Constituents of the Cuban Endemic Species *Calophyllum pinetorum*

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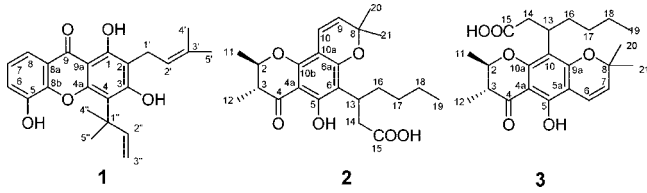
A new prenylated xanthone, pinetoxanthone (**1**), and two new pyranochromanones, pinetoric acid I (**2**) and pinetoric acid II (**3**), together with 10 known compounds, namely, the triterpenes friedelin and canophyllol, the xanthone macluraxanthone, the pyranochromanone derivatives calophyllic acid, isocalophyllic acid, calolongic acid, apetalic acid, and isoapetalic acid, and the flavonoids amentoflavone and apigenin, were isolated from the stem bark and leaves of *Calophyllum pinetorum*, an endemic species in Cuba. The structures of **1**−**3** were elucidated by spectroscopic methods including 1D and 2D NMR experiments as well as HRESIMS analysis.

The genus *Calophyllum* is comprised of about 130 species, mostly trees, distributed in tropical areas of the world. Previous phytochemical studies of this genus have revealed it to be a rich source of secondary metabolites such as xanthones,[1,2] coumarins,[3,4] chromenes,[5,6] flavonoids,[7] and triterpenoids.[8] Some of these compounds exhibit biological effects such as imunomodulatory,[9] antifungal,[10,11] antimicrobial,[12] antimalarial,[13] and anti-HIV[14,15] activities. Calanolides, a series of pyranocoumarins isolated from *Calophyllum* species, are representatives of a class of non-nucleoside HIV-1-specific reverse-transcriptase inhibitors under development as AIDS chemotherapeutic agents.[4]

As part of an ongoing study on species from the Guttiferae,[16–19] we report herein the first phytochemical investigation on *Calophyllum pinetorum* Bisse (Guttiferae), a rare endemic tree growing in Pinar del Rio Province of Cuba and used by local populations as a cicatrisant agent.[20] Three new natural products, a xanthone (**1**) and two pyranochromanone acids (**2** and **3**), were isolated and characterized from the stem bark and leaves of this plant, respectively, along with 10 compounds of previously known structure.



1 **2** **3**

The dried and powered stem bark of *C. pinetorum* was extracted sequentially with *n*-hexane and EtOAc. Both extracts were fractionated by repeated column chromatography on silica gel to obtain four compounds (see Experimental Section). Three of these were identified as the known compounds friedelin,[21] canophyllol,[21] and macluraxanthone[22] on the basis of interpretation of their spectroscopic data and by comparison of their NMR values with those in the literature. The fourth compound was identified as a new xanthone named pinetoxanthone (**1**). The isolate was obtained as a yellow, amorphous solid, and its (+)-HRESIMS showed a pseudomolecular ion at *m*/*z* 381.1695 $[M + H]^+$, indicating a molecular formula of $C_{23}H_{24}O_5$. The UV spectrum exhibited six absorption bands characteristic of a xanthone (λ_{max} 235, 250, 282, 290, 310, and 375 nm). In the ^{1}H NMR spectrum, **1** displayed a chelated hydroxyl signal at δ 13.50. When the ^{1}H−^{1}H COSY spectrum was analyzed, an ABC spin system formed by two double doublets at δ 7.29 (1H, J = 2.4, 7.8 Hz) and 7.70 (1H, J = 2.4, 7.8 Hz) and a triplet at δ 7.25 (1H, J = 7.8 Hz), corresponding to a 1,2,3-trisubstituted benzene ring, was observed. These three mutually coupled aromatic protons were readily assigned to H-6, H-8, and H-7, respectively, from the HMBC correlations evident (H-6/C-5, C-8b; H-7/C-5, C-8a; H-8/C-7, C-9). The ^{1}H NMR spectrum did not show any other aromatic signal, indicating ring A to be completely substituted. The ^{1}H NMR spectrum also exhibited the presence of four methyl groups (δ 1.69, 6H, s and δ 1.76 and 1.84, 3H each, s) and four olefinic protons at δ 5.16, 5.25, 5.31, and 6.66, respectively. The ^{13}C NMR spectrum of **1** showed a carbonyl signal at δ 181.4, 12 aromatic carbon signals, five of which were oxygenated at δ 145.1, 146.2, 151.4, 158.9, and 161.9, and two C_5 groups (Table 1). The aforementioned data were found to be in conformity with a diprenylated pentasubstituted xanthone.[23]

