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Antiproliferative Homoisoflavonoids and Bufatrienolides from *Urginea depressa*

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Abstract

Investigation of the South African plant *Urginea depressa* Baker (Asparagaceae Juss.) for antiproliferative activity against the A2780 ovarian cancer cell line led to the isolation of the six new homoisoflavonoids urginanins A-F (**1** - **6**), the two known bufatrienolides **7** and **9**, and the new bufatrienolides urginins B and C (**8** and **10**). Structures were elucidated based on analysis of their 1D and 2D NMR spectra, electronic circular dichroism, and mass spectrometric data. Five of the six new homoisoflavonoids had good antiproliferative activity against the A2780 ovarian cancer, A2058 melanoma, and H522-T1 human non-small cell lung cancer cells, and urginanin A (**1**) had submicromolar activity against all three cell lines. The four bufatrienolides **7** – **10** had strong antiproliferative activity against the same cell line, with IC₅₀ values of 24.1, 11.2, 111 and 40.6 nM, respectively.

Natural products have been a major source of new drugs for the last 70 years, with almost 50% of all anticancer drugs discovered since 1940 being natural products or modified natural products.¹ Until recently the Merck pharmaceutical company maintained a strong program in natural products research, which resulted in the discovery of the antifungal agent CANCIDAS[®], the antibacterial MEFOXIN[®] (cefoxitin), and the statin MEVACOR[®], among others, but the company halted in-house natural products research in 2008.² The superb Merck natural products library, which is considered to be one of the most diverse and best curated natural products libraries in the world, was donated to The Institute for Hepatitis and Virus Research (IHVR) and its Natural Products Discovery Institute (NPDI) in 2011.³ A collaborative research project was then established between Virginia Tech and the NPDI to interrogate this library for potential antiproliferative and antimalarial activities, and this paper reports the first results from these investigations.

Screening of several thousand extracts from the NPDI collection indicated that a dichloromethane extract of the whole plant *Urginea depressa* (Asparagaceae, formerly Hyacinthaceae) exhibited antiproliferative activity against the A2780 human ovarian cancer cell line, with an IC₅₀ value of 2.4 µg/mL. The Hyacinthaceae family is a family of

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ASSOCIATED CONTENT

Supporting Information

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herbaceous perennial bulbous flowering plants, which are distributed predominantly in Mediterranean climates including South Africa, Central Asia, and South America.⁴ *Urginea*, a genus of this family, is a rich source of stilbenoids, homoisoflavonoids, bufadienolides, sesquiterpenoids, and alkaloids, which are well-known for their broad range of bioactivities, including antimicrobial, anti-oxidant, cytotoxic, and anti-inflammatory activities, etc.^{5–9} The genus *Urginea* has been explored by other investigators, and in addition four bufadienolides were isolated from *Drimys depressa*, a botanical synonym of *Urginea depressa*.^{10,11} These previous studies did not however report any biological data, and so the extract of *U. depressa* was selected for bioassay-guided fractionation to isolate its bioactive components.

RESULTS AND DISCUSSION

Isolation and Structure Elucidation of Bioactive Homoisoflavonoids

An EtOH extract of the whole plant of *Urginea depressa* was subjected to liquid-liquid partitioning to give active hexanes, CH₂Cl₂, and aqueous MeOH fractions with the IC₅₀ values to the A2780 ovarian cancer cell line of 2.6, 0.68, and 0.72 µg/mL, respectively. Bioassay-guided separation of the hexanes and CH₂Cl₂ fractions, including Sephadex LH-20 size-exclusion, silica gel normal-phase chromatography and C₁₈ reverse-phase HPLC, yielded six new homoisoflavonoids which were named urgineanins A - F (**1** - **6**); urgineanins C and F (**3** and **6**) are enantiomers of known compounds. Compounds **1** - **5** displayed good antiproliferative activity against A2780 ovarian cancer cells, while compound **6** was much less active. Herein, we report the structural elucidation of these compounds.

Urgineanin A (**1**), [α]_D²¹ +26 (c 0.12, MeOH), was isolated as a light yellow oil. Its composition was established as C₂₀H₂₂O₇ by positive ion HRESIMS. Its IR spectrum exhibited absorption bands at 3464 and 1678 cm⁻¹, indicating the presence of hydroxy and conjugated carbonyl groups. Its ¹H NMR spectrum displayed the typical splitting pattern of an eucomol-type homoisoflavonoid, with two pairs of geminal coupled proton signals at δ_H 3.93 and 3.96 (each a doublet, J = 11.4 Hz), and δ_H 2.79 and 2.83 (each a doublet, J = 10.8 Hz).¹² The observation of 12 aromatic carbon signals, a carbonyl carbon resonance at δ_C 191.8, as well as a methylene carbon signal at δ_C 38.6 in the ¹³C NMR spectrum, corroborated the homoisoflavonoid structure. The basic skeleton of 3-hydroxy-3-benzyl-4-chromanone was confirmed by the HMBC correlations between H-2 and C-3, H-9 and C-3, H-9 and C-2, H-2 and C-4, H-9 and C-4, and H-9 and C-1'. The presence of four proton singlets (each 3H) located at δ_H 3.69, 3.72, 3.74, and 3.85 in the ¹H NMR spectrum, corresponding by HMQC to the four aromatic carbon resonances located at δ_C 60.8, 54.9, 61.2, and 56.2, indicated the presence of four methoxy groups. Two doublets at δ_H 6.84 and 7.13 (2H each, J = 8.7 Hz) indicated a *para*-disubstituted B-ring. A singlet at δ 6.49 was assigned to H-8 of the A-ring, based on the HMBC correlations between H-8 and C-8a and between H-8 and C-4a. The stereochemistry at C-3 was determined by comparison of its electronic circular dichroism (ECD) spectrum with those of previously reported homoisoflavonoids. Thus, the positive $\pi \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon$ = 10.8) at 280 nm and the negative $n \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon$ = -7.2) at 315 nm indicated the *R* configuration, in analogy to caesalpinaphenol A,¹³ and in agreement with the statement that a positive Cotton effect in the 287–295 nm region of the ECD curves of homoisoflavonoids is indicative of a 3*S*-configuration for compounds lacking a C-3 hydroxy group,¹⁴ corresponding to the 3*R* configuration for C-3 hydroxylated compounds.

