compounds, are structurally related to the more widely distributed flavonoids. In comparison with flavonoids, homoisoflavonoids have one additional carbon atom in their skeleton. More than 240 natural homoisoflavonoids have been reported with a wide range of biological activities, including antimicrobial, antimutagenic, anti-oxidant, and anti-inflammatory effects.¹ Homoisoflavonoids can be classified into five types: 3-benzyl-4-chromanones, 3-benzyl-3-hydroxy-4-chromanones, 3-benzylidene-4-chromanones (*E* or *Z*), 3-benzylchrom-2-en-4-ones, and the scillascillins.²

Being a floral bridge between the continents of Asia, Africa, and Europe, with ecologically diverse natural habitats, Jordan is richer in biodiversity than many realize.^{3,4} More than 2000 plant species were reported to grow in the wild,³ with only a small percentage explored for bioactive secondary metabolites. As part of a long-term research project to examine the flora of Jordan for anticancer leads,⁵⁻⁸ *Bellevalia eigii* Feinbrun was investigated. *B. eigii* is a perennial plant belonging to the family Asparagaceae, subfamily Scilloideae, which may also be treated as a separate family Hyacinthaceae.^{2,9} Eleven species were reported to grow in the wild in Jordan.³ *B. eigii*, which is known locally as “the Jordan Valley onion”, is known for its toxicity among local people. Promising cytotoxic activity of the organic extract of the bulbs led to the isolation and characterization of compounds **1-18**, including 16 homoisoflavonoids, nine known (**1**, **2**, **4**, **5**, **7**, **8**, **11**, **12**, and **16**) and seven new analogues 7-*O*-methyl-3'-hydroxy-3,9-dihydropunctatin (**3**), 6-hydroxy-7-*O*-methyl-3,9-dihydropunctatin (**6**), 7,4'-di-*O*-methyl-3'-hydroxy-3,9-dihydropunctatin (**9**), 7-*O*-methylpunctatin (**10**), 7-*O*-methyl-3'-hydroxypunctatin (**13**), 5-hydroxy-7,8-dimethoxychroman-4-one (**14**), and 7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihydropunctatin (**15**). The known phenolic amide, *N-trans*-feruloyltyramine (moupinamide) (**17**) and a new methylthioacrylate derivative, bellegimycin (**18**) were also isolated. The absolute configurations of compounds **1-9**, **15**, and **16** were determined using ECD spectroscopy, while for **18** a modified Mosher's ester method was used. There has been growing interest in homoisoflavonoids because of their broad range of biological activities, including cytotoxic and antimicrobial effects.¹ Hence, these compounds were tested for their cytotoxicity in the MDA-MB-435 (melanoma) and HT-29 (colon) cancer cell lines and for antimicrobial activity in an assemblage of bacteria and fungi.

RESULTS AND DISCUSSION

The CH₂Cl₂ extract of the air-dried bulbs of *B. eigii* was reconstituted in a mixture of 5:4:1 H₂O:CHCl₃:MeOH. The organic layer was separated and dried under reduced pressure. To the dried organic extract was added equal volumes of CH₃CN-MeOH and hexanes. The CH₃CN-MeOH fraction was evaporated to dryness. The brine shrimp test was utilized^{5,10,11} as an initial screen for cytotoxic activity; a promising LC₅₀ value of 28.1 μ g/mL was

observed. The organic extract was then fractionated using flash chromatography to produce 10 fractions. Of these, fractions 3–8 showed interesting HPLC profiles, and hence were further purified using HPLC (preparative and semipreparative). Compounds **1**–**18** were isolated, the purities of which were evaluated by UPLC (Figure S1).

Sixteen homoisoflavonoids **1**–**16** were isolated and identified using HRMS, 1D/2D NMR, and ECD data. The experimental and reported spectroscopic and spectrometric data were used to define the structures of the known analogues. Compound **1** (68.6 mg), obtained as a vitreous solid, had a molecular formula of $C_{18}H_{18}O_6$ as established by HRESIMS and ^{13}C NMR data. The NMR, HRMS, and ECD data¹² (Figures S2 and S3, Supporting Information) identified **1** as the known 3-benzylchroman-4-one-type homoisoflavonoid, 7-*O*-methyl-3,9-dihydropunctatin, which was described for the first time by Adinolfi and coauthors in 1984 from the bulbs of *Muscari comosum*.¹³ The specific rotation of **1** was determined as $[\alpha]_D^{24} -92$ (*c* 0.1, MeOH) for the first time.

