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Diversity of Antimicrobial Pyrenophorol Derivatives from an Endophytic Fungus, *Phoma* sp.[‡]

Wen Zhang,[a,b] Karsten Krohn,*[b] Hans Egold,[b] Siegfried Draeger,[c] and Barbara Schulz[c]

Dedicated to Professor Dr. Gerald Henkel on the occasion of his 60th birthday

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Pyrenophorol (**1**) and (–)-dihydropyrenophorin (**3**), two known macrodiolides, were isolated together with four new analogues (**2**, **4**–**6**) and three ring-opened derivatives (**7**–**9**) from *Phoma* sp., an endophytic fungus isolated from *Lycium intricatum* from Gomera. The structures of the new compounds were elucidated by detailed spectroscopic analysis, comparison with reported data, and chemical interconversion. The absolute configurations were determined by chemical correlations and a modified Mosher's method. The diversity of these seven newly discovered metabolites not only extends the pyrenophorol macrocyclic family, but also gives insight into the biosynthetic interconnections, in particular by isolation of the open-chain precursors. Preliminary studies showed antimicrobial activity of these compounds against the fungus *Microbotryum violaceum*, the alga *Chlorella fusca*, and the bacteria *Escherichia coli* and *Bacillus megaterium*.

Introduction

Naturally occurring macrodiolides, which can be isolated from various fungi and marine sponges, are an interesting family of secondary metabolites with pronounced biological activity. A prominent example from marine sponges is swinholide A, possessing a large 44-membered ring structure.[1] The macrodiolides can roughly be divided into two groups: They form either homodimers, constructed of two identical monomeric units, or heterodimers with two different subunits. Examples with a symmetrical 16-membered ring system are pyrenophorol (**1**),[2–7] vermiculine,[8–10] and elaiophylin;[11–13] representatives of heterodimeric 14-membered rings include colletodiol[14–18] and grahamimycin A_1.[19] Within the homodimers, pyrenophorol (**1**) and analogues such as pyrenophorin (**10**)[20–23] or (–)-dihydropyrenophorin (**3**)[24] represent the simplest group within the cluster of 16-membered macrocyclic dilactones. A typical arrangement of these naturally occurring diolide 16-membered rings is the head-to-tail connection of the two C_8 hydroxy acid subunits. Many of these diolides show strong antifungal,[2,7,22] antihelmintic,[4,6,25] or phytotoxic activity.[5,23,24] This broad spectrum of bioactivity and the unique structure of pyrenophorol (**1**) and its analogues have also attracted great attention from synthetic chemists.[26–44]

As part of our continuing investigations to elucidate new bioactive metabolites from fungi, we previously analyzed the culture extract of an endophytic *Phoma* sp., isolated from the plant *Fagonia cretica*. This work resulted in the isolation of tetrahydropyrenophorol, a hydrogenated pyrenophorol derivative.[7] The structure of the tetrahydropyrenophorol was unambiguously confirmed by X-ray single-crystal structure analysis with its absolute configuration solved by the solid-state CD/TDDFT method.[7] In connection with our ongoing screening for biologically active secondary metabolites from fungi,[45] we have now investigated another endophytic *Phoma* sp. (internal strain no. 8874) isolated from *Lycium intricatum*, a Mediterranean plant from the island of Gomera, Spain. The crude ethyl acetate extract of the biomalt agar culture showed good antifungal activity against *Microbotryum violaceum* and moderate algicidal activity against *Chlorella fusca*. Fractionation of the ethyl acetate extract led to the isolation and structural determination of known pyrenophorol (**1**) and (–)-dihydropyrenophorin (**3**), together with four new analogues (**2**, **4**–**6**) and three novel ring-opened derivatives (**7**–**9**) (Figure 1). The four new analogues are 4-acetylpyrenophorol (**2**), 4′-acetyldihydropyrenophorin (**4**), *cis*-dihydropyrenophorin (**5**), and tetrahydropyrenophorin (**6**), while the three ring-opended deriv-

