Notes

Antioxidative Constituents from the Leaves of *Hypericum styphelioides*

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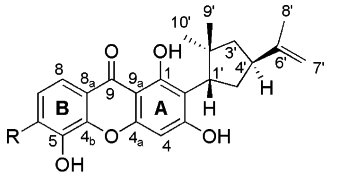
Two new compounds have been isolated from the leaves of *Hypericum styphelioides*. Their structures have been established on the basis of mass spectrometry and 2D NMR techniques as 1,3,5-trihydroxy-2-(2′,2′-dimethyl-4′-isopropenyl)cyclopentanylxanthone (**1**) and 3,5-dihydroxybenzophenon-4-*β*-D-glucoside (**2**). Known compounds 5-*O*-demethylpaxanthonin (**3**) and 3-geranyl-1-(3-methylbutanoyl)phloroglucinol (**4**) were also isolated and characterized. Compounds **1**−**4** were evaluated for their antioxidative properties in Trolox equivalent antioxidant activity (TEAC) and chemiluminescence (CL) assays.

Plants of the genus *Hypericum* have been used as traditional medicinal plants in various parts of the world.[1−4] *H. styphelioides* has been employed in traditional Cuban herbal medicine as a depurative, diaphoretic, diuretic, and tonic, against blennorrhea, cold, cough, and dysmenorrhoea, and for treatment of arthritis, rheumatism, hepatitis, herpes, and syphilis.[5] Flavonoids, xanthones, bisanthraquinone glycosides, and phloroglucinols have been isolated from the genus *Hypericum*.[6−8] In this paper, we report the isolation and spectroscopic identification of some chemical constituents of *Hypericum styphelioides* (Clusiaceae) leaves, a Cuban endemic plant.

The leaves of *H. styphelioides* were extracted successively with hexane, $CHCl_3$, and MeOH. The MeOH extract was subjected to chromatography on Sephadex LH-20 and HPLC, which yielded two new compounds, 1,3,5-trihydroxy-2-(2′,2′-dimethyl-4′-isopropenyl)cyclopentanylxanthone (**1**) and 3,5-dihydroxybenzophenone-4-*β*-D-glucoside (**2**), and two known compounds, 5-*O*-demethylpaxanthonin (**3**) and 3-geranyl-1-(3-methylbutanoyl)phloroglucinol (**4**).



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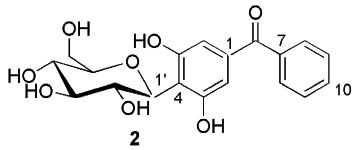
3 R = OH



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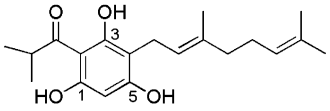

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The molecular formula of **1** ($C_{23}H_{24}O_5$) was deduced using ESIMS, ^{13}C NMR, and DEPT analyses. The UV spectrum of **1** exhibited maxima at 207, 221, 242, and 315 nm, and the IR spectra showed broad absortion in the 3500−3000 cm^{-1} region, together with an intense peak at 1640 cm^{-1}, consistent with a xanthone derivative. Analysis of the 1D and 2D NMR spectra with homo- and heteronuclear direct or long-range correlations allowed assignment of ^1H and ^{13}C NMR signals (see Experimental Section).

The ^1H NMR spectrum exhibited two doublets at δ 7.20 and 7.64, a doublet of doublet at δ 7.17, and a singlet at δ 6.50, indicating the existence of two different aromatic spin systems that were confirmed by DQF-COSY. The ^{13}C NMR spectrum and DEPT confirmed the presence of four aromatic methine groups (δ 94.8, 116.6, 121.3, and 124.7), a carbonyl group at δ 182.3, and signals at δ 146.5, 147.3, 157.2, 163.5, and 166.6, suggesting three hydroxyl groups and two ether groups. Multiple-bond heteronuclear correlation (HMBC) data unambiguously established a 1,3,5-trihydroxyxanthone derivative for **1** having a substituent group at C-2.

The ^1H and ^{13}C NMR and HSQC spectra of **1** showed the presence of three tertiary methyl groups, two quaternary carbons, a terminal double bond [δ_H 4.65 (H-7′b) and 4.75 (H-7′a); δ_C 108.3 (C-7′)], two methylenes, and two methynes. These data were consistent with the presence of a 2,2-dimethyl-4-isopropenyl cyclopentanyl group by DQF-COSY and HMBC experiments. The relative stereochemistry at C-1′ and C-4′ was obtained by NOEDIF (NOE difference) experiments, and significant results were observed irradiating the C-10′ methyl protons at δ 0.94 and observing enhancement of the C-4′ methine signal at δ 3.03 (5.8%), which revealed their *syn* relationship. Moreover,