In addition to **1**, the following known homoisoflavonoids were isolated and identified, 8-*O*-demethyl-7-*O*-methyl-3,9-dihydropunctatin^{12–14} (**2**) [4.6 mg, $[\alpha]_D^{24} -82$ (*c* 0.1, MeOH)]; 5,7-dihydroxy-3-(3'-hydroxy-4'-methoxybenzyl)-4-chromanone (**4**) (1.9 mg);^{12,14,15} 5,6-dihydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone (**5**) (0.9 mg);¹⁶ 4',5,7-trihydroxyhomoisoflavanone (**7**) (1.1 mg);^{14,16} 3',4',5,7-tetrahydroxyhomoisoflavanone (**8**) [1.0 mg, $[\alpha]_D^{24} -90$ (*c* 0.1, MeOH)];¹⁷ demethyleucomin (**11**) (0.9 mg);¹⁸ punctatin (**12**) (0.5 mg);¹⁹ and isomuscomosin (**16**) (0.5 mg).²⁰ The specific rotations of **2** and **8** were not reported previously and are hence provided herein.

Compound **3** (10.2 mg) was isolated as a yellowish oil with a molecular formula of $C_{18}H_{18}O_7$ as determined by HRESIMS and NMR data (Tables 1 and 2, Figure S5, Supporting Information). Analysis of the NMR data of **3** indicated a 3-benzylchroman-4-one homoisoflavonoid with structural similarity to **1**. However, the aromatic A₂B₂ system of ring B in **1** was replaced by an ABM spin system in **3** (δ_H 6.73, s; 6.79, d, *J* = 7.5; and 6.62,

d, $J = 7.5$, for H-2', H-5', and H-6', respectively), indicating the presence of a 1,3,4-trisubstituted benzene ring. These data along with a deshielded C-3' resonance in **3** (δ_C 144.0) relative to **1** (δ_C 115.7) and a 16 amu difference in the HRMS data indicated oxygenation at C-3' in **3**. Further examination of the 2D NMR data established the structure of **3** (Figure 1) as 7-*O*-methyl-3'-hydroxy-3,9-dihydropunctatin. The absolute configurations of 3-benzylchroman-4-one-type homoisoflavonoids have been determined by electronic circular dichroism (ECD) spectroscopy,¹² where a negative Cotton effect in the 287–295 nm region of the ECD curves is indicative of a 3*R* configuration.¹² As such, a negative Cotton effect at 299 nm in the ECD spectrum of compound **3** ($\epsilon = -17.7$) supported an *R*-configuration at C-3 (Figure 2).

Compound **6** (0.3 mg), which was obtained as a yellow amorphous powder, had a molecular formula of $C_{18}H_{18}O_7$ as determined by HRESIMS and NMR data (Tables 1 and 2, Figure S8, Supporting Information). The NMR data of **6** indicated a 3-benzylchroman-4-one-type homoisoflavonoid with structural similarity to **1**. However, the aromatic proton singlet of ring A (δ_H 6.09 for H-6) in **1** was replaced by an exchangeable proton singlet (δ_H 5.15) in **6**. The deshielded C-6 (δ_C 130.9) resonance in **6** relative to that in **1** (δ_C 93.1), along with a 16 amu difference in the HRMS data between **6** and **1**, indicated hydroxylation at C-6, which was confirmed by HMBC correlations from the 6-OH proton to C-5 and C-7. The structure of compound **6** was inferred by further analysis of the 2D NMR data (Figure 1). The trivial name 6-hydroxy-7-*O*-methyl-3,9-dihydropunctatin was assigned to compound **6**. A negative Cotton effect at 293 nm in the ECD spectrum of compound **6** ($\epsilon = -6.1$), indicated an *R*-configuration at C-3 (Figure 2).

Compound **9** (1.1 mg) was obtained as a white amorphous powder. HRESIMS and NMR data indicated a molecular formula of $C_{19}H_{20}O_7$ (Tables 1 and 2, Figure S11, Supporting Information). Analysis of the NMR data indicated compound **9** as a 3-benzylchroman-4-one-type of homoisoflavonoid. The compound showed high structural similarity to **3**. Compound **9** showed an extra methoxy group, as indicated by 1H and ^{13}C NMR data (δ_H/δ_C 3.87/56.2), consistent with the 14 amu difference in the HRMS data of **9** relative to **3**. An HMBC correlation from the 7'- OCH_3 protons to C-4' (δ_C 145.6) confirmed its connectivity. The structure of compound **9** was deduced by further inspection of the 2D NMR, including COSY and HMBC spectra (Figure 1). Compound **9** was given the trivial name 7,4'-di-*O*-methyl-3'-hydroxy-3,9-dihydropunctatin. The ECD spectrum of **9** showed a negative Cotton effect ($\epsilon = -21.1$) at 297 nm, indicating an *R*-configuration at C-3 (Figure 2).