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[a] Research Center for Marine Drugs, School of Pharmacy, Second Military Medical University, 325 Guo-He Road, Shanghai 200433, P. R. China
[b] Department of Chemistry, Universität Paderborn, Warburger Straße 100, 33098 Paderborn, Germany
Fax: +49-5251-603245
E-mail: k.krohn@uni-paderborn.de
[c] Institut für Mikrobiologie, Technische Universität Braunschweig, Spielmannstraße 7, 31806 Braunschweig, Germany

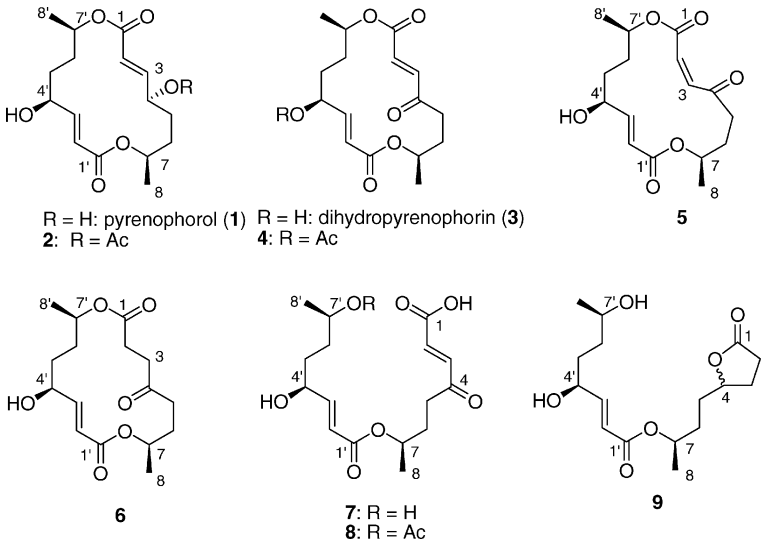


Figure 1. Structures **1**–**9** isolated from *Phoma* sp.

atives are structurally correlated to (–)-dihydropyrenophorin (**3**) and named *seco*-dihydropyrenophorin (**7**), 7′-acetyl-*seco*-dihydropyrenophorin (**8**), and *seco*-dihydropyrenophorin-1,4-lactone (**9**). We herein report on the isolation, structural elucidation, and bioactivities of these compounds.

Results and Discussion

The fungus *Phoma* sp. was cultivated on biomalt agar medium for four weeks, and then extracted with ethyl acetate. The crude extract was fractionated on a silica gel column, followed by Sephadex LH-20 column chromatography to yield pure compounds **1**, **4**, **7**, **8**, and mixtures containing compounds **2**, **3**, **5**, **6** and **9**, respectively. Further silica column chromatography gave compounds **2**, **3**, **5**, **6** and **9** in pure form. The structures were elucidated by spectroscopic analysis in combination with chemical interconversions.

The structure of pyrenophorol (**1**), the major metabolite of the title fungus, was determined by detailed spectroscopic analysis and comparison with reported data. Pyrenophorol (**1**) was previously isolated from the fungus *Byssochlamys nivea*,[2] and subsequently found in *Stemphylium radicinum*,[3] *Alternaria alternata*,[4] *Drechslera avenae*,[5] *Byssochlamys nivea*,[6] and *Phoma* sp.[7] The relative stereochemistry of pyrenophorol (**1**) was confirmed by X-ray diffraction analysis.[6] Its absolute configuration was determined by enantioselective syntheses[42,43] and also by the synthesis of *ent*-**1**.[44] (–)-Dihydropyrenophorin (**3**) had been previously isolated from *Drechslera avenae*.[24] The structure was elucidated by spectroscopic analysis and oxidation to the diketone pyrenophorin (**10**) by using Jones' reagent. Pyrenophorin (**10**) is a good antifungal and herbicidal agent and has been isolated from *Pyrenophora avenae*,[20] *Stemphylium radicinum*,[21,22] and *Drechslera avenae*.[23]

Interestingly, some ^{1}H NMR signals of pyrenophorol (**1**) vary considerably with the concentrations above 20 mg/mL, whereas the ^{13}C NMR signals remain virtually unchanged. When the concentration is increased from 20 to 60 mg/mL, a noticeable upfield shift of the ^{1}H NMR signals is observed, probably due to intermolecular interactions at higher concentration (Table 1).

