Chromones from the Endophytic Fungus *Pestalotiopsis* sp. Isolated from the Chinese Mangrove Plant *Rhizophora mucronata*

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Six new chromones, named pestalotiopsones A−F (**1**−**6**), and the known derivative 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone (**7**) were obtained from the mycelia and culture filtrate of the mangrove endophytic fungus *Pestalotiopsis* sp., which was isolated from leaves of the Chinese Mangrove plant *Rhizophora mucronata*. Their structures were elucidated on the basis of spectroscopic data. Pestalotiopsones A−F are chromones having both an alkyl side chain substituted at C-2 and a free or substituted carboxyl group at C-5. Compound **6** exhibited moderate cytotoxicity against the murine cancer cell line L5178Y, whereas the other investigated compounds proved to be inactive.

Fungi of the genus *Pestalotiopsis* are known as endophytes of tropical higher plants.[1−3] Previous chemical investigations of marine endophytes afforded a variety of bioactive natural products such as polyketides and terpenoids.[4−12] During our ongoing search for new bioactive metabolites from plant endophytes,[13,14] six new chromones, named pestalotiopsones A−F (**1**−**6**), in addition to 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone (**7**), a previously known metabolite from medicinal rhubarb *Rhei Rhizoma* (*Rheum officinale*),[15] were obtained from endophytic fungus *Pestalotiopsis* sp., which had been isolated from leaves of the Chinese Mangrove plant *Rhizophora mucronata* and cultured on solid rice medium. Their structures were elucidated on the basis of spectroscopic data. Pestalotiopsones A−F are chromones featuring both an alkyl side chain substituted at C-2 and a free or substituted carboxyl group at C-5. This is the first report of chromones from the genus *Pestalotiopsis*. Details of the isolation, structure elucidation, and biological activity of these chromones are reported herein.

Results and Discussion

The mycelia and culture medium of the fungus *Pestalotiopsis* sp. were extracted with ethyl acetate. The resulting ethyl acetate extract was dried and chromatographed on silica gel and Sephadex LH-20 followed by preparative HPLC to yield pestalotiopsones A−F (**1**−**6**) and 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone (**7**).[15] Pestalotiopsones A−F were identified as 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone (**1**), 5-carboethoxymethyl-2-heptyl-7-hydroxychromone (**2**), 5-carboxymethyl-2-heptyl-7-hydroxychromone (**3**), 5-carbomethoxymethyl-7-hydroxy-2-(6-hydroxyheptyl)chromone (**4**), 5-carboethoxymethyl-7-hydroxy-2-(6-hydroxyheptyl)chromone (**5**), and 5-carbomethoxymethyl-7-hydroxy-2-pentylchromone (**6**).

Pestalotiopsone A (**1**), a colorless amorphous solid, has the molecular formula $C_{19}H_{24}O_5$ established by HR-ESIMS (*m*/*z* 333.1697). Consequently, **1** had eight degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **1** (Tables 1 and 2) indicated that six of the eight units of unsaturation come from four carbon−carbon double bonds and two carbonyls. Therefore, the other two units of unsaturation come from two rings. The UV absorption maxima at 219, 243, and 250 nm indicated that **1** should be a chromone derivative. DEPT experiments showed that the compound had two methyl groups, including a methoxy and a terminal alkyl methyl, seven methylenes, three olefinic methines, and seven quaternary carbons, including two carbonyls. The ^{1}H and ^{13}C NMR data of **1** (Tables 1 and 2) and its ^{1}H−^{1}H COSY and HSQC spectra showed the presence of a methoxy substituent (δ_H 3.60, s, δ_C 52.6, CH_3), an olefinic methine (δ_H 5.92, s, δ_C 111.9, CH-3), a methylene connecting a phenyl ring and a carboxyl group (δ_H 4.12, s, δ_C 41.9, CH_2-1″), two *meta*-coupled aromatic methines [δ_H 6.75 (d, J = 2.2 Hz), δ_C 119.6, CH-6; δ_H 6.81 (d, J = 2.2 Hz), δ_C 103.8,