Compound **10** (6.7 mg), with a molecular formula of $C_{18}H_{16}O_6$, was isolated as a yellow amorphous powder (Tables 1 and 2; Figure S12, Supporting Information). Analyses of the spectroscopic and spectrometric data suggested **10** as a homoisoflavonoid derivative showing structural similarity with **1**. Key differences were replacement of the signals for H-3/C-3 and H₂-9/C-9 in **1** by a quaternary olefinic carbon (δ_C 127.6) and an olefinic methine (δ_H/δ_C 7.79/137.6, t, $J = 1.2$ Hz) in **10**, respectively. The H₂-2 chemical shift (δ_H 5.34, d, $J = 1.2$ Hz) in **10** was deshielded relative to that in **1**. The carbonyl carbon in **10** (δ_C 185.8) was shielded relative to **1** (δ_C 198.5), indicating conjugation with a double bond. These data, along with a 2 amu difference between **1** and **10**, indicated the presence of a ^{3,9}

double bond in **10**. The geometry of the double bond was established as *E*, deduced from the long-range coupling between H₂-2 and H-9 and by the unusual deshielding of the olefinic proton H-9 (δ_{H} 7.79).^{18,21} The structure of **10** was established as a 3-benzylidene-4-chromanone-type homoisoflavonoid by further examination of the 2D NMR data (Figure 1). The trivial name 7-*O*-methylpunctatin was given to compound **10**.

Compound **13** (0.7 mg), with a molecular formula of C₁₈H₁₆O₇, was obtained as a yellow amorphous powder (Tables 1 and 2, Figure S15, Supporting Information). The NMR data of **13** indicated a 3-benzylidene-4-chromanone-type homoisoflavonoid with structural similarity to **10** including the *E*-configuration of the ^{3,9} double bond. However, **13** lacked the aromatic A₂B₂ spin system of ring B, which was replaced by an ABC aromatic proton spin system (δ_{H} 6.85, s; 6.87, d, *J* = 8.6; and 6.81, d, *J* = 8.6, for H-2', H-5', and H-6', respectively), indicative of a 1,3,4-trisubstituted benzene ring. These data, along with a deshielded C-3' resonance in **13** (δ_{C} 147.0) relative to **10** (δ_{C} 116.1) and a 16 amu difference in the HRMS data, indicated oxygenation at C-3' in **13**. The structure of **13** was established by further inspection of the 2D NMR data such as COSY and HMBC experiments (Figure 1), and was named as 7-*O*-methyl-3'-hydroxypunctatin.

Compound **14** (0.4 mg) was isolated as a white amorphous powder with a molecular formula of C₁₁H₁₂O₅ (Tables 1 and 2, Figure S16, Supporting Information). Key structural features of **14** include two mutually coupled methylenes (δ_{H} 4.52, t, *J* = 6.3 Hz and 2.79, t, *J* = 6.3 Hz, for H₂-2 and H₂-3, respectively). The deshielded singlet, δ_{H} 11.99, was attributed to the hydrogen-bonded 5-hydroxy group in ring A, which also bore the proton responsible for the singlet at δ_{H} 6.08 and the two methoxy groups (δ_{H} 3.88 and 3.77 for 9- and 10-OCH₃, respectively). The structure of **14** was established by further examination of the HMBC NMR data (Figure 1), and was defined as 5-hydroxy-7,8-dimethoxychroman-4-one.

Compound **15** (0.4 mg) was obtained as a white amorphous powder with a molecular formula of C₁₇H₁₆O₆ (Tables 1 and 5; Figure S17, Supporting Information), establishing an index of hydrogen deficiency of 10. Analyses of the HRMS and NMR data suggested **15** as a homoisoflavonoid derivative with structural similarity with **7**, including ¹H NMR signals of an aromatic A₂B₂ system (δ_{H} 7.07 and 6.71, 2H, d, *J* = 8.5 Hz) and two *meta* coupled aromatic protons (δ_{H} 6.08, d, *J* = 2.1 Hz and 6.06, d, *J* = 2.1 Hz, for H-6 and H-8, respectively). A key difference was replacement of the H-3/C-3 ($\delta_{\text{H}}/\delta_{\text{C}}$ 2.81/48.2) functionality in **7** by a tertiary oxygenated carbon (δ_{C} 73.7) in **15** indicating hydroxylation at C-3 in **15** relative to **7**. Compound **15** showed a methoxy group ($\delta_{\text{H}}/\delta_{\text{C}}$ 3.83/56.5), located at C-7 as indicated by an HMBC correlation from H₃-10 to C-7. The structure of **15** was deduced by further examination of the 2D NMR data such as COSY and HMBC spectra (Figure 1), and was named 7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihydropunctatin. The ECD spectrum of **15** showed a negative Cotton effect (ϵ = -22.4) at 291 nm, indicating an *S*-configuration for C-3 (Figure 2).¹²

In addition to compounds **1–16**, one known phenolic amide derivative *N*-*trans*-feruloyltyramine (**17**) was isolated and its structure was identified by comparison of its recorded and reported²² NMR and HRMS data.