The presence of a 3-methylbut-2-enyl moiety was deduced from the ^{1}H−^{1}H COSY cross-peaks, H-1′ (δ 3.50, 2H, d, J = 6.8 Hz)/H-2′ (δ 5.25, 1H, m), and HMBC correlations H-2′/C-4′ (δ 17.9), C-5′ (δ 25.9), H-4′ (1.84, 3H, s) and H-5′ (1.76, 3H, s)/C-3′ (δ 131.2), C-2′ (δ 123.0). In turn, the two-methyl singlet at δ 1.69 and the coupling pattern (*cis*−*trans*) between the olefinic proton signals at δ 6.66 (dd, J = 17.6 and 10.6 Hz, H-2″), 5.16 (dd, J = 10.6 and 1.2 Hz, H-3″a), and 5.31 (dd, J = 17.6 and 1.2 Hz, H-3″b) suggested the presence of a terminal olefin as a part of a 1,1-dimethylallyl group. Analysis of the 1D and 2D NMR spectra with homo- and heteronuclear direct or long-range correlations allowed the assignment of all ^{1}H and ^{13}C NMR signals (see Experimental Section). The definition of ring A was deduced from the HMBC spectrum. The chelated hydroxyl signal showed HMBC correlations to three quaternary carbon signals at δ 158.9 (C-1), 102.9 (C-2), and 114.3 (C-9a), indicating its attachment to C-1 of the xanthone skeleton. The 3-methylbut-2-enyl moiety was located at C-2 by the key correlations H-1′/C-1, C-3 and H-2′/C-2, whereas the HMBC correlation between H-2″/C-4 confirmed the attachment of the 1,1-dimethylallyl group to C-4. From all these data the structure of **1**, named pinetoxanthone, was assigned as shown. From a structural point of view, pinetoxanthone is very closely related to inoxanthone, a compound isolated from the root bark of *C. inophyllum*.[12]

The dried leaves of *C. pinetorum* were extracted successively with *n*-hexane and EtOAc. Fractionation of the dried residues on silica gel and Sephadex LH-20 columns and final purification by HPLC gave two new pyranochromanone acids, named pinetoric acid I (**2**) and pinetoric acid II (**3**). Friedelin,[21] calophyllic acid,[24] isocalophyllic acid,[24] calolongic acid,[15] apetalic acid,[6] isoapetalic

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Table 1. NMR Data of Compounds **1**−**3** in $CDCl_3$[a]

	pinetoxanthone (**1**)			pinetoric acid I (**2**)			pinetoric acid II (**3**)	
position	δ_C	δ_H (J_{H-H} in Hz)		δ_C	δ_H (J_{H-H} in Hz)		δ_C	δ_H (J_{H-H} in Hz)
1	158.9		2	78.7	4.18 m	2	78.8	4.12 m
2	102.9		3	45.5	2.56 dq (11.0, 7.0)	3	45.7	2.53 dq (11.0, 7.0)
3	151.4		4	199.1		4	199.3	
4	104.9		4a	101.9		4a	101.9	
4a	161.9		5	161.9		5	159.9	
5	146.2		6	110.2		5a	102.2	
6	119.6	7.29 dd (2.4, 7.8)	6a	159.9		6	116.7	6.61 d (10)
7	123.9	7.25 t (7.8, 7.8)	8	77.8		7	125.6	5.47 d (10)
8	116.3	7.70 dd (2.4, 7.8)	9	124.7	5.47 d (9.9)	8	78.1	
8a	121.0		10	115.8	6.55 d (9.9)	9a	157.0	6.55 d (9.9)
8b	145.1		10a	101.1		10	109.9	
9	181.4		10b	155.0		10a	158.2	
9a	114.3		11	19.4	1.51 d (6.4)	11	19.5	1.48 d (6.2)
1′	21.6	3.50 d (6.8)	12	10.1	1.21 d (7.0)	12	10.4	1.20 d (7.0)
2′	123.0	5.25 m	13	30.6	3.70 m	13	30.6	3.68 m
3′	131.2		14	37.7	2.70 dd (7.3, 15.5) 2.88 s (broad)	14	38.5	2.70 dd (7.3, 15.5) 2.82 s (broad)
4′	17.9	1.84 s	15	177.4		15	178.2	
5′	25.9	1.76 s	16	32.9	1.59 overlapped 1.88 m	16	32.9	1.58 overlapped 1.84 m
1″	41.6		17	29.8	1.11 m 1.20 overlapped	17	29.7	1.10 m 1.21 overlapped
2″	154.2	6.66 dd (10.6, 17.6)	18	21.2	1.26 m 1.31 m	18	22.5	1.26 m 1.34 m
3″	106.5	5.16 dd (10.6, 1.2) 5.31 dd (17.6, 1.2)	19	13.6	0.83 t (6.8)	19	14.0	0.84 t (6.8)
4″	28.1	1.69 s	20	28.3	1.44 s	20	28.3	1.44 s
5″	28.1	1.69 s	21	28.2	1.44 s	21	28.4	1.46 s
OH-1		13.50 s	OH-5		12.71 s (broad)	OH-5		12.78 s (broad)