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when the C-9′ methyl protons (δ 1.08) were irradiated, the H-1′ signal was enhanced by 6.7%, suggesting their *syn* relationship and the proximity of this methyl group to H-1′. This information is consistent with the relative stereochemistry of the cyclopentanyl moiety suggested previously for paxanthonin.[9] ^{1}H and ^{13}C NMR data were similar to those reported for 5-*O*-demethylpaxanthonin, a xanthone isolated from the roots of *Hypericum roeperanum*.[10] The main difference was associated with the B-ring, where only a single hydroxyl group was present. The hydroxyl group on C-1 was confirmed with an additional ^{1}H NMR spectrum (DMSO), which showed a chelated proton at δ 13.2. Thus, compound **1** was identified as 1,3,5-trihydroxy-2-(2′,2′-dimethyl-4′-isopropenyl)cyclopentanylxanthone, a new natural product named 6-deoxy-5-*O*-demethylpaxanthonin.

The molecular formula of compound **2** ($C_{19}H_{20}O_8$) was deduced on the basis of ESIMS, ^{13}C NMR, and DEPT analyses. Compound **2** exhibited UV absortion maxima at 212, 223, and 259 nm. The IR spectrum suggested the presence of hydroxyl (3500−3000 cm^{-1}) and conjugated carbonyl (1633 cm^{-1}) groups. The ^{1}H NMR spectrum indicated the presence of a sugar moiety as a substituent of an aromatic compound. The sugar substituent was identified as β-D-glucopyranosyl from the 1D-TOCSY, DQF-COSY, and HSQC spectra. The ^{1}H and ^{13}C NMR data of the key hydrogens and carbons (C-2′, C-3′, and C-5′) indicated a β-configuration at the anomeric position ($J_{H-1'-H-2'} = 7.5$ Hz).

Two aromatic rings were inferred considering the existence of four signals at δ 6.60 (2H, s, H-2 and H-6), 7.29 (t, $J = 7.2$, H-10), 7.38 (2H, dd, $J = 7.6$, 7.2, H-9 and H-11), and 7.51 (2H, d, $J = 7.6$, H-8 and H-12); these four signals revealed the presence of one monosubstituted and one tetrasubstituted ring. The simplicity of the NMR data and the molecular weight deduced by ESIMS suggested a symmetric substitution in both aromatic rings. Both the anomeric proton at δ 4.94 and the aromatic proton at δ 6.60 (H-2 and H-6) showed cross-peaks in the HMBC with C-3, C-4, and C-5, confirming the location of a glucose moiety at C-4. Also, diagnostic correlation was observed between the proton appearing as a singlet at δ 6.60 and the carbonyl group at δ 193.0, which indicated that the two aromatic protons were linked at the C-2 and C-6 positions. The connectivity pattern inferred by the HMBC spectrum was compatible only with the structure **2**. Thus, **2** was established as 3,5-dihydroxybenzophenone-4-β-D-glucoside, named hyperinone.

The free-radical-scavenging activity of compounds **1**−**4** was evaluated in the antioxidant (TEAC) and chemiluminescence (CL) assays. The first measures the relative ability of antioxidant substances to scavenge the radical cation 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonate) ($ABTS^{\bullet+}$) as compared to a standard amount of the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).[11] The CL assay measures the inhibition of iodophenol-enhanced chemiluminescence by a horseradish peroxidase/perborate/luminol system.[12] Trolox was used as the reference antioxidant. The results (Table 1) showed that xanthones **1** and **3** exhibited free-radical-scavenging activity at potency levels comparable to reference antioxidant compounds quercetin and rutin, while **2** and **4** had more moderate activities.

Table 1. Antioxidant Activity of Compounds **1**−**4** in the TEAC and CL Assay[a]

compound	TEAC assay (mM) ± SD[b]	CL assay (mM) ± SD[b]
1	2.92 ± 0.09	1.94 ± 0.06
2	1.93 ± 0.11	1.24 ± 0.05
3	2.75 ± 0.12	1.78 ± 0.07
4	1.10 ± 0.08	0.96 ± 0.05
quercetin	3.33 ± 0.06	3.09 ± 0.05
rutin	2.78 ± 0.05	2.54 ± 0.07

[a] For protocols used, see Experimental Section. [b] $n = 3$. Results are expressed in terms of mM Trolox equivalent.