Compound **18** (1.2 mg) was isolated as a colorless oil with a molecular formula of $C_{12}H_{14}O_3S$ as revealed by HRESIMS, 1H , ^{13}C and edited-HSQC NMR data (Figure S20, Supporting Information), indicating an index of hydrogen deficiency of six. Inspection of the NMR data showed signals characteristic of six aromatic carbons and five aromatic protons constituting an AA'BB'C spin system suggestive of a monosubstituted benzene ring (Table 3, Figure S20, Supporting Information). COSY data identified two spin systems H-2'/H-3'/H-4'/H-5' and 1-OH/H-1/H₂-2 with C-1 and C-2 being oxygenated and C-1 attached to the benzene ring. HMBC correlation from 1-OH to C-1' confirmed C-1 as the attachment point of the aliphatic side chain with the aromatic ring. ^{13}C NMR data indicated an ester functionality (δ_C 165.6), which was conjugated with the 5,6 double bond. The two olefinic protons showed a coupling constant that supported an *E* configuration ($J_{H-5/H-6} = 15.1$ Hz). Further analysis of the NMR data indicated a methyl group (δ_H/δ_C 2.33/14.6) that was attached to a sulfur atom. An HMBC correlation from 8-CH₃ to C-6 confirmed the sulfur atom as the attachment point of the S-CH₃ group to C-6. Also, an HMBC correlation from H₂-2 to C-4 confirmed the connectivity of C-2 to the ester functionality. Further examination of the NMR data yielded the planar structure of **18**, which was ascribed the trivial name, bellegimycin. The absolute configuration of **18** was assigned via a modified Mosher's ester method,²³ establishing the (1*R*) configuration (Figure 3). This is the first report of a sulphur-containing compound in the Hyacinthaceae. Although the compound may be biosynthesized in *B. eigii*, there is a possibility it could arise as an artifact. However, we could not prove this one way or the other. The compound was detected in the crude organic extract by HRESIMS, which excludes the possibility of contamination during the fractionation/purification processes.

The cytotoxicities of compounds **1–18** were measured against the MDA-MB-435 (melanoma) and HT-29 (colon) cancer cell lines. Compounds **4** and **9** were the most potent on the MDA-MB-435 cell line with IC₅₀ values of 1.0 and 1.1 μ M, respectively (Table 4). Compound **4** was reported previously to be active against colon cancer (HT-29 cell line ED₅₀ = 2.78 μ M) and breast cancer (MDA-MB-435 cell line ED₅₀ = 1.33 μ M).²⁴ The cytotoxicity data of the compounds enabled preliminary structure-activity relationships considerations. Introducing an exo-double bond increased the activity significantly. Although compounds **1** and **3** were inactive, compounds **10** and **13** with exo-double bonds showed IC₅₀ values of 13.2 and 4.6 μ M, respectively, against the MDA-MB-435 cell line. Introducing a 3'-OH group to compound **10** (13.2 μ M) increased the cytotoxic activity (IC₅₀ = 4.6 μ M) for compound **13** three fold. Reducing the polarity of highly oxygenated compounds increased the activity as well. For example, while the highly oxygenated compound **8** was inactive, compound **4**, with a 4'-methoxy substituent was active (IC₅₀ = 1.0 μ M) against the MDA-MB-435 cell line. The same trend was observed for compounds **3** and **9**, where the latter with a 4'-methoxy substituent was more active (IC₅₀ = 1.1 μ M) against the MDA-MB-435 cell line. In general, less polar substituents contributed to the activity more than the exo-double bond, as seen in compounds **11** and **12**, both having exo-double bonds, but they were inactive.

Compounds **1–18** were evaluated for antimicrobial activity using a group of bacteria and fungi (Table 5). Compounds **4** and **12** showed promising activity against the bacterium,

Mycobacterium smegmatis, with MIC values of 17 and 24 µg/mL, respectively. As *M. smegmatis* is used as a surrogate for the pathogenic *Mycobacterium* species (e.g., *M. tuberculosis* and *M. avium*) whose susceptibility to antimicrobials can differ from that of *M. smegmatis*,²⁵ the susceptibility of pathogenic *Mycobacterium* species to these novel compounds will be measured in the future studies.

EXPERIMENTAL SECTION

General Experimental Procedures

Description of the NMR, HRESIMS, UV, ECD, IR, UPLC, and HPLC instrumentation were as reported previously.^{25,26}

Plant Material

Bulbs of *B. eigii* were collected in March/May 2012 from the campus of the Jordan University of Science and Technology (JUST), Irbid, Jordan. The plant material was identified by Mohammad Al-Gharaibeh, Plant Taxonomist, Faculty of Agriculture, JUST. A voucher specimen (PHS-118) was deposited in the herbarium of the Faculty of Pharmacy, JUST. The bulbs were cleaned of mud, sliced into small pieces, and air dried at rt in a well-ventilated area.