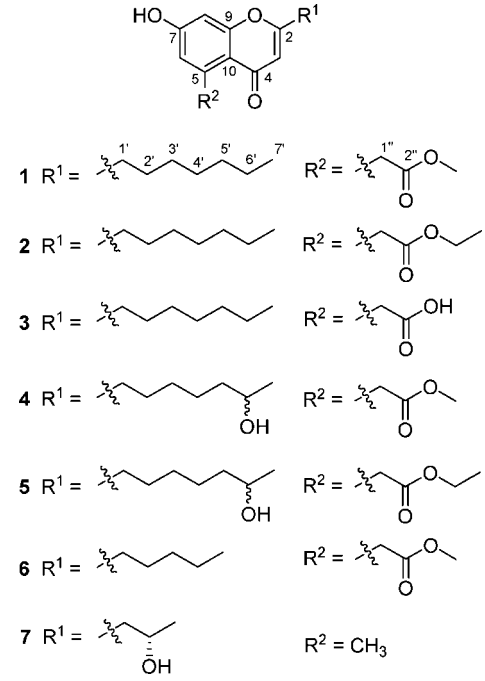

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Table 1. ^{1}H NMR (500 MHz) Spectroscopic Data for Pestalotiopsones A−F (**1**−**6**) and 5-Carbomethoxymethyl-2-heptyl-7-hydroxychromone (**7**)

position	**1**[a]	**2**[a]	**3**[b]	**4**[a]	**5**[a]	**6**[a]	**7**[b]
3	5.92, s	5.93, s	6.03, s	5.92, s	5.92. s	5.92, s	5.97, s
6	6.75, d (2.2)	6.78, d (2.2)	6.71, s	6.73, s	6.74, d (2.2)	6.73, s	6.47, s
8	6.81, d (2.2)	6.84, d (2.5)	6.78, s	6.81, s	6.82, d (2.2)	6.80, s	6.50, s
1′	2.59, t (7.5)	2.62, t (7.6)	2.63, t (7.5)	2.59, dt (7.5, 2.5)	2.59, dt (7.5, 2.5)	2.58, t (7.3)	2.65, m
2′	1.73, m	1.74, m	1.74, m	1.73, m	1.73, m	1.72, m	4.17, m
3′	1.35−1.45, m	1.22−1.43, m	1.35−1.45, m	1.36−1.47, m	1.36−1.47, m	1.28−1.49, m	1.25, d (6.3)
4′	1.35−1.45, m	1.22−1.43, m	1.35−1.45, m	1.36−1.47, m	1.36−1.47, m	1.28−1.49, m	
5′	1.31, m	1.33, m	1.32, m	1.36−1.47, m	1.36−1.47, m	0.9, brs	
6′	1.31, m	1.33, m	1.32, m	3.70, m	3.70, m		
7′	0.88, t (6.5)	0.90, t (7.0)	0.96, t (6.5)	1.11, d (6.1)	1.11, d (6.1)		
1″	4.12, s	4.14, s	4.12, s	4.12, s	4.10, s	4.10, s	2.66, s
2″-OCH_3	3.60, s			3.60, s		3.59, s	
2″-$OC\underline{H_2}CH_3$		4.10, q (7.1)			4.07, q (7.1)		
2″-$OCH_2C\underline{H_3}$		1.24, t (7.1)			1.20, t (7.1)		

[a] In acetone-d_6. [b] In methanol-d_4.

Table 2. ^{13}C NMR (125 MHz) Spectroscopic Data for Pestalotiopsones A and C−E

no.	**1**[a]	**3**[b]	**4**[a]	**5**[a]
2	169.3, qC	170.5, qC	170.0, qC	170.0, qC
3	111.9, CH	110.6, CH	112.0, CH	112.0, CH
4	181.0, qC	181.6, qC	181.0, qC	181.0, qC
5	139.8, qC	139.3, qC	140.0, qC	140.0, qC
6	119.6, CH	119.5, CH	120.0, CH	120.0, CH
7	162.8, qC	163.3, qC	162.8, qC	162.8, qC
8	103.8, CH	102.9, CH	104.0, CH	104.0, CH
9	161.4, qC	161.4, qC	161.0, qC	161.0, qC
10	117.2, qC	115.8, qC	117.0, qC	117.0, qC
1′	35.1, CH_2	34.6, CH_2	35.0, CH_2	35.0, CH_2
2′	28.5, CH_2	27.9, CH_2	29.0, CH_2	29.0, CH_2
3′	30.5, CH_2	30.1, CH_2	30.5, CH_2	30.5, CH_2
4′	30.5, CH_2	30.1, CH_2	30.5, CH_2	30.5, CH_2
5′	33.0, CH_2	32.9, CH_2	41.0, CH_2	41.0, CH_2
6′	24.3, CH_2	23.7, CH_2	68.0, CH	68.0, CH
7′	15.3, CH_3	14.4, CH_3	23.5, CH_3	23.5, CH_3
1″	41.9, CH_2	42.1, CH_2	42.0, CH_2	42.0, CH_2
2″	172.8, qC	176.2, qC	172.5, qC	172.5, qC
2″-OCH_3	52.6, CH_3		52.6, CH_3	
2″-$O\underline{C}H_2CH_3$				61.0, CH_2
2″-$OCH_2\underline{C}H_3$				14.5, CH_3