Hence, the structure of compound **1** was assigned as (3*R*)-3-hydroxy-3-(4'-methoxybenzyl)-5,6,7-trimethoxychroman-4-one.

Urgineanin B (**2**), $[\alpha]_D^{21} +18$ (c 0.12, MeOH), was also isolated as a light yellow oil; its composition was established as $C_{19}H_{20}O_7$ by positive ion HRESIMS. Comparison of 1H and ^{13}C NMR spectroscopic data of **2** with those of **1** (Table 1) indicated that both compounds had the same skeleton and substitution pattern, and differed only in that **2** had a hydroxy group in place of one of the *O*-methyl groups of **1**. The 1H NMR spectrum of compound **2** showed three singlets at δ_H 3.68, 3.74 and 3.86 (each 3H) for the three methoxy groups of ring A. The presence of a hydroxy group instead of an *O*-methyl group at C-4' was confirmed by the absence of the NMR resonances (δ_H 3.72/ δ_C 54.9) and 2D correlations of the 4'-*O*-methyl group of **1**. Similar to compound **1**, a positive $\pi \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = 10.4$) at 281 nm and a negative $n \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = -7.3$) at 316 nm indicated the *R* configuration of C-3. Thus, the structure of compound **2** was concluded to be (3*R*)-3-hydroxy-3-(4'-hydroxybenzyl)-5,6,7-trimethoxychroman-4-one.

Urgineanin C (**3**), $[\alpha]_D^{21} +9$, was identified as the homoisoflavonoid (3*S*)-5,6,7-trimethoxy-3-(4-methoxybenzyl)chroman-4-one by comparison of its experimental and reported physical and spectroscopic data.^{14,15} Based on the positive $\pi \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = 5.6$) at 286 nm and a negative $n \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = -2.8$) at 251 nm, the absolute configuration of C-3 was assigned as *S*, enantiomeric with the reported compound.¹⁴

Urgineanin D (**4**, $[\alpha]_D^{21} +23$, light yellow oil) had the molecular formula $C_{20}H_{20}O_8$ as determined by the HRESIMS. Comparison of the NMR spectroscopic data of **4** with those of **1** indicated that the only difference was the B-ring substitution pattern. The 1H NMR spectrum of **4** had signals for three aromatic protons of the B-ring with an ABX-type spin system [δ_H 6.79 (1H, d, $J = 1.6$ Hz), 6.81 (1H, d, $J = 7.9$ Hz) and 6.65 (1H, dd, $J = 7.9, 1.6$ Hz)]. The presence of a methylenedioxy group ($-OCH_2O-$) in **4** was confirmed by the typical methylene carbon signal at δ_C 100.6 and two proton resonances at δ_H 5.97 and 5.98 (2H, d, $J = 1.0$ Hz).¹⁶ This group was assigned to C-3' and C-4' based on HMBC correlations between the methylene protons and C-3'/C-4', and between H-2' and C-4'. As with compounds **1** and **2**, the absolute configuration of C-3 was determined to be *R* based on the positive $\pi \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = 6.8$) at 280 nm and a negative $n \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = -5.2$) at 316 nm. The structure of compound **4** was thus assigned as (3*R*)-3-hydroxy-3-(3',4'-methylenedioxy)-5,6,7-trimethoxychroman-4-one.

Urgineanin E (**5**) was obtained as a light yellow oil with $[\alpha]_D^{21} +12$ (c 0.12, MeOH). Its molecular formula was deduced as $C_{20}H_{20}O_7$ by HREIMS. Comparison of the 1H NMR spectroscopic data of compound **5** with those of compound **4** indicated that both compounds possessed the same A- and B-ring substitutions but differed in the C-ring. The 1H NMR spectrum of compound **5** displayed the typical splitting pattern of eucomin-type homoisoflavonoids, characterized by two sets of doublet of doublets (δ_H 4.08/4.27 and δ_H 2.57/3.01) assignable to the two protons of C-2 and C-9, respectively; and the prominent complex multiplet (δ_H 2.85) signal of the proton at C-3.¹⁷ The absence of an OH group at C-3 was confirmed by the upfield shift of the C-3 resonance (δ_C 47.7 ppm). The basic skeleton of 3-benzyl-4-chromanone was corroborated by HMBC correlations between H-2 and C-3, H-3 and C-9, H-9 and C-3, H-9 and C-2, H-2 and C-4, and H-9 and C-1'. The absolute configuration of compound **5** was determined to be *S*, based on its positive $\pi \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = 5.9$) at 288 nm and the negative $n \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = -0.1$) at 309 nm.¹⁸ The structure of compound **5** was thus assigned as (3*S*)-3-(3',4'-methylenedioxy)-5,6,7-trimethoxychroman-4-one.