[a] Chemical shift values are in ppm referring to the solvent peak, and *J* values in Hz are presented in parentheses. All signals were assigned by DQF-COSY, HSQC, and HMBC experiments.

acid,[6] amentoflavone,[25] and apigenin[25] were also isolated and identified by comparison of their spectroscopic data with literature values. The NMR and HRESIMS data of **2** indicated a molecular formula of $C_{23}H_{30}O_6$ ([M − H]$^-$ 401.1956) and nine degrees of unsaturation. The UV spectrum exhibited three absorption bands characteristic of a pyranochromanone (λ_{max} 264, 288, 346 nm). The ^{1}H NMR spectrum of **2** (Table 1) showed signals for two tertiary and two secondary methyl groups, one primary methyl group, two olefinic protons, an oxygen-bearing methine proton at δ 4.18 (m, H-2), two methine protons at δ 2.56 (dq, J = 11.0, 7.0 Hz, H-3) and 3.70 (m, H-13), and four pairs of methylene protons. The ^{13}C NMR data of **2** (Table 1) displayed resonances for a carbonyl at δ 199.1, six quaternary aromatic carbons at δ 101.1, 101.9, 110.2, 155.0, 159.9, and 161.9, along with a CH at δ 45.5, a CH-O at δ 78.7, and two CH_3 groups at δ 19.4 and 10.1, attributed to a dimethyl chromanone moiety. The two methyls at δ_H 1.51 (3H, d, J = 6.4 Hz) and 1.21 (3H, d, J = 7.0 Hz) in the ^{1}H NMR spectrum were assigned to H-11 and H-12, attached to C-2 and C-3 of the chromanone moiety. The relative configuration in the 2,3-dimethylchromanone ring was defined by considering the magnitude of the coupling constants of H-2 (m) and H-3 (dq, J = 11.0, 7.0 Hz) protons, which revealed their *trans*-diaxial relationship. Observation of NMR data for the dimethyl chromanone derivatives isolated in this study (**2** and the known compounds calophyllic acid, isocalophyllic acid, calolongic acid, apetalic acid, and isoapetalic acid), with different configurations at C-2 and C-3, revealed several features useful in establishing the relative configuration in the 2,3-dimethylchromanone ring of **2**. First, the methyl groups at C-2 and C-3 showed two ranges of ^{13}C NMR chemical shifts: if they are in a *cis* configuration, as in isocalophyllic acid and apetalic acid, the ranges will be δ 16.0−16.6 for Me-11 and δ 9.1−9.6 for Me-12, whereas if they are in a *trans* configuration, as in compound **2**, calophyllic acid, calolongic acid, and isoapetalic acid, the ranges will be δ 19.4−19.8 for Me-11 and δ 10.1−10.6 for Me-12. Second, the H-3 proton showed $^3J_{H2-H3}$ = 10.5−11.0 when the Me-12 substituent is in an α-position (calophyllic acid, calolongic acid, and isoapetalic acid), whereas the H-3 has $^3J_{H2-H3}$ = 3.0−3.6 Hz when the Me-12 is β-oriented (isocalophyllic acid and apetalic acid).