[a] In acetone-d_6. [b] In methanol-d_4.

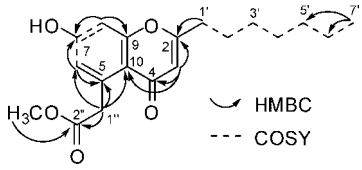

Figure 1. Selected HMBC and ^{1}H−^{1}H COSY correlations of pestalotiopsone A (**1**).

CH-8], and a seven-membered alkyl chain (CH_2-1′ to CH_3-7′). Comparison of the ^{1}H and ^{13}C NMR data of **1** with those of 2-methyl-5-carboxymethyl-7-hydroxychromone[15] revealed that both had the same chromone core. The strong HMBC correlation (Figure 1) from H-1′ (δ 2.59) to C-2 (δ 169.3) revealed that the seven-membered alkyl side chain was attached to C-2 of the chromone core. Moreover, HMBC correlations (Figure 1) from the protons of the methyl ester group (δ 3.60, s) and H_2-1″ (δ 4.12, s) to C-2″ (δ 172.8), combined with that from H_2-1″ (δ 4.12, s) to C-5 (δ 139.8), disclosed the presence of the CH_2COOCH_3 group at C-5. This finding was further supported by the fragments *m*/*z* 301 and 273 observed in the positive ESIMS (Figure 2) that originate from the subsequent loss of methanol and of carbon monoxide. In addition, the shift of C-7 (δ 162.8) in the ^{13}C NMR spectrum revealed that this carbon was oxygenated. The attachment of a hydroxyl group to C-7 was deduced from the molecular formula of compound **1**. On the basis of the above results, the structure of pestalotiopsone A (**1**) was identified as 5-carbomethoxymethyl-2-heptyl-7-hydroxychromone.

Pestalotiopsone B (**2**) was found to have the molecular formula $C_{20}H_{26}O_5$ (i.e., differing from that of **1** by an additional CH_2 group), which was established by HR-ESIMS (*m*/*z* 347.1860). The ^{1}H NMR data of **2** were similar to those of **1**. This suggests that **2** might have the same basic molecular framework as **1**. However, the signal of the methyl ester group of **1** was absent in the ^{1}H NMR spectrum of **2**. Instead, signals of an ethoxy group (δ_H 1.24, t, J = 7.1 Hz; δ_H 4.10, q, J = 7.1 Hz) appeared, indicating that an ethoxy group had replaced the methoxy substituent of **1**. Confirming evidence was obtained from the ^{1}H−^{1}H COSY correlation and from characteristic ESIMS fragments. The presence of the ethoxy group was corroborated by the ^{1}H−^{1}H COSY correlation from the protons of the methyl group (δ_H 1.24, t, J = 7.1 Hz) to the oxygenated methylene (δ_H 4.10, q, J = 7.1 Hz). Connection to the carbonyl C-2″ was established by fragments *m*/*z* 301 and 273 in the positive ESIMS of **2** (Figure 2) that originate from a sequential loss of one molecule of ethanol and of carbon monoxide. Therefore, the structure of pestalotiopsone B was characterized as 5-carboethoxymethyl-2-heptyl-7-hydroxychromone.