Extraction and Isolation

Air-dried bulbs of *B. eigii* were ground to a powder using a Retsch Mühle mill (RETSCH GmbH, Haan, Germany). About 0.5 kg of powdered bulbs were extracted exhaustively with CH₂Cl₂ using a Soxhlet apparatus. The solvent was evaporated under reduced pressure to yield about 3 g of CH₂Cl₂ extract, which was reconstituted in a 2.5 L mixture of 5:4:1 H₂O:CHCl₃:MeOH. The mixture was stirred for 30 min and left to separate in a separatory funnel. The organic layer was collected and evaporated to dryness under reduced pressure. To the dried organic extract was added 200 mL of 1:1 MeOH/CH₃CN and 200 mL of hexanes and shaken vigorously in a separatory funnel. The dried MeOH/CH₃CN layer (1.1 g) was dissolved in CHCl₃ and mixed with Celite 545. Normal-phase flash chromatography was run using a gradient solvent system of hexanes-CHCl₃-MeOH, the flow rate used was 40 mL/min, and column volumes were 53.3 over a total run time of 63.9 min to yield 10 fractions. Fraction 4 (96.0 mg) was purified using preparative HPLC linked with a YMC column using a gradient system of 70:30 to 90:10 of MeOH-H₂O (0.1% formic acid) over 15 min at a flow rate of 12 mL/min to yield compounds **1** (65.7 mg) and **10** (5.8 mg). Fraction 5 (54.3 mg) was subjected to preparative HPLC over a Gemini column using a gradient system of 50:50 to 60:40 of MeOH-H₂O (0.1% formic acid) over 30 min at a flow rate of 21.24 mL/min to yield four sub-fractions. Further semipreparative HPLC purifications of the sub-fractions yielded compounds **2** (2.7 mg), **4** (1.9 mg), **5** (0.9 mg), **6** (0.3 mg), **15** (0.4 mg), and **16** (0.5 mg). Preparative HPLC purification of fraction 6 (53.4 mg) over a Gemini column, at a flow rate of 21.24 mL/min and a gradient system of 50:50 to 60:40 of MeOH-H₂O (0.1% formic acid) over 30 min yielded six subfractions. Further semipreparative HPLC purifications of the subfractions yielded compounds **2** (1.9 mg), **3** (10.2 mg), **12** (0.5 mg), and **13** (0.74 mg). Preparative HPLC purification of fraction 3 (99.1 mg) over a Gemini column using a gradient system of 50:50 to 60:40 of MeOH-H₂O (0.1%

formic acid) over 30 min at a flow rate of 21.24 mL/min yielded five subfractions. Further semipreparative purifications of the subfractions yielded compounds **1** (2.9 mg), **9** (1.1 mg), **14** (0.9 mg), and **18** (1.2 mg). Fraction 7 (84.6 mg) was purified using preparative HPLC that was connected with a Gemini column. The mobile phase used was a gradient system of 50:50 to 60:40 over 30 min to 100:00 over 10 min of MeOH-H₂O (0.1% formic acid) at a flow rate of 21.24 mL/min. The three subfractions were purified to afford compounds **7** (1.1 mg) and **11** (0.9 mg). Fraction 8 (118.1 mg) was purified using preparative HPLC over a Gemini column and a gradient system of 50:50 to 100:00 over 15 min of MeOH-H₂O (0.1% formic acid) at a flow rate of 21.24 mL/min. The four subfractions were further purified to yield compounds **8** (1.0 mg) and **17** (0.6 mg).

7-O-Methyl-3,9-dihydropunctatin (1): vitreous solid; $[\alpha]_D^{24} -92$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 343 (3.26), 289 (3.79), 224 (3.72) nm; ECD (0.9×10^{-4} M, MeOH), see Figure S2, Supporting Information; HRESIMS m/z 331.1169 [M + H]⁺ (calcd for C₁₈H₁₈O₆, 331.1176).

8-O-Demethyl-7-O-methyl-3,9-dihydropunctatin (2): light yellow amorphous powder; $[\alpha]_D^{24} -82$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 366 (3.29), 293 (3.72), 245 (3.62), 223 (3.63) nm; ECD (0.9×10^{-4} M, MeOH), see Figure S2, Supporting Information; HRESIMS m/z 317.1012 [M + H]⁺ (calcd for C₁₇H₁₆O₆, 317.1020).

7-O-Methyl-3'-hydroxy-3,9-dihydropunctatin (3): yellowish oil; $[\alpha]_D^{24} = -86$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 343 (3.31), 289 (3.76), 224 (3.67) nm; ECD (c 0.9×10^{-4} M, MeOH) λ (ϵ) 238 (+5.2) nm, 272 (+6.8) nm, 299 (−17.8) nm (Figure 2); IR (diamond) $\nu_{\max}$ 3325, 2934, 1627, 1518, 1496, 1445, 1374, 1255, 1200, 1104, 1010, 954, 749 cm^{−1}; ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz), see Tables 1 and 2; HRESIMS m/z 347.1118 [M + H]⁺ (calcd for C₁₈H₁₉O₇, 347.1125).