In the ^{1}H NMR spectrum of **2** the two-methyl singlet at δ 1.44 (6H, s) and the two endocyclic olefinic doublets at δ 5.47 and 6.55 (each 1H, d, J = 9.9 Hz) were attributable to a 2,2-dimethylpyrene ring fused to a benzene ring. This was supported by the ^{13}C NMR data, which revealed a two-methyl signal at δ 28.2 and two olefinic carbons at δ 115.8 and 124.7, together with a quaternary carbon signal at δ 77.8. The HMBC correlations observed between H-9/C-8, C-10a and H-10/C-6a, C-10b indicated the attachment of the dimethylpyrene moiety to C-6a/C-10a. Inspection of the ^{1}H−^{1}H DQF-COSY and 1D-TOCSY spectra of **2** allowed the detection of a distinct spin system connecting H-13 to H_2-14 and H-13 to Me-19 (t, J = 6.8 Hz) and consistent with the presence of a 3-substituted heptanoic acid unit (C-13 to C-19). The assignments of all proton resonances for this unit also allowed the assignment of the resonances of the linked carbon atoms in the HSQC spectrum, while data arising from the HMBC spectrum were used to interconnect the partial structure. Diagnostic correlations were observed between H_2-14/C-6 and C-16, between H_2-16/C-6, C-14, and C-18, between H-13/C-15, C-6a, C-5, and C-17, and between the hydrogen-bonded hydroxyl group at δ 12.7 and C-5, C4a, and C-6, confirming that CH-13 of the heptanoic acid unit is attached to C-6 of the dimethylchromanone moiety. These data suggested that the structure of **2** is similar to calolongic acid, a chromene acid isolated from *C. caledonicum*[10] and *C. brasiliense*,[15] except for the 3-substituted heptanoic acid moiety at C-6 in **2** rather than a hexanoic acid unit.

The isomeric relationship between **2** and **3** was evident from the HRESIMS data, which indicated the same molecular formula of $C_{23}H_{30}O_6$ ([M − H]$^-$ *m*/*z* 401.1960). From the NMR spectroscopic data, identifiable constitutive residues of compound **3** included the 2,3-dimethylchromanone ring, the 2,2-dimethylpyrene ring, and the 3-substituted heptanoic acid unit. The NMR data for **3**, when compared to those of compound **2** (Table 1), showed slight chemical shift differences only for the three oxygenated carbons of the aromatic ring (C-5, C-9a, C-10a), while the carbon connectivity of **3**, established by COSY, HMQC, and HMBC experiments, was found to be different from that of **2**. In the HMBC spectrum, the chelated hydroxyl group (δ 12.78) was correlated to the quaternary carbons at δ 101.9 (C-4a), 159.9 (C-5), and 102.2

(C-5a). The latter resonance at δ 102.2 also gave cross-peaks with one of the *cis*-olefinic protons of the chromene ring at δ 5.47 (H-7), while the other *cis*-olefinic proton at δ 6.61 (H-6) was correlated with the quaternary carbon at δ 159.9 (C-5). These results demonstrated clearly that the *gem*-dimethylpyrene moiety was fused in a linear manner to the aromatic ring of the pyranochromanone skeleton bearing the chelated hydroxyl group. Other correlations were also in agreement with the structure **3**. These findings indicated that the new pinetoric acid II (**3**) is related to isoapetalic acid, except for the 3-substituted heptanoic acid moiety at C-10 instead of a hexanoic acid unit. The 3-substituted hexanoic acid unit has been found previously in apetalic acid, isoapetalic acid, calolongic acid, isocalolongic acid, recedensic acid, brasiliensic acid, isobrasiliensic acid, and inocalophyllin B,[26] while the 3-substituted heptanoic acid unit has not been previously reported.

Experimental Section

General Experimental Procedures. 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 Biorad FTS 155 FT-IR spectophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 MHz for ^{13}C, using the UXNMR software package, was used for NMR experiments in $CDCl_3$. Chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ_H 7.27 and δ_C 77.0 for $CDCl_3$, with coupling constants, *J*, in hertz. ^{1}H−^{1}H DQF-COSY, TOCSY, ^{1}H−^{13}C HSQC, and HMBC experiments were obtained using conventional pulse sequences. The selective excitation spectra, 1D TOCSY, were acquired using waveform generator-based GAUSS-shaped pulses, with mixing times ranging from 100 to 120 ms, and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse. Electrospray ionization mass spectrometry (ESIMS) was performed using a LCQ Advantage ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with Xcalibur 3.1 software. Exact masses were measured by a Q-TOF premier (Waters, Milford, MA) high-resolution mass spectrometer. Column chromatography was performed over Sephadex LH-20 (1 m × 3 cm i.d.; Pharmacia, Uppsala, Sweden) and silica gel 60 (0.040−0.063 mm; Carlo Erba, Italy). HPLC separations were performed employing two procedures: method a and method b. Method a was performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Waters 10 μm μ-Bondapak C_{18} column (300 × 7.8 mm). Method b was carried out on an Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328a Rheodyne injector (20 μL loop), a G-1322A degasser, and a G-1315A photodiode array detector (PDA), equipped with a Kromasil 10 μm C_{18} column (300 × 10 mm). The elution solvents used were water and methanol. The flow rate was 1 mL/min, and PDA data were recorded with a 200−600 nm range with two preferential channels as the detection wavelength, 254 and 278 nm.