The molecular formula of pestalotiopsone C (**3**) was determined as $C_{18}H_{22}O_5$ by HR-ESIMS (*m*/*z* 319.1540). The ^{1}H and ^{13}C NMR data of **3** were similar to those of **1** except for the signals of the OCH_3 substituent within **1**, which were absent in **3**. Instead, compound **3** featured a free carboxyl group, as established by the ESIMS fragments *m*/*z* 301 and 273 (Figure 2), which originated from the subsequent loss of one molecule of water and of carbon monoxide. Thus, pestalotiopsone C was identified as 5-carboxymethyl-2-heptyl-7-hydroxychromone.

The molecular weight of pestalotiopsone D (**4**) was 16 mass units larger than that of **1**, suggesting the presence of an additional hydroxyl group. The ^{1}H and ^{13}C NMR data of **4** clearly indicated that this hydroxyl group was attached at C-6′. Supporting evidence for this assignment was obtained from the downfield chemical shifts of H-6′ (δ 3.70, m) and C-6′ (δ 68.0, CH) in **4** (δ_H 1.32, m for H-6′ and δ_C 24.3, CH_2 for C-6′ in **1**, respectively). Moreover, the presence of a hydroxyl group at C-6′ was corroborated by the observed ^{1}H−^{1}H COSY correlation from the doublet H_3-7′ to H-6′. Therefore, the structure of pestalotiopsone D was characterized as 5-carbomethoxymethyl-7-hydroxy-2-(6-hydroxyheptyl)chromone.

The molecular weight of pestalotiopsone E (**5**) was 14 mass units larger than that of **4**, indicating the presence of an additional CH_2 group in **5**. The ^{1}H and ^{13}C NMR data of **5** were similar to those of **4**. However, the proton and carbon signals of the methoxy group in **4** were absent in **5**. Instead, the signals of an ethoxy group [δ_H 1.20 (t, J = 7.1 Hz), 4.07 (q, J = 7.1 Hz); δ_C 61.0, CH_2, 14.5, CH_3] appeared, indicating that an ethyl substituent had replaced the methyl group of **4**. The presence of the ethoxy group was further corroborated by the ^{1}H−^{1}H COSY correlations from the protons

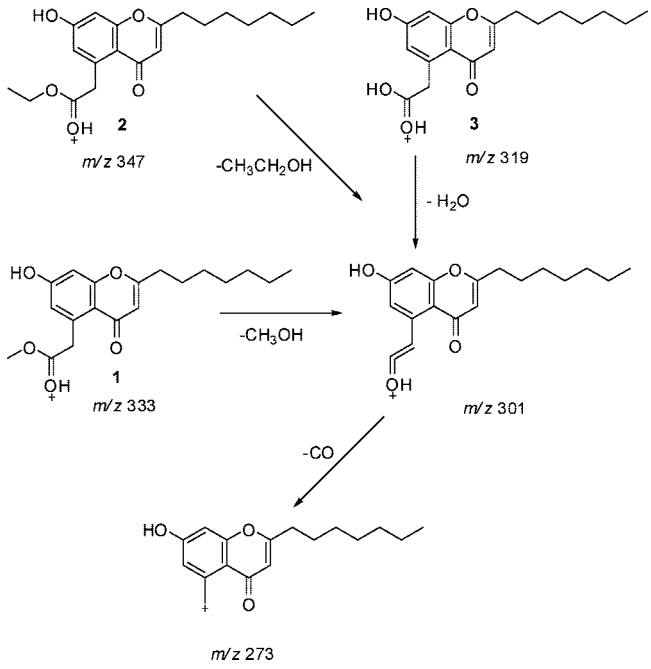

Figure 2. ESIMS fragments of compounds **1**−**3**.

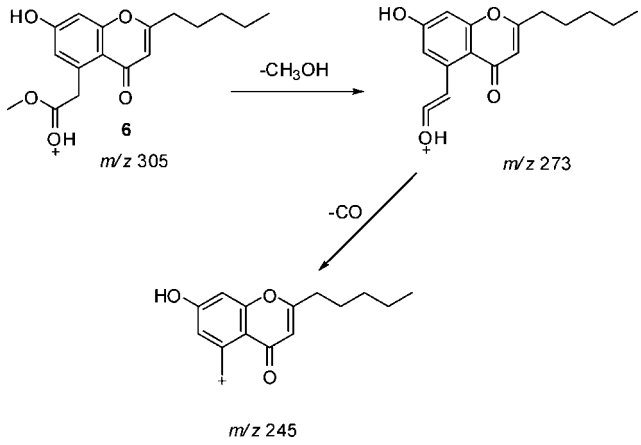

Figure 3. ESIMS fragments of compound **6**.

of the methyl group (δ_H 1.20, t, J = 7.1 Hz) to the oxygenated methylene (δ_H 4.07, q, J = 7.1 Hz). The connection of the latter to the carbonyl carbon C-2″ was established by the HMBC correlations from the CH_2 group to C-2. On the basis of these findings, the structure of pestalotiopsone E was identified as 5-carboethoxymethyl-7-hydroxy-2-(6-hydroxyheptyl)chromone.