Urgineanin F (**6**), $[\alpha]_D^{21} +6$, was identified as the homoisoflavonoid (3*S*)-5,7-dihydroxy-3-(4-methoxybenzyl)chroman-4-one by comparison of its experimental and reported physical and spectroscopic data.^{19,20} As with **3**, compound **6** differs from the known compound by its

configuration at C-3. A positive $\pi \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = 5.8$) at 288 nm and a negative $n \rightarrow \pi^*$ Cotton effect ($\Delta\epsilon = -2.2$) at 251 nm indicated the 3*S* configuration.

Isolation and Structure Elucidation of Bioactive Bufatrienolides

The bioactive aqueous methanol fraction from the initial liquid-liquid partition described above was subjected to bioassay-guided separation using Diaion HP-20, size-exclusion Sephadex LH-20, C¹⁸ flash chromatography, and C¹⁸ reverse-phase HPLC to afford the two known compounds **7** and **9**, and the two new bioactive bufatrienolides, named urginin B (**8**) and urginin C (**10**).

Compound **7** was identified as altoside (14 β -hydroxy-19 β -oxobufa-4,20,22-trienolide-3 β -*O*- β -D-glucopyranoside), a known bufatrienolide isolated in 1959 from *Urginea altissima*.²¹ To complete the partially reported NMR spectroscopic data reported in the literature, and to enable comparison with the NMR data of **8** and **10**, we list its complete ¹H and ¹³C NMR spectroscopic data (Table 2), as well as its key HMBC correlations (Figure 2a).

Urginin B (**8**), [α]_D²¹ +27 (*c* 0.12, MeOH), was isolated as a light yellow powder, and its composition was determined as C₃₂H₄₂O₁₂ by HREIMS. A ¹³C NMR resonance at δ_C 170.2 ppm, and a singlet at δ_H 1.75 (3H) in the ¹H NMR spectrum suggested the presence of an *O*-acetyl group. In addition, a formyl group was revealed by the observation of a carbonyl absorption (1710 cm⁻¹), a ¹³C NMR resonance at δ_C 203.9, and a singlet signal at δ_H 9.72 in the ¹H NMR spectrum. Meanwhile, the glycosidic nature of compound **8** was indicated by the presence of a proton signal at δ_H 4.33 (d, *J* = 7.8 Hz, H-1'), as well as six resonances observed in the range of δ_H 2.90–3.80. In addition to the methyl and carbonyl carbons of the *O*-acetyl group, there were 30 carbon signals in the ¹³C NMR spectrum, among which 24 (including a formyl carbon) were assigned to a bufatrienolide aglycone, and the remaining six to a monosaccharide moiety.²²

The ¹H and ¹³C NMR spectra of compounds **8** and **7** were similar except that the spectra of **8** contained signals for an *O*-acetyl group and one additional oxygen-bearing carbon (δ_H 5.38, δ_C 74.2), indicating that **8** is a bufatrienolide. The three oxygen-bearing carbons C-3 (δ_C 74.1), C-16 (δ_C 74.2) and C-14 (δ_C 82.9) were assigned based on the interpretation of the HSQC, COSY and HMBC spectra (Figure 2b), indicating the additional group to be at C-16. The *O*-acetyl group was deduced to be attached to C-16, as indicated by the HMBC correlations between H-17 and C-16, H-17 and C-20, as well as between H-16 (δ_H 2.87) and the carbonyl carbon of the *O*-acetyl group.

The coupling constant between H-17 and H-16 (*J* = 8.8 Hz) indicated a *trans* configuration of the two protons, and thus an α -orientation of the *O*-acetyl group.²³ The orientation of H-3 was also determined to be α , based on the NOESY crosspeaks between H-1b and H-19, and H-3 and H-1a. (Figure 2b). The absolute configuration at C-14 was assigned as *S* based on the similarities of structure and origin of **7** and **8**, and on the common occurrence of this configuration for 14-hydroxybufadienolides.²⁴

The anomeric signals at δ_H 4.33 (d, *J* = 7.8 Hz)/ δ_C 102.1 were assigned to a β -glucopyranosyl unit, based on their similarity with those of the sugar moiety of **7** (Table 1). In order to determine the absolute configuration of the glucose, compound **8** was hydrolyzed with 3 M hydrochloric acid to yield D-glucose, as indicated by its identical *R_f* value compared to the D-glucose standard in TLC-analysis and its positive optical rotation. Based on the above evidence, the structure of compound **8** was assigned as 14 β -hydroxy-19 β -oxobufa-4,20,22-trienolide-16 α -*O*-acetyl-3 β -*O*- β -D-glucopyranoside.

Compound **9** was identified as urginin, (14 β -hydroxybufa-4,20,22-trienolide-3 β -*O*-{ α -L-rhamnopyranosyl-[(1 $\rightarrow$ 4)- β -D-glucopyranosyl]-(1 $\rightarrow$ 3)- α -L-rhamnopyranoside}), by comparison of its experimental and reported physical and spectroscopic data.²⁵

Urginin C (**10**), [α]_D²¹ -14 (c 0.12, MeOH), was obtained as a light yellow powder. Its composition was assigned as C₄₂H₆₀O₁₈, based on its positive ion HRESIMS spectrum. Comparison of the ¹H and ¹³C NMR spectroscopic data of compound **10** with those of compounds **7** and **9** indicated that compounds **7** and **10** possessed the same 3 β ,14 β -dihydroxy-19 β -oxobufa-4,20,22-trienolide aglycone,^{26,27} and that compounds **9** and **10** had identical sugar moieties at C-3.