Experimental Section

General Experimental Procedures. Melting points were determined using a Bausch & Lomb apparatus. Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 1 cm microcell. UV spectra were obtained with a Beckman DU 670 spectrophotometer and IR spectra with a Bruker IFS-48 spectophotometer. A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C, using the UXNMR software package was used for NMR experiments in $CDCl_3$. ^{1}H−^{1}H DQF-COSY (double quantum filtered COSY), ^{1}H−^{13}C HSQC, HMBC, and NOEDIF experiments were obtained using conventional pulse sequences. The selective excitation spectra, 1D TOCSY,[13] were acquired using waveform generator-based Gauss-shaped pulses, mixing times ranging from 100 to 120 ms, and MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse; chemical shifts are expressed in δ (ppm) referring to solvent peaks: δ_H 3.34 and δ_C 40.0 for CD_3OD. Electrospray ionization mass spectrometry (ESIMS) in the positive ion mode was performed using a Finnigan LC-Q Deca instrument from Thermoquest (San Jose, CA) equipped with Excalibur software. Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump; the flow rate was 3 μL/min. The capillary voltage was 5 V, the spray voltage 5 kV, and the tube lens offset 35 V. The capillary temperature was 220 °C. Data were acquired in the MS1 scanning mode (m/z 150−700). Exact masses were measured by a Q-Star Pulsar (Applied Biosystems) triple-quadrupole orthogonal time-of-flight (TOF) instrument. Electrospray ionization was used in TOF mode at 8.500 resolving power. Samples were dissolved in MeOH, mixed with the internal calibrant, and introduced directly into the ion source by direct infusion. Calibration was performed on the peaks of cesium iodide and synthetic peptide (TOF positive ion calibration solution, Bachem) at m/z 132.9054 and 829.5398, respectively. Sodium-containing molecular ions of analytes were measured. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Waters μ-Bondapak C18 column.

Biological Material. Leaves of *Hypericum styphelioides* were collected in Guane, Pinar del Rio (Cuba), and identified by Dr. Armando Urquiola-Cruz. A voucher specimen (HPPR-9190) was deposited in the Herbario del Instituto Pedagogico de Pinar del Rio.

Extraction and Isolation. Ground, air-dried leaves of *H. styphelioides* (500 g) were extracted successively with hexane, $CHCl_3$, and MeOH employing a Dionex Accelerated Solvent Extractor ASE-200. After evaporation of the solvent, the concentrated MeOH extract (22.2 g) was partitioned between n-BuOH and H_2O, and a portion of the BuOH fraction (5.9 g) was chromatographed on a Sephadex LH-20 column eluting with MeOH to yield six fractions. Fractions 4−6 were submitted to RP-HPLC on a C18 μ-Bondapack column (30 cm × 7.8 mm, flow rate 2.5 mL min^{-1}) using MeOH/H_2O (4:6) as the eluent to yield 1,3,5-trihydroxy-2-(2′,2′-dimethyl-4′-isopropenyl)cyclopentanylxanthone (**1**) (174.2 mg; t_R, 25.6 min), hyperinone (**2**) (30.2 mg; t_R, 15.1 min), 5-*O*-demethylpaxanthonin (**3**) (25.3 mg; t_R, 22.4 min), and 3-geranyl-1-(3-methylbutanoyl)-phloroglucinol (**4**) (7.8 mg; t_R, 33.5 min).

1,3,5-Trihydroxy-2-(2′,2′-dimethyl-4′-isopropenyl)cyclopentanylxanthone (1): brown, solid residue; $[\alpha]^{25}_D$ −1.13 (c 1.74, MeOH); UV MeOH λ_{max}(log ϵ) 315 (4.02), 242 (4.81), 221 (3.88), and 207 (3.41) nm; IR (KBr) ν_{max} 3254, 2951, 1640,

1580, 1455, 1024 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 7.64 (1H, d, J = 7.5 Hz, H-8), 7.20 (1H, d, J = 7.7 Hz, H-6), 7.17 (1H, dd, J = 7.5 and 7.7 Hz, H-7), 6.50 (1H, s, H-4), 4.75 (1H, br s, H-7′b), 4.65 (1H, br s, H-7′a), 3.65 (1H, dd, J = 7.5 and 11 Hz, H-1′), 3.03 (1H, m, H-4′), 2.96 (1H, m, H-5′b), 1.77 (3H, s, CH_3-8′), 1.71 (1H, t, J = 11 Hz, H-3′a), 1.65 (1H, m, H-5′a), 1.60 (1H, d, J = 11 Hz, H-3′b), 1.08 (3H, s, CH_3-9′), 0.94 (3H, s, CH_3-10′); ^{13}C NMR (CD_3OD, 600 MHz) δ 182.3 (C, C-9), 166.6 (C, C-3), 163.5 (C, C-1), 157.2 (C, C-4a), 150.8 (C, C-6′), 147.3 (C, C4b), 146.5 (C, C-5), 124.7 (CH, C-7), 122.7 (C, C8a), 121.3 (CH, C-6), 116.6 (CH, C-8), 112.2 (C, C-2), 108.3 (CH_2, C-7′), 104.3 (C, C-9a), 94.8 (CH, C-4), 49.2 (CH_2, C3′), 46.2 (C, C-2′), 45.4 (CH, C-4′), 44.8 (CH, C-1′), 33.7 (CH_2, C5′), 30.7 (CH_3, C-9′), 25.3 (CH_3, C-10′), 21.3 (CH_3, C-8′); ESI-MS m/z 381 $[M + H]^+$, m/z 379 $[M - H]^+$; HREIMS m/z 380.1618 (calcd for $C_{23}H_{24}O_5$, 380.1624).