Pestalotiopsone F (**6**) was found to have the molecular formula $C_{17}H_{20}O_5$ (differing from that of **1** by the loss of a C_2H_4 unit), which was established by HR-ESIMS (*m/z* 305.1384). Comparison of its ^{1}H NMR data to those of **1** revealed that the substituents at C-5 for both compounds were the same. Therefore, the alkyl side chain at C-2 in **6** differed from that of **1** by loss of a C_2H_4 unit. Therefore, the structure of pestalotiopsone F was characterized as 5-carbomethoxymethyl-7-hydroxy-2-pentylchromone.

Compounds **1**−**7** were evaluated for their cytotoxicity against the murine cancer cell line L5178Y.[13] Pestalotiopsone F (**6**) exhibited moderate cytotoxicity with an EC_{50} value of 8.93 μg/mL. Compounds **1**−**5** and **7** had no cytotoxic activity.

Pestalotiopsones A−F are new chromones featuring both an alkyl side chain substituted at C-2 and a free or esterified carboxyl group at C-5. To our knowledge, these compounds belong to a rare subtype of chromones found in nature. Previously reported examples include 5-acetonyl-7-hydroxy-2-methylchromone and 5-carboxymethyl-7-hydroxy-2-methylchromone from medicinal rhubarb *Rhei Rhizoma* (*Rheum officinale*)[15] and phaeochromycins D and E isolated from the soil actinomycete *Streptomyces phaeochromogenes*.[17] The above findings demonstrate that the Mangrove-derived endophytic fungus *Pestalotiopsis* sp. is a promising source for new natural products.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a Perkin-Elmer model 341 LC polarimeter. UV spectral data were obtained from online UV spectra measured by photodiode array detection (Gynkotek, Germany). ^{1}H and ^{13}C NMR (chemical shifts in ppm) spectra were recorded on Bruker ARX 600 or DRX 500 NMR spectrometers in acetone-d_6 or methanol-d_4. ESIMS spectra were recorded on a Finnigan MAT TSQ 7000 mass spectrometer. High-resolution ESIMS were recorded on a Micromass Q-Tof-2 mass spectrometer using peak matching.

Isolation and Cultivation of the Fungus. *Pestalotiopsis* sp. was isolated from fresh healthy leaves of *Rhizophora mucronata* (Rhizophoraceae) collected in October 2005 in Dong Zhai Gang-Mangrove Garden on Hainan Island, China. The fungus (strain no. JCM2A4) was isolated under sterile conditions from the inner tissue of the twigs following an isolation protocol, cultured on solid rice medium as described previously,[13] and identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region (GenBank accession no. FJ465172).[18] A voucher strain was deposited at one of the authors' laboratory (P.P.). Mass growth of the fungus for the isolation and identification of new metabolites was carried out in Erlenmeyer flasks (1 L each). The fungus was grown on rice solid medium (to 100 g commercially available rice was added 110 mL of distilled water and kept overnight prior to autoclaving, 2 flasks) at room temperature under static conditions and daylight for 40 days.