6-Hydroxy-7-O-methyl-3,9-dihydropunctatin (6): yellowish amorphous powder; $[\alpha]_D^{24} = -14$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 376 (2.79), 288 (3.42), 223 (3.48) nm; ECD (c 0.9×10^{-4} M, MeOH) λ (ϵ) 261 (+2.9) nm, 293 (−6.1) nm (Figure 2); ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz), see Tables 1 and 2; HRESIMS m/z 347.1116 [M + H]⁺ (calcd for C₁₈H₁₉O₇, 347.1125).

3',4',5,7-Tetrahydroxyhomoisoflavanone (8): yellow amorphous powder; $[\alpha]_D^{24} -89$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 290 (3.72), 230 (3.66) nm; ECD (1.0×10^{-4} M, MeOH), see Figure S2, Supporting Information; HRESIMS m/z 303.0859 [M + H]⁺ (calcd for C₁₆H₁₄O₆, 303.0863).

7,4'-di-O-Methyl-3'-hydroxy-3,9-dihydropunctatin (9): white amorphous powder; $[\alpha]_D^{24} = -66$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 342 (3.21), 289 (3.71), 225 (3.60) nm; ECD (c 0.8×10^{-4} M, MeOH) λ (ϵ) 236 (+7.5) nm, 272 (+4.8) nm, 297 (−21.1) nm (Figure 2); ¹H NMR (CDCl₃, 500 MHz) and ¹³C NMR (CDCl₃, 125 MHz), see Tables 1 and 2; HRESIMS m/z 361.1276 [M + H]⁺ (calcd for C₁₉H₂₁O₇, 361.1282).

7-O-Methylpunctatin (10): yellow amorphous powder; UV (MeOH) $\lambda_{\max}$ (log ϵ) 391 (3.49), 378 (3.51), 237 (3.42) nm; ^1H NMR (CDCl_3 , 500 MHz) and ^{13}C NMR (CDCl_3 , 100 MHz), see Tables 1 and 2; HRESIMS m/z 329.1017 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{18}\text{H}_{17}\text{O}_6$, 329.1020).

7-O-Methyl-3'-hydroxypunctatin (13): dark yellow amorphous powder; UV (MeOH) $\lambda_{\max}$ (log ϵ) 378 (3.75), 264 (3.47), 222 (3.63) nm; ^1H NMR (methanol- d_4 , 500 MHz) and ^{13}C NMR (methanol- d_4 , 125 MHz), see Tables 1 and 2; HRESIMS m/z 345.0963 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{18}\text{H}_{17}\text{O}_7$, 345.0969).

5-Hydroxy-7,8-dimethoxychroman-4-one (14): white amorphous powder; UV (MeOH) $\lambda_{\max}$ (log ϵ) 339 (2.44), 288 (3.01), 237 (2.83), 215 (2.93) nm; ^1H NMR (CDCl_3 , 500 MHz) and ^{13}C NMR (CDCl_3 , 125 MHz), see Tables 1 and 2; HRESIMS m/z 225.0758 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{11}\text{H}_{13}\text{O}_5$, 225.0758).

7-O-Methyl-8-demethoxy-3-hydroxy-3,9-dihydropunctatin (15): white amorphous powder; $[\alpha]_{\text{D}}^{24} = -63$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 289 (3.66), 226 (3.65) nm; ECD (c 0.9×10^{-4} M, MeOH) $\lambda_{\text{ext}}(\epsilon)$ 261 (+12.8) nm, 291 (−22.4) nm, 310 (+3.7) nm, 333 (−6.5) nm (Figure 2); ^1H NMR (methanol- d_4 , 700 MHz) and ^{13}C NMR (methanol- d_4 , 175 MHz), see Tables 1 and 2; HRESIMS m/z 317.1011 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{17}\text{H}_{17}\text{O}_6$, 317.1020).

Bellegimycin [(*R*)-2-hydroxy-2-phenylethyl-(*E*)-3-(methylthio)acrylate (**18**)]: colorless oil; $[\alpha]_{\text{D}}^{24} = -8$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 274 (3.25), 212 (2.73) nm; ^1H NMR (CDCl_3 , 500 MHz) and ^{13}C NMR (CDCl_3 , 100 MHz), see Tables 1 and 2; HRESIMS m/z 239.0732 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{12}\text{H}_{15}\text{O}_3\text{S}$, 239.0736).