The presence of trisaccharide units was suggested by the three sets of anomeric signals at δ_H 4.72/ δ_C 100.9, δ_H 4.89 (d, J = 1.7 Hz)/ δ_C 104.6, and δ_H 4.48 (d, J = 7.9 Hz)/ δ_C 104.4, respectively. In addition, the two doublets in the ¹H NMR spectrum at δ_H 1.20 (3H, J = 6.2 Hz) and δ_H 1.21 (3H, J = 6.2 Hz) indicated the presence of two rhamnose units. The third sugar moiety was identified as glucose, based on the proton splitting patterns of the seven proton resonances of H-1''' to H-6''' in the ¹H NMR spectrum (Table 2). 2D NMR spectroscopic data, including HMBC, HSQC, and NOESY, were employed to determine the linkages between the sugars and the aglycone and between the sugars. One of the rhamnopyranosyl units was connected to C-3, as indicated by ³J-HMBC correlations between H-1' and C-3. The HMBC crosspeaks between H-1'' and C-3', and H-3' (δ_H 3.64, dd, J = 9.5, 3.4 Hz) and C-1' indicated that the second rhamnopyranosyl unit was connected to C-3'. The glucopyranosyl unit was determined to be connected to C-4', based on the HMBC correlation between H-1''' and C-4', as well as H-6' and C-4' (Figure 2c). The coupling constant between H-1''' and H-2''' (J = 7.9 Hz) of the glucose indicated the β -configuration of the glucopyranosyl unit, and the configurations of the two rhamnopyranosyl units were both deduced to be α , as indicated by the coupling constants between H-1' and H-2', and H-1'' and H-2'' (both J = 1.7 Hz). Compound **10** was hydrolyzed with 3 M hydrochloric acid to yield L-rhamnose and D-glucose, as indicated by the identical R_f -values compared to the L-rhamnose and D-glucose standards, respectively, in the TLC-analysis, the positive optical rotation of the glucose, and the negative optical rotation of the rhamnose. Compound **10** was thus assigned as 14 β -hydroxy-19 β -oxobufa-4,20,22-trienolide-3 β -*O*-{ α -L-rhamnopyranosyl-[(1 $\rightarrow$ 4)- β -D-glucopyranosyl]-(1 $\rightarrow$ 3)- α -L-rhamnopyranoside}.

Biological Activities

The isolated compounds were tested for antiproliferative activity against the A2780 ovarian cancer cell line. As listed in Table 3, the five homoisoflavonoids urginanins A - E (**1** - **5**) showed strong antiproliferative activity against the A2780 ovarian cancer cell line with IC₅₀ values of 0.32, 3.4, 1.35, 0.35 and 1.44 μ M, respectively, while the less oxygenated analogue **6** had a much lower activity. Additional testing against the A2058 melanoma and H522-T1 human non-small cell lung cancer cells lines showed some interesting differential activities, with compound **1** being about fivefold more potent to the A2058 and H522-T1 cell lines than to the A2780 line, compound **4** being approximately equipotent to all three cell lines, and compounds **2** and **5** being significantly less potent to the A2058 and H522-T1 cell lines than to the A2780 cell line.

To date, more than 110 homoisoflavonoids distributed between eight different structural types have been isolated from plants, varying by the number and position of oxygenation of the A- and/or B- rings.²⁸ Previous work showed that not all homoisoflavonoids are cytotoxic to cancer cells,²⁹ and the present results suggest two factors that contribute to increased antiproliferative activity. The first is the degree of methylation. This is suggested by the observation that the two compounds with the highest overall antiproliferative activities are **1**,

with four methoxy groups, and **4**, with three methoxy and one methylenedioxy group. This result may be due to the fact that methylation increases lipophilicity, which facilitates cellular uptake.^{30,31} The second factor is that hydroxylation at C-3 increases activity; higher antiproliferative activity was observed for the eucomol-type homoisoflavonoids **1** and **4**, with a hydroxy group at C-3, than for the corresponding eucomin-type homoisoflavonoids **3** and **5**, lacking a hydroxy group at C-3.

The bufatrienolides were also tested for antiproliferative activity against the A2780 ovarian cancer, the A2058 melanoma, and the H522-T1 human non-small cell lung cancer cells lines. As listed in Table 3, the four bufatrienolides, altoside (**7**), uginin B (**8**), uginin (**9**), and uginin C (**10**) all showed strong antiproliferative activity, with IC₅₀ values of 24, 11, 111, and 41 nM, respectively. Bufadienolides are polyhydroxy C-24 steroids and their glycosides which have been found in both plant and animal sources.^{24,32} They inhibit the α -unit of the enzyme Na⁺, K⁺-ATPase to further induce cell apoptosis.³³ Previous research on the structure-activity relationship of the bufadienolides indicated that the presence of the 19-oxo group increases the antiproliferative activity against primary liver carcinoma PLC/PRF/5 cells compared to those bufadienolides with 19-CH₃.³⁴ Our data corroborate this statement since uginin C (**10**) showed a higher activity compared to uginin (**9**), and the two compounds only differ in their C-10 substitution. Moreover, uginin B (**8**) displayed a higher cytotoxicity than altoside (**7**), indicating that the introduction of an *O*-acetyl group increases the potency, as previously described.³⁵ In addition, the number of sugars attached to the aglycone affects the cytotoxicity of the bufatrienolide, since the monoglucosides **7** and **8** were more potent than the trisaccharide bufatrienolides **9** and **10**. It is possible that the greater number of hydroxy groups from the sugars causes decreased lipophilicity, blocking the cell uptake.³⁶

EXPERIMENTAL SECTION

General Experimental Procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. UV and IR spectroscopic data were measured on a Shimadzu UV-1201 spectrophotometer and a MIDAC M-series FTIR spectrophotometer, respectively. ECD spectra were obtained on a JASCO J-815 circular dichroism spectrometer. NMR spectra were recorded in DMSO-*d*₆ on Bruker Avance 500 or 600 spectrometers. The chemical shifts are given in δ (ppm), and coupling constants (*J*) are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion mode.