Plant Material. The stem bark and the leaves of *Calophyllum pinetorum* Bisse were collected separately in the Botanic Garden of the University of Havana, in October 2005. Plant materials were identified by Dr. C. Victor Fuentes Fiallo. A voucher specimen has been deposited at HAJB Herbarium (Havana, Cuba) under number 84364.

Extraction and Isolation. The dried and powered stem bark of *C. pinetorum* (472 g) was extracted successively with *n*-hexane (2 L) and EtOAc (2 L) for 7 days each. After concentration, 5.2 g of *n*-hexane extract and 21.0 g of EtOAc extract were obtained, respectively. Part of the *n*-hexane (1 g) extract was fractioned by vacuum-liquid chromatography (VLC) on silica gel G, eluted with *n*-hexane, using a gradient of EtOAc (0−100%), followed by EtOAc−MeOH mixtures, to yield 22 fractions (A1−A22). Fractions A2 and A9 yielded friedelin (146.7 mg) and macluraxanthone (23.0 mg), respectively. Part of the EtOAc extract (2 g) was fractioned by VLC (silica gel G) employing *n*-hexane, EtOAc, and MeOH as solvents to afford 11 fractions (B1−B11). From fractions B1 and B4 were obtained friedelin (84.2 mg) and canophyllol (160.1 mg) by crystallization procedures, respectively. Fraction B6 was chromatographed over silica gel by VLC with EtOAc and MeOH as solvents to yield macluraxanthone (18.3 mg) and pinetoxanthone (**1**, 1.6 mg).

The dried and powdered leaves (600 g) of *C. pinetorum* were extracted in the same manner to give *n*-hexane (11.2 g) and EtOAc (21.0) extracts. Part of the dried *n*-hexane extract (2 g) was subjected to column chromatography over silica gel using 100% *n*-hexane to 100% EtOAc in 10% stepwise elutions and then 100% EtOAc to 100% MeOH in 5% stepwise elutions and afforded 22 fractions (C1−C22). Further purification of fraction C7 by VLC (silica gel G) with *n*-hexane and EtOAc as eluents gave friedelin (186.3 mg). Fraction C10 was chromatographed over silica gel and HPLC (method b) to yield calophyllic acid (28.6 mg) and isocalophyllic acid (49.1 mg). Further separation of fraction C11 by VLC (silica gel G), Sephadex LH-20 column chromatography (100 × 5 cm) using methanol as solvent, and HPLC (method a) yielded pinetoric acid I (**2**, 3.0 mg), pinetoric acid II (**3**, 3.1 mg), calolongic acid (2.5 mg), apetalic acid (5.8 mg), and isoapetalic acid (3.3 mg). Part of the EtOAc extract (2 g) was separated on silica gel G by VLC with EtOAc and MeOH as solvents to afforded 17 fractions (D1−D17). From D3 was obtained friedelin (61 mg), and D11 was chromatographed over a silica gel column with EtOAc and MeOH as eluents to yield amentoflavone (66 mg) and apigenin (4.6 mg).

Pinetoxanthone (1): yellow, amorphous solid; UV (MeOH) λ_{max} (log ϵ) 235 (4.15), 250 sh, 282 sh, 290 (4.89), 310 sh, 375 (3.27) nm; IR (KBr) ν_{max} 3423, 3290, 2959, 2910, 1640, 1624, 1580 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (+)-HRESIMS *m/z* 381.1695, calcd for $C_{23}H_{25}O_5$, 381.1701.

Pinetoric acid I (2): yellow, amorphous solid; $[\alpha]^{30}_D$ −30.3 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 264 (4.57), 288 (4.36), 346 (4.16) nm; IR (KBr) ν_{max} 3310, 3290, 1710, 1660 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS (negative mode) *m/z* 401 [M − H]$^-$; MS/MS *m/z* 383, 357, 319, 273; (positive mode) *m/z* 403 [M + H]$^+$, *m/z* 385, 343, 287; (−)-HRESIMS *m/z* 401.1956, calcd for $C_{23}H_{29}O_6$, 401.1204.

Pinetoric acid II (3): yellow, amorphous solid; $[\alpha]^{30}_D$ −24.0 (*c* 1.0, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 266 (4.51), 280 (4.31), 346 (4.14) nm; IR (KBr) ν_{max} 3300, 3290, 1710, 1664 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS (negative mode) *m/z* 401 [M − H]$^-$; MS/MS *m/z* 383, 357, 319, 273; (positive mode) *m/z* 403 [M + H]$^+$, *m/z* 385, 343, 287; (−)-HRESIMS *m/z* 401.1960, calcd for $C_{23}H_{29}O_6$, 401.1204.

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