Hyperinone (2): brown, solid residue; $[\alpha]^{25}_D$ −5.5 (*c* 0.302, MeOH); UV MeOH λ_{max} (log ϵ) 259 (4.77), 223 (4.32), and 212 nm (3.66); IR (KBr) ν_{max} 3333, 2923, 1633, 1591,1573, 1417, 1338, 1273, 1195 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 7.51 (2H, d, J = 7.6 Hz, H-8 and H-12), 7.38 (2H, dd, J = 7.6 and 7.2 Hz, H-9 and H-11), 7.29 (1H, dd, J = 7.2 and 7.2 Hz, H-10), 6.60 (2H, s, H-2 and H-6), 4.94 (1H, d, J = 9.8 Hz, H-1′), 3.93 (1H, dd, J = 9.8 and 9.8 Hz, H-2′), 3.87 (1H, dd, J = 12.1 and 1.9 Hz, H-6′a), 3.78 (1H, dd, J = 12.1 and 4.1 Hz, H-6′b), 3.52 (1H, dd, J = 9.9 and 9.8 Hz, H-4′), 3.50 (1H, dd, J = 9.9 and 9.8 Hz, H-3′), 3.42 (1H, m, H-5′); ^{13}C NMR (CD_3OD, 600 MHz) δ 193.0 (C═O, C-13), 158.4 (2C, C-3 and C-5), 143.8 (C, C-1), 142.1 (C, C-7), 129.6 (2CH, C-9 and C-11), 128.4 (CH, C-10), 127.6 (2CH, C-8 and C-12), 111.6 (C, C-4), 107.4 (2CH, C-2 and C-6), 82.5 (CH, C-5′), 79.9 (CH, C-3′), 76.8 (CH, C-1′), 73.7 (CH, C-2′), 71.3 (CH, C-4′), 62.4 (CH_2, C-6′); ESI-MS m/z 377 $[M + H]^+$, m/z 375 $[M - H]^+$; HREIMS m/z 376.1202 (calcd for $C_{19}H_{20}O_8$, 376.1159).

Compounds 3 and 4. These were identified as 5-*O*-demethylpaxanthonin (**3**) and 3-geranyl-1-(3-methylbutanoyl)-phloroglucinol (**4**) by ^1H and ^{13}C NMR in comparison with literature.[14,15]

ABTS Radical Cation Decolorization Assay. Evaluation of free-radical-scavenging activity was performed with the TEAC assay. TEAC value is based on the ability of the antioxidant to scavenge $ABTS^+$, the preformed radical monocation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), with spectrophotometric analysis, according to Re et al.[11] Samples were diluted with MeOH to obtain 0.3, 0.5, 1.0, 1.5, and 2.0 mM solutions. The reaction was enhanced by the addition of 1.0 mL of diluted ABTS to 10 μL of each solution of sample or Trolox (standard) or 10 mL of MeOH (control). The determination was repeated three times for each sample solution. The percentage inhibition of absorbance at 734 nm was calculated for each concentration as a function of the control's absorbance, 1 min after initial mixing. The antioxidant activity was expressed as TEAC (Trolox equivalent antioxidant activity), which is the concentration of standard Trolox solution with percentage inhibition equivalent to a 1 mM solution of the tested compounds.

Chemiluminescence Assay. Total antioxidant capacity was assayed by chemiluminescence according to Whitehead et al.[12] Enhanced chemiluminescent signal reagent (Amersham, UK), comprising assay buffer and tablets A and B (containing luminol, *p*-iodophenol enhancer, and perborate oxidant), was prepared by adding tablets A and B to the buffer solution. Signal reagent (0.4 mL) was added to distilled water (1 mL) in a glass cuvette containing a magnetic stirrer. The cuvette was placed in a Perkin-Elmer Wallac Victor 2 chemiluminometer, and the reaction commenced by addition of 25 μL of horseradish peroxidase (4 μg mL^{-1} in H_2O). Compounds (100 μL of 0.5 mg/mL dissolved in PBS, pH 7.4) were added to the cuvette, and the time for which light output was suppressed was determined. Comparison was made with a standard curve generated using Trolox (20 μg mL^{-1} in H_2O).

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