Antiproliferative Bioassays

Antiproliferative activities were obtained at Virginia Polytechnic Institute and State University against the drug-sensitive A2780 human ovarian cancer cell line as previously described.³⁷ Antiproliferative activities against the A2058 melanoma and the H522-T1 lung cancer cell lines were determined at Eisai Inc. by similar procedures to those used for the H460 cell line.³⁸

Plant Material

Plant collection was made in the Pilgrims Rest district, State of Mpumalanga, South Africa, by Prof. P. C. Zietsman (National Museum, Bloemfontein, South Africa) under the auspices of the New York Botanical Garden, accession number Z03749a. A voucher specimen is deposited in the New York Botanical Garden.

Extraction and Isolation

The dried and powdered whole plant of *Urginea depressa* (100 g) was exhaustively extracted with EtOH (2×1 L) in two 24-hour percolation steps to give 20 g of extract; successive partition of the concentrated extract with hexanes and CH_2Cl_2 gave an active CH_2Cl_2 extract. For purposes of fractionation and purification, 0.75 g of the original EtOH extract designated 60070-6B was shipped to Virginia Tech for bioassay-guided isolation. A 0.60 g sample of 60070-6B (IC_{50} 2.4 $\mu\text{g/mL}$) was suspended in aqueous MeOH (MeOH- H_2O 9:1, 100 mL), and extracted with hexanes (3×100 mL). The aqueous layer was diluted to 60% MeOH (v/v) with H_2O and extracted with CH_2Cl_2 (3×150 mL). The hexanes fraction was evaporated in vacuo to leave 89.3 mg of material with IC_{50} = 2.6 $\mu\text{g/mL}$. The residue from the CH_2Cl_2 fraction (86.2 mg) had an IC_{50} of 0.68 $\mu\text{g/mL}$, and the remaining aqueous MeOH fraction was centrifuged to give a supernatant (311 mg) with an IC_{50} of 0.72 $\mu\text{g/mL}$.

The CH_2Cl_2 fraction was subjected to size exclusion open column chromatography on Sephadex LH-20 to yield three fractions, of which the most active fraction F3 (21.2 mg) had an IC_{50} value of 0.37 $\mu\text{g/mL}$. Fraction F3 was applied to a silica gel column with elution by CHCl_3 :MeOH, 20:1 to give 14 fractions. Fraction F3.2 (1.8 mg), the most active fraction (IC_{50} 0.14 $\mu\text{g/mL}$), was further applied on C_{18} HPLC and eluted by 35% MeCN in H_2O to yield compound **1** (0.8 mg) and compound **4** (0.5 mg), with retention times of 20.3 and 19.2 minutes, respectively. Using the same C_{18} HPLC column, fraction F3.3 (5.9 mg, IC_{50} 1.3 $\mu\text{g/mL}$) was eluted with 40% MeCN in H_2O to give compound **2** (0.25 mg) and compound **6** (0.25 mg), with retention times of 9.8 and 20.4 minutes, respectively. In the same manner, Sephadex LH-20 size exclusion open column chromatography of the hexanes fraction yielded six fractions, of which the most active fraction F5 (22.4 mg) had an IC_{50} values of 0.53 $\mu\text{g/mL}$. Fraction F5 was applied to a silica gel column and eluted with hexanes:EtOAc, 7:1 to give 13 fractions. Fraction F5.12 (2.5 mg), the most active fraction (IC_{50} 0.15 $\mu\text{g/mL}$), was further separated on C_{18} HPLC eluted by 35% MeCN in H_2O to yield compound **5** (0.2 mg) and compound **3** (1.0 mg), with retention times of 26.2 and 27.4 minutes, respectively.

The MeOH-soluble fraction was separated on an HP-20 Diaion open column to give three fractions, of which the most active fraction F3 (111.1 mg) had an IC_{50} value of 0.39 $\mu\text{g/mL}$. F3 was applied to a Sephadex LH-20 size-exclusion open column with elution by CH_2Cl_2 :MeOH = 1:1 to give four fractions. Fraction 3.2 (54.2 mg), the most active (IC_{50} 0.14 $\mu\text{g/mL}$) fraction, was applied on C_{18} SPE eluted by 40, 60, 80 and 100% MeOH in H_2O to give a 40% fraction (IC_{50} 0.12 $\mu\text{g/mL}$), and a 60% fraction (IC_{50} 0.36 $\mu\text{g/mL}$). The 40% fraction was subjected to C_{18} HPLC eluted by 40% MeOH in H_2O and yielded compound **9** (1.8 mg) and compound **10** (2.6 mg), with retention times of 13.9 and 7.2 minutes, respectively. In the same manner, the 60% fraction was subjected to C_{18} HPLC eluted by 60% MeOH in H_2O to yield compound **7** (1.0 mg) and compound **8** (1.2 mg), with retention times of 20.6 and 6.2 minutes, respectively.