Compound **2** was isolated as an optically active, colorless oil. The molecular formula $C_{18}H_{26}O_7$ was established by HRESIMS. The IR spectrum of **2** indicated the presence of a hydroxy group (3603 cm^{-1}) and an α,β-unsaturated ester group (1717, 1244, 1134 cm^{-1}). The presence of α,β-unsaturated esters was also supported by signals for two pairs of olefinic protons (δ_H = 6.77, 5.93; 6.85, 5.93 ppm) in the

Table 1. NMR spectroscopic data for **1** in different concentrations (*c*).

No.	a: *c* = 2 and 20 mg/mL		b: *c* = 60 mg/mL		$\Delta\delta = \delta_a - \delta_b$	
	δ_H, m, *J* [Hz]	δ_C, m[a]	δ_H, m, *J* [Hz]	δ_C, m[a]	$\Delta\delta_H$	$\Delta\delta_C$
1,1′		165.0, s		165.3, s		–0.3
2,2′	5.98, dd, 15.3, 1.5	122.2, d	5.98, dd, 15.8, 1.0	122.0, d	0	0.2
3,3′	6.90, dd, 15.3, 5.0	149.4, d	6.83, dd, 15.8, 5.0	149.1, d	0.07	0.3
4,4′	4.30, m	69.8, d	4.19, dd, 12.3, 6.3	69.9, d	0.11	–0.1
5a,5a′	1.84, m	30.5, t	1.79, dd, 13.3, 7.3	30.5, t	0.05	0
5b,5b′	1.91, m		1.79, dd, 13.3, 7.3		0.12	
6a,6a′	1.65, m	29.0, t	1.57, m	28.8, t	0.08	0.2
6b,6b′	1.74, m		1.67, m		0.07	
7,7′	5.13, m	70.4, d	5.04, m	70.3, d	0.09	0.1
8,8′	1.28, d, 6.5	18.3, q	1.21, d, 6.5	18.4, q	0.07	–0.1

[a] By DEPT sequence.

downfield area of the ^{1}H NMR spectrum. The ^{1}H NMR spectrum of **2** was similar to that of **1**, except for the presence of an additional methyl signal at δ_H = 2.06 (s) ppm (Table 2). This signal, in conjunction with two carbon resonances at δ_C = 169.8 (s) and 21.1 (q) ppm in its ^{13}C NMR spectrum (Table 2), suggested the presence of an acetyl group in **2**. The location of the acetoxy group at C-4 was shown by the downfield shift of the respective proton signal at C-4 at δ_H = 4.30 ppm in **1** to δ_H = 5.20 ppm in **2**. An additional structural proof came from chemical interconversion: Acetylation of both **1** and **2** gave an identical 4,4′-diacetylpyrenophorol (**11**)[4] (Scheme 1). Thus, compound **2** was established as 4-acetylpyrenophorol.