Extraction and Isolation. The mycelia and solid rice medium were extracted with EtOAc. The extract was evaporated under reduced pressure to yield 3.0 g of residue. This residue was subjected to vacuum liquid chromatography (VLC) on a short silica gel column employing a step gradient of dichloromethane/methanol. Thirty-seven fractions were collected and examined by TLC on premade silica Si 60 F_{254} (Merck, Germany) using a dichloromethane/methanol-based solvent system to yield eight fractions, F1−F8. Moreover, each fraction obtained was analyzed by HPLC using a reversed-phase column and employing a linear gradient of methanol and water (adjusted to pH 2.0 by addition of phosphoric acid). Promising fraction F2 (38 mg) was subjected to further chromatographic separation using Sephadex LH-20 with methanol as solvent. Subfraction F2-3 (7.77 mg) was further purified by semipreparative reversed-phase HPLC (MeOH/H_2O (7:3), 5 mL/min) to obtain compounds **1** (1.18 mg) and **6** (1.0 mg). F4 (89.7 mg) was processed in the same way as F2 on Sephadex LH-20 using methanol as eluent to afford compound **4** (1.08 mg). Compounds **2** (0.67 mg), **5** (0.26 mg), and **7** (0.19 mg) were isolated from subfraction F4-1 (13.7 mg) by semipreparative reversed-phase HPLC (MeOH/H_2O (5:4), 5 mL/min). F7 (29.7 mg) was purified on an ODS column eluting with MeOH/H_2O (4:1) to furnish compound **3** (2.67 mg).

Cytotoxic Assay. Cytotoxic activity was evaluated against L5178Y (mouse lymphoma cell line) by the MTT method.[16] The cell line was grown in RPMI-1640 culture medium with Na-carbonate (pH 7.2) supplemented with 10% FCS under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. An aliquot (180 μL) of these cell suspensions at a density of 1500 cell mL^{-1} was pipetted into 96-well microtiter plates. Subsequently, 180 μL of the test compounds (in DMSO) at different concentrations was added to each well and incubated for 72 h at the above conditions in a CO_2 incubator. MTT solution (20 μL of 5 mg/mL in RPMI-1640 medium) was added to each well and further incubated for 3 h. After addition of 100 μL of DMSO and incubation for 1 h, the cells were lysed to liberate the formed formazan crystals. Absorbance was then determined on a Multiscan plate reader at 595 nm. As negative controls, media with 0.1% ethylene glycol monomethyl ether (EGMME)/DMSO were included in all experiments.

Pestalotiopsone A (1): colorless, amorphous residue (MeOH); UV (MeOH) λ_{max} 219, 243, 250 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESIMS *m/z* 333.1697 [M + H]$^+$ (calcd for $C_{19}H_{25}O_5$, 333.1702).

Pestalotiopsone B (2): colorless, amorphous residue (MeOH); UV (MeOH) λ_{max} 220, 242, 250 nm; ^{1}H NMR data, see Table 1; HR-ESIMS *m*/*z* 347.1860 $[M + H]^+$, *m*/*z* 369.1678 $[M + Na]^+$ (calcd for $C_{20}H_{27}O_5$, 347.1859).

Pestalotiopsone C (3): colorless, amorphous residue (MeOH); UV (MeOH) λ_{max} 222, 243, 250 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESIMS *m*/*z* 319.1540 $[M + H]^+$, *m*/*z* 341.1359 $[M + Na]^+$ (calcd for $C_{18}H_{23}O_5$, 319.1545).

Pestalotiopsone D (4): colorless, amorphous residue (MeOH); $[\alpha]^{20}_D$ +16 (*c* 0.02, MeOH); UV (MeOH) λ_{max} 210, 242, 250 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESIMS *m*/*z* 349.1646 $[M + H]^+$ (calcd for $C_{19}H_{25}O_6$, 349.1651).

Pestalotiopsone E (5): colorless, amorphous residue (MeOH); $[\alpha]^{20}_D$ +37 (*c* 0.02, MeOH); UV (MeOH) λ_{max} 208, 242, 250 nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively; HR-ESIMS *m*/*z* 363.1802 $[M + H]^+$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

Pestalotiopsone F (6): colorless, amorphous residue (MeOH); UV (MeOH) λ_{max} 225, 243, 250 nm; ^{1}H NMR data, see Table 1; HR-ESIMS *m*/*z* 305.1384 $[M + H]^+$ (calcd for $C_{17}H_{21}O_5$, 305.1389).

5-Carbomethoxymethyl-2-heptyl-7-hydroxychromone (7):[15] colorless, amorphous residue (MeOH); $[\alpha]^{20}_D$ +32 (*c* 0.02, MeOH); UV (MeOH) λ_{max} 227, 244, 251 nm; ^{1}H NMR data, see Table 1; HR-ESIMS *m*/*z* 235.0965 $[M + H]^+$ (calcd for $C_{13}H_{15}O_4$, 235.0970).

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