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of Bellegimycin (**18**)

To 0.13 mg of compound **18** were added 400 μL of pyridine- d_5 , and the solution was transferred into an NMR tube. To initiate the reaction, 20 μL of *S*(+)- α -methoxy- α -(trifluoromethyl)phenylacetyl (MTPA) chloride were added with careful shaking and then monitored immediately by ^1H NMR at the following time points: 0, 5, 10, 15, and 30 min. The reaction was found to be complete in 30 min, yielding the mono (*R*)-MTPA ester derivative (**18b**) of **18**. ^1H NMR data of **18b** (500 MHz, pyridine- d_5): δ_{H} 6.67 (1H, dd, $J = 8.6, 3.4$, H-1), 4.64 (2H, m, H₂-2), 5.81 (1H, d, 14.9, H-5), 7.98 (1H, d, 14.9, H-6). In an analogous manner, 0.13 mg of compound **18** dissolved in 400 μL pyridine- d_5 was reacted in a second NMR tube with 20 μL (*R*)(−)- α -MTPA chloride for 30 min, to afford the mono (*S*)-MTPA ester (**18a**). ^1H NMR data of **18a** (500 MHz, pyridine- d_5): δ_{H} 6.64 (1H, dd, $J = 8.6, 4.0$, H-1), 4.67 (2H, m, H₂-2), 5.90 (1H, d, 14.9, H-5), 8.08 (1H, d, 14.9, H-6).

Cytotoxicity Assay

Compounds (**1–18**) were tested for cytotoxicity against the MDA-MB-435²⁷ human melanoma (HTB-129, ATCC) and the HT-29 human colon cancer (HTB-38, ATCC) cell lines as described previously.^{26,28} Briefly, the cell lines were propagated at 37 °C in 5% CO₂ in RPMI 1640 medium, which was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells were harvested by trypsinization while in their log phase and washed twice to remove all traces of enzyme. In every well of a

96-well plate (Microtest 96, Falcon), about 5,000 cells were seeded and incubated overnight in 5% CO₂ at 37 °C. Tested compounds dissolved in DMSO were incubated with the cells for 72 h at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, WI). IC₅₀ values are expressed in μ M relative to the solvent (DMSO) control. Vinblastine was used as a positive control.

Antimicrobial Assay

Minimal inhibitory concentrations (MICs) of compounds (**1–15**) were measured against a collection of bacteria and fungi by broth microdilution in 96-well microtitre plates as described previously.²⁶ Briefly, in 96-well microtitre plates, a 2-fold dilution series of the tested compounds was prepared in a 50 μ L volume of 1/10-strength BHIB+S. The resulted dilution series was inoculated with 50 μ L of each cell suspension. After incubation for 4 days, the growth, as noted by turbidity, was scored visually. MIC, which is defined as the lowest concentration of drug resulting in absence of turbidity compared with negative control, was measured for each compound in triplicate. All measurements were made in duplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Key COSY and HMBC correlations of 3, 6, 9, 10, 13–15, and 18.

Figure 2.

ECD spectra for A) (**3**) [0.09 mM, MeOH, cell length 2 cm], B) (**6**) [0.09 mM, MeOH, cell length 2 cm], C) (**9**) [0.08 mM, MeOH, cell length 2 cm], and D) (**15**) [0.09 mM, MeOH, cell length 2 cm].

18a: R = (*S*)-MTPA
18b: R = (*R*)-MTPA

Figure 3.

δ_{H} values [δ (in ppm) = $\delta_{\text{S}} - \delta_{\text{R}}$] obtained for (*S*)- and (*R*)-MTPA esters **18a** and **18b**, respectively of bellegimycin (**18**) in pyridine-*d*₅.

Table 1
¹H NMR Data for Compounds 3, 6, 9, 10, 13, and 14 (500 MHz in CDCl₃) and 15 (700 MHz in Methanol-d₄)^a

position	3 ^b	6 ^b	9 ^b	10 ^b	13 ^c	14 ^b	15 ^c
2	4.17, dd (11.5, 6.9)	4.17, dd (11.5, 7.5)	4.18, dd (11.5, 7.5)	5.34, d (1.2)	5.40, s	4.52, t (6.3)	3.98, d (11.3) 4.08, d (11.3)
3	4.30, dd (11.5, 3.4)	4.31, dd (11.5, 4.0)	4.33, dd (11.5, 4.6)			2.79, t (6.3)	
6	2.77, m	2.83, m	2.83, m				
	6.09, s		6.09, s	6.11, s	6.19, s	6.08, s	6.08, d (2.1)
8							6.06, d (2.1)
9	2.64, dd (13.8, 10.3)	2.72, dd (13.8, 10.3)	2.66, dd (13.8, 10.3)	7.79, t (1.2)	7.73, s	3.88, s	2.89, d (14.1)
	3.07, dd (13.8, 4.0)	3.14, dd (13.8, 4.6)	3.16, dd (13.8, 4.6)				2.91, d (14.1)
10	3.87, s	4.09, s	3.88, s	3.88, s	3.88, s	3.77, s	3.83, s
11	3.76, s	3.80, s	3.76, s	3.76, s	3.72, s		
2'	6.73, s	7.10, d (8.6)	6.80, d (2.3)	7.20, d (7.5)	6.85, s		7.07, d (8.5)
3'		6.79, d (8.6)		6.91, d (7.5)			6.71, d (8.5)
5'	6.79, d (7.5)	6.79, d (8.6)	6.78, d (8.6)	6.91, d (7.5)	6.87, d (8.6)		6.71, d (8.5)
6'	6.62, d (7.5)	7.10, d (8.6)	6.69, dd (8.6, 2.3)	7.20, d (7.5)	6.81, d (8.6)		7.07, d (8.5)
7'			3.87, s				
5-OH	12.02, s	11.66	12.05, s	12.68, s		11.99, s	
9-OH		5.15, s					
3'-OH			5.59, s				
4'-OH		4.75, s		5.72, br. s			