Urgineanin A (1): (3*R*)-3-hydroxy-3-(4'-methoxybenzyl)-5,6,7-trimethoxychroman-4-one light yellow oil; $[\alpha]_D^{21} +26$ (c 0.12, MeOH); UV (MeOH) λ_{max} (e) 229 (1.6), 274 (1.2); IR ν_{max} cm^{-1} : 3464, 2932, 1678, 1600, 1483, 1265, 1121 cm^{-1} ; ECD (c 0.031 mM, MeOH) $\Delta\epsilon_{280} +10.8$, $\Delta\epsilon_{315} -7.2$; ^1H NMR (600 MHz, DMSO- d_6), and ^{13}C NMR (150 MHz, DMSO- d_6), see Table 1; HRESIMS m/z 397.1258 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{20}\text{H}_{22}\text{NaO}_7$, 397.1263) and 375.1438 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{20}\text{H}_{23}\text{O}_7$, 375.1444).

Urgineanin B (2): (3*R*)-3-hydroxy-3-(4'-hydroxybenzyl)-5,6,7-trimethoxychroman-4-one light yellow oil; $[\alpha]_D^{21} +23$ (c 0.12, MeOH); UV (MeOH) λ_{max} (e) 229 (1.2), 274 (0.9); IR ν_{max} cm^{-1} : 3485, 2924, 1677, 1598, 1477, 1263, 1119 cm^{-1} ; ECD (c 0.031 mM, MeOH) $\Delta\epsilon_{281} +10.4$, $\Delta\epsilon_{316} -7.3$; ^1H NMR (600 MHz, DMSO- d_6), and ^{13}C NMR (150 MHz,

DMSO-*d*₆), see Table 1; HRESIMS *m/z* 383.1102 [M+Na]⁺ (calcd for C₁₉H₂₀NaO₇, 383.1107) and 361.1277 [M+H]⁺ (calcd for C₁₉H₂₁O₇, 361.1287).

Urigineanin C (4): (3*R*)-3-hydroxy-3-(3',4'-methylenedioxy)-5,6,7-trimethoxychroman-4-one light yellow oil; [α]_D²¹ +18 (c 0.12, MeOH); UV (MeOH) λ_{max} (ε) 229 (1.2), 274 (0.9); IR ν_{max} cm⁻¹: 3497, 2897, 1679, 1600, 1479, 1246, 1111 cm⁻¹; ECD (c 0.031 mM, MeOH) Δε₂₈₀ +6.8, Δε₃₁₆ -5.2; ¹H NMR (600 MHz, DMSO-*d*₆), and ¹³C NMR (150 MHz, DMSO-*d*₆), see Table 1; HRESIMS *m/z* 411.1053 [M+Na]⁺ (calcd for C₂₀H₂₀NaO₈, 411.1056), and [M+H]⁺ *m/z* 389.1231 (calcd for C₂₀H₂₁O₈, 389.1236).

Urigineanin D (5): (3*S*)-3-(3',4'-methylenedioxy)-5,6,7-trimethoxychroman-4-one light yellow oil; [α]_D²¹ +12 (c 0.12, MeOH); UV (MeOH) λ_{max} (ε) 229 (1.8), 280 (1.3); IR ν_{max} cm⁻¹: 2898, 1646, 1591, 1483, 1251, 1028 cm⁻¹; ECD (c 0.031 mM, MeOH) Δε₂₈₈ +5.9, Δε₃₀₉ -0.1; ¹H NMR (600 MHz, DMSO-*d*₆), and ¹³C NMR (150 MHz, DMSO-*d*₆), see Table 1; HRESIMS *m/z* 395.1100 [M+Na]⁺ (calcd for C₂₀H₂₀NaO₇, 395.1107) and 373.1279 [M+H]⁺ (calcd for C₂₀H₂₁O₇, 373.1287).

Altoside (7): light yellow powder; [α]_D²¹ +23 (c 0.12, MeOH); UV (MeOH) λ_{max} (ε) 208 (1.8), 298 (0.6); IR ν_{max} cm⁻¹: 3385, 2924, 2855, 1738, 1710, 1455, 1250, 1064 cm⁻¹. ¹H NMR (600 MHz, MeOH-*d*₄), and ¹³C NMR (150 MHz, methanol-*d*₄), see Table 1; HRESIMS *m/z* 583.2482 [M+Na]⁺ (calcd for C₃₀H₄₀NaO₁₀, 583.2519).

Urginin B (8): light yellow powder; [α]_D²¹ +27 (c 0.12, MeOH); UV (MeOH) λ_{max} (ε) 211 (1.9), 288 (0.6); IR ν_{max} cm⁻¹: 3362, 2924, 2850, 1735, 1710, 1450, 1245, 1068 cm⁻¹. ¹H NMR (600 MHz, MeOH-*d*₄), and ¹³C NMR (150 MHz, methanol-*d*₄), see Table 1; HRESIMS *m/z* 641.2646 [M+Na]⁺ (calcd for C₃₂H₄₂NaO₁₂, 641.2574).

Urginin C (10): light yellow powder; [α]_D²¹ -14 (c 0.12, MeOH); UV (MeOH) λ_{max} (ε) 211 (1.9), 296 (0.7); IR ν_{max} cm⁻¹: 3348, 2925, 2860, 1734, 1710, 1459, 1268, 1124 cm⁻¹. ¹H NMR (600 MHz, MeOH-*d*₄), and ¹³C NMR (150 MHz, methanol-*d*₄), see Table 1; HRESIMS *m/z* 875.3738 [M+Na]⁺ (calcd for C₄₂H₆₀NaO₁₈, 875.3677).

Hydrolysis of Compounds 7, 8, and 10

Compound **10** (1.0 mg) was hydrolyzed with 3 M HCl for 4 h at 100 °C. The solution was extracted with EtOAc (3 × 50 mL), and both the organic and the water layers were evaporated to dryness under reduced pressure. The structure of the white powder (1.4 mg) derived from the organic layer was determined to be 3β,14β-dihydroxy-19β-oxobufa-4,20,22-trienolide by ¹H and ¹³C NMR spectroscopy. The semi-solid carbohydrate mixture from the water layer (0.45 mg) was applied to silica gel column, eluted by CHCl₃:MeOH:H₂O = 15:7:2 (lower phase). Two fractions (monosaccharide 1 and monosaccharide 2) were obtained; each of which was dissolved in 1 mL of H₂O and kept overnight before TLC analysis and determination of optical rotations. On the silica gel TLC plate, monosaccharide 1 (*R_f* = 0.36) had an identical *R_f* value to L-rhamnose, developed by CHCl₃:MeOH:H₂O = 13:7:2 (lower phase). Moreover, it had an [α]_D²² -7.0, (c 0.1, H₂O). Monosaccharide 2 (*R_f* = 0.20) had an identical *R_f* value to D-glucose on the same TLC system as that of monosaccharide 1, with [α]_D²² +34, c 0.1, H₂O.

Compounds **7** and **8** were hydrolyzed and analyzed in the same manner, and D-glucose was obtained from both of the aqueous portions of the hydrolytes, with [α]_D²² +36 and +38, (c 0.1, H₂O).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
HMBC correlation of compounds **1,2,4** and **5**

Figure 2a

Figure 2b

Figure 2c

Figure 2.

Figure 2a. HMBC and NOESY correlations of compound **7**.

Figure 2b. HMBC and NOESY correlations of compound **8**.

Figure 2c. HMBC and NOESY correlations of compound **10**.

Table 1
NMR Spectroscopic Data for Compounds 1, 2, 4 and 5 in DMSO-*d*₆ (600 MHz)

position	1		2		4		5	
	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type
2a	3.96 d (11.4)	71.5, CH ₂	3.96 d (11.3)	71.2, CH ₂	3.99 d (10.6)	71.4, CH ₂	4.27 dd (11.3, 4.5)	69.4, CH ₂
2b	3.93 d (11.4)		3.92 d (11.3)		3.95 d (10.6)		4.08 dd (11.3, 8.8)	
3	-	72.1, C	-	72.7, C	-	72.1, C	2.85 m	47.7, CH
4	-	191.8, C	-	191.9, C	-	191.7, C	-	190.8, C
4a	-	106.8, C	-	106.8, C	-	107.7, C	-	108.7, C
5	-	153.6, C	-	153.7, C	-	153.6, C	-	154.0, C
6	-	137.1, C	-	137.3, C	-	137.1, C	-	137.4, C
7	-	159.0, C	-	159.1, C	-	159.0, C	-	159.2, C
8	6.49 s	96.5, CH	6.49 s	96.6, CH	6.49 s	96.5, CH	6.45 s	96.9, CH
8a	-	158.8, C	-	158.7, C	-	158.8, C	-	158.9, C
9a	2.83 d (10.8)	38.6, CH ₂	2.77 d (10.7)	38.7, CH ₂	2.83 d (10.5)	38.7, CH ₂	3.01 dd (14.1, 5.1)	32.3, CH ₂
9b	2.79 d (10.8)		2.73 d (10.7)		2.79 d (10.5)		2.57 dd (14.1, 9.4)	
1'	-	127.2, C	-	125.8, C	-	129.0, C	-	132.1, C
2'	7.13 d (8.7)	131.5, CH	7.00 d (8.5)	131.4, CH	6.79 d (1.6)	114.9, CH	6.84 d (1.6)	108.6, CH
3'	6.84 d (8.7)	113.2, CH	6.66 d (8.5)	114.6, CH	-	145.8, C	-	145.7, C
4'	-	157.9, C	-	156.3, C	-	146.9, C	-	146.4, C
5'	6.84 d (8.7)	113.2, CH	6.66 d (8.5)	114.6, CH	6.81 d (7.9)	110.6, CH	6.83 d (8.0)	109.8, CH
6'	7.13 d (8.7)	131.5, CH	7.00 d (8.5)	131.4, CH	6.65 dd (7.9, 1.6)	123.4, CH	6.68 dd (8.0, 1.6)	122.0, CH
5-OMe	3.74 s	61.2, CH ₃	3.74 s	61.2, CH ₃	3.74 s	61.2, CH ₃	3.74 s	61.2, CH ₃
6-OMe	3.69 s	60.8, CH ₃	3.68 s	60.8, CH ₃	3.69 s	60.8, CH ₃	3.66 s	60.8, CH ₃
7-OMe	3.85 s	56.2, CH ₃	3.86 s	56.2, CH ₃	3.85 s	56.2, CH ₃	3.84 s	56.2, CH ₃
4'-OMe	3.72 s	54.9, CH ₃						
3'-OCH ₂ O-4'					5.97 d (1.0) 5.98 d (1.0)	100.6, CH ₂	5.97 d (1.0) 5.98 d (1.0)	100.6, CH ₂

* Carbon-NMR data for compound **2** and **5** were obtained from HMBC and HSQC or HMQC spectra.