^a δ in ppm, mult (*J* in Hz).

^b In CDCl₃.

^c In methanol-*d*₄.

Table 2
¹³C NMR Data for 10 (100 MHz), 3, 6, 9, 13, 14 (125 MHz) and for 15 (175 MHz) in CDCl₃

position	6 ^c	9 ^a	10 ^a	13 ^b	14 ^a	15 ^b
2	69.4	69.6	69.5	67.8	67.1	73.1
3	47.0	47.6	47.0	127.6	36.8	73.7
4	198.5	199.6	198.3	185.8	196.2	200.5
4a	102.4	103.7	102.5	103.2	103.3	102.3
5	160.5	145.2	160.4	161.1	160.3	165.7
6	93.2	130.9	93.1	93.4	94.1	96.2
7	161.6	148.9	161.5	161.5	162.8	169.9
8	129.3	133.2 ^c	129.6	129.5	129.6	95.0
8a	153.8	147.7	153.9	153.0	154.5	164.4
9	32.4	32.0	32.3	137.6	56.4	40.8
10	56.5	61.5	56.4	56.4	56.9	56.5
11	61.6	61.9	61.6	61.6	61.7	
1'	130.6	130.0	130.9	127.1	127.2	126.9
2'	116.3	130.5	115.3	132.5	118.4	132.9
3'	144.0	115.8	145.9	116.1	147.0	116.0
4'	142.7	154.5	145.6	157.3	149.8	157.7
5'	115.7	115.8	110.9	116.1	116.7	116.0
6'	121.8	130.4	120.9	132.5	125.2	132.9
7'			56.2			

^a In CDCl₃.

^b methanol-*d*₄.

^c obtained from HMBC

Table 3NMR Data for 18 (500 MHz for ^1H , 100 MHz for ^{13}C) in CDCl_3

position	δ_{C}	δ_{H} mult (<i>J</i> in Hz)
1	72.9	4.98, ddd (8.7, 3.2, 3.2)
2	69.5	4.21, dd (11.9, 8.7) 4.33, dd (11.9, 3.2)
4	165.6	
5	112.5	5.69, d (15.1)
6	148.5	7.79, d (15.1)
8	14.6	2.33, s
1'	139.9	
2', 6'	126.4	7.38, dd (7.5, 1.7)
3', 5'	128.8	7.36, dd (7.5, 6.9)
4'	128.4	7.31, tt (6.9, 1.7)
1-OH		2.57, d (3.2)

Table 4

Cytotoxic Activity Evaluation of Active Compounds 4, 9, 10, and 13 Against Colon and Breast Cancer Cell Lines.

compound ^a	IC ₅₀ values in μM ^b	
	MDA-MB-435	HT-29
4	1.0±0.1	>20
9	1.1±0.1	17.3±3.1
10	13.2±0.4	>20
13	4.6±0.1	>20
Vinblastine^c	0.0005	0.0045

^a Compounds **1–3**, **5–8**, **11**, **12**, **14–18** were inactive, IC₅₀ values >20 μM .

^b IC₅₀ is the concentration to inhibit 50% of growth with a 72 h incubation.

^c Positive control.

Table 5

Antimicrobial Activities of Compounds 4, 7, 12, and 13

compound	Minimal inhibitory concentration ($\mu\text{g/mL}$) ^a				
	<i>S. aureus</i>	<i>E. coli</i>	<i>M. smegmatis</i>	<i>C. albicans</i>	<i>A. niger</i>
4	>138	>138	17	>138	>138
7	>125	>125	63	>125	>125
12	>48	>48	24	>48	>48
13	93	>93	47	>93	>93
Vancomycin ^b	0.25	---	---	---	---
Ampicillin ^b	---	8	---	---	---
Ciprofloxacin ^b	---	---	2	---	---
Amphotericin B ^b	---	---	---	16	31

^aCompounds **1–3**, **5–6**, **8–11**, **14–18** were inactive^bPositive controls