Table 2

NMR Spectroscopic Data for 7-8, 10 in Methanol- d_4 (600 MHz)

	7	8			10		
	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type	
1a	2.13 m	28.1, CH ₂	2.16 m	27.7, CH ₂	2.16 m	29.6, CH ₂	
1b	1.08 m		1.04 m		1.10 m		
2a	1.99 m	27.6, CH ₂	2.00 m	27.2, CH ₂	2.08 m	28.5, CH ₂	
2b	1.28 m		1.36 m		1.30 m		
3	4.18 m	74.2, CH	4.19 m	74.1, CH	4.05 m	74.1, CH	
4	5.74 brs	126.2, CH	5.76 brs	126.4, CH	5.63 brs	127.2, CH	
5	-	138.2, C	-	138.4, C	-	139.7, C	
6a	2.29 m	32.9, CH ₂	2.31 m	32.7, CH ₂	2.33 m	34.2, CH ₂	
6b	2.23 m		2.25 m		2.24 m		
7a	2.35 m	28.4, CH ₂	2.34 m	28.3, CH ₂	2.36 m	29.4, CH ₂	
7b	1.27 m		1.30 m		1.29 m		
8	1.78 m	43.0, CH	1.81 m	42.9, CH	1.80 m	44.4, CH	
9	1.23 m	49.4, CH	1.21 m	49.3, CH	1.25 m	51.0, CH	
10	-	53.6, C	-	53.4, C	-	55.0, C	
11a	1.60 m	21.5, CH ₂	1.62 m	21.3, CH ₂	1.63 m	23.1, CH ₂	
11b	1.30 m		1.32 m		1.32 m		
12a	1.44 m	40.2, CH ₂	1.50 m	39.0, CH ₂	1.46 m	41.5, CH ₂	
12b	1.31 m		1.28 m		1.33 m		
13	-	47.7, C	-	49.2, C	-	49.0, C	
14	-	84.0, C	-	82.9, C	-	85.8, C	
15a	1.88 m	31.4, CH ₂	1.67 d (15.6)	39.5, CH ₂	1.91 m	32.8, CH ₂	
15b	1.53 m		2.43 dd (15.6, 8.8)		1.55 m		
16a	2.07 m	28.2, CH ₂	5.38 t (8.8)	74.2, CH	1.91 m	29.7, CH ₂	
16b	1.65 m				1.66 m		
17	2.45 dd (9.7, 6.9)	50.6, CH	2.87 d (8.8)	56.6, CH	2.47 dd (9.6, 7.0)	52.0, CH	
18	0.62 s	15.7, CH ₃	0.70 s	15.5, CH ₃	0.64 s	17.1, CH ₃	
19	9.72 s	204.1, C	9.72 s	203.9, C	9.75 s	204.4, C	

	7	8	10	
	δ_H (J in Hz)	δ_C , type	δ_H (J in Hz)	δ_C , type
20	-	123.7, C	-	117.6, C
21	7.33 d (1.7)	149.1, CH	7.34 d (1.7)	151.3, CH
22	7.90 dd (9.5, 1.7)	147.8, CH	8.15 dd (9.5, 1.7)	150.6, CH
23	6.19 d (9.5)	114.0, CH	6.10 d (9.5)	111.8, CH
24	-	163.5, C	-	163.6, C
Acetyl				
CO				170.2, C
CH ₃			1.75 s	19.6, CH ₃
	C-3- <i>O</i> -Glucopyranosyl		C-3- <i>O</i> -Glucopyranosyl	
1'	4.32 d (7.9)	102.0, CH	4.33 d (7.8)	102.1, CH
2'	3.04 dd (9.1, 7.9)	73.6, CH	3.05 dd (9.2, 7.8)	73.7, CH
3'	3.17 m	76.6, CH	3.18 m	76.6, CH
4'	3.15 m	70.2, CH	3.16 m	70.3, CH
5'	3.26 m	76.7, CH	3.26 m	76.7, CH
6'a	3.55 dd (4.7, 11.1)	61.5, CH	3.56 dd (6.5, 11.4)	61.4, CH
6'b	3.76 dd (2.1, 11.5)		3.77 dd (2.1, 11.5)	
				C-3'- <i>O</i> -Rhamnopyranosyl
1''				4.89 d (1.7)
2''				3.94 dd (3.4, 1.7)
3''				3.64 dd (9.5, 3.4)
4''				3.30 t (9.5)
5''				3.73 dq (9.5, 6.2)
6''				1.20 d (6.2)
				C-4'- <i>O</i> -Glucopyranosyl
1'''				4.48 d (7.9)
2'''				3.07 dd (9.1, 7.9)
3'''				3.26 dd (9.1, 8.8)
4'''				3.21 t (8.8)
5'''				3.13 ddd (2.4, 5.8, 8)
6'''				3.58 dd (5.6, 11.8)

6'''	7		8		10	
	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type	δ_{H} (J in Hz)	δ_{C} , type
					3.75 dd (2.4, 11.9)	

* Carbon-NMR data for compound **7** were obtained from HMBC and HSQC spectra.

Table 3

Bioactivities of Homoisoflavonoids 1-6

Compound									
1	2	3	4	5	6	Vinblastine	Taxol		
A2780 IC ₅₀ (μM)	0.32 ± 0.05	3.4 ± 0.16	1.35 ± 0.10	0.35 ± 0.06	1.44 ± 0.08	23 ± 1.2	NT	0.028 ± 0.002	
A2058 IC ₅₀ (μM)	0.068	>10	0.69	0.38	>10	>10	0.0037	NT	
H522-T1 IC ₅₀ (μM)	0.071	6.78	0.74	0.43	>10	>10	0.0052	NT	

Table 4

Bioactivities of Bufatrienolides 7-10

	Compound					Vinblastine	Taxol
	7	8	9	10			
A2780 IC ₅₀ (μM)	0.024 ± 0.006	0.011 ± 0.002	0.111 ± 0.008	0.041 ± 0.003	NT	0.028 ± 0.002	
A2058 IC ₅₀ (μM)	0.048	0.060	0.18	0.076	0.0037	NT	
H522-T1 IC ₅₀ (μM)	0.034	0.044	0.11	0.051	0.0052	NT	