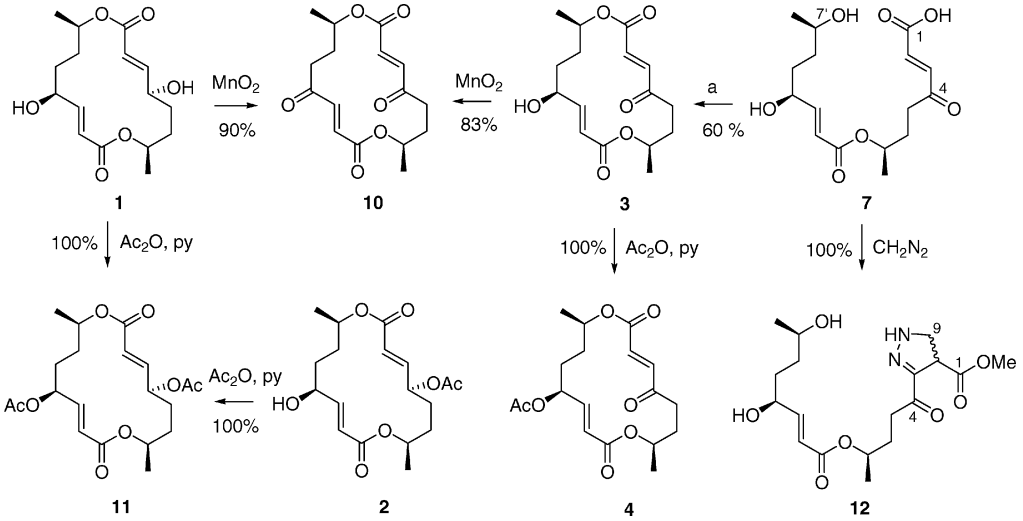
Table 2. NMR spectroscopic data for compounds **2** and **4**.

No.	4-Acetylpyrenophorol (**2**)		4′-Acetyldihydropyrenophorin (**4**)	
	δ_H, m, *J* [Hz]	δ_C, m[a]	δ_H, m, *J* [Hz]	δ_C, m[a]
1		164.8, s		164.6, s
2	5.93, dd, 15.5, 1.0	124.1, d	6.58, d, 16.0	131.6, d
3	6.77, dd, 15.5, 6.7	143.8, d	6.95, d, 16.0	139.5, d
4	5.20, m	72.0, d		200.8, s
5a	1.86, m	27.9, t	2.58, ddd, 13.5, 8.0, 4.5	36.0, t
5b	1.86, m		2.71, ddd, 13.5, 9.0, 4.5	
6a	1.58, m	28.8, t	2.01, m	32.3, t
6b	1.70, m		2.03, m	
7	5.07, m	69.6, d	5.04, m	70.8, d
8	1.23, d, 7.0	18.6, q	1.26, d, 6.5	19.6, q
1′		165.2, s		165.3, s
2′	5.93, dd, 15.5, 1.0	122.5, d	5.94, dd, 15.5, 1.5	122.3, d
3′	6.85, dd, 15.5, 6.5	148.5, d	6.98, dd, 15.5, 4.0	145.7, d
4′	4.21, m	70.7, d	5.31, m	70.7, d
5a′	1.80, m	30.7, t	1.93, m	27.7, t
5b′	1.80, m		1.93, m	
6a′	1.58, m	28.9, t	1.67, m	28.1, t
6b′	1.70, m		1.73, m	
7′	5.07, m	70.4, d	5.13, m	70.8, d
8′	1.24, d, 7.0	18.6, q	1.26, d, 6.5	18.2, q
OAc	2.06, s	21.1, q	2.10, s	21.0, q
		169.8, s		169.7, s

[a] By DEPT sequence.

Compound **4** was obtained as an optically active colorless oil with the molecular formula $C_{18}H_{24}O_7$, established by HREIMS. The ^{1}H and ^{13}C NMR spectra of **4** (Table 2) resembled those of **3**, revealing their similar structures. The presence of signals at δ_H = 2.10 ppm and δ_C = 169.7, 21.0 ppm in combination with the remarkable downfield shift of the methine proton signal (δ_H = 5.31 ppm in **4** and δ_H = 4.39 ppm in **3**) indicated the presence of an acetoxy group in **4**. Thus, compound **4** was established as 4′-acetyldihydropyrenophorin. This was further confirmed by acetylation of **3** to **4**, identical with the natural product (Scheme 1).

Compound **5** was isolated as an optically active colorless oil. Its molecular formula $C_{16}H_{22}O_6$, established by HREIMS, was the same as that of **3**. ^{1}H and ^{13}C NMR of **5** showed great similarity to those of (–)-dihydropyrenophorin (**3**),[24] suggesting the same macrocyclic skeleton. However, a difference was observed in the NMR resonances of the ketone subunit from C-2 to C-4 (Table 3), involving one carbonyl group and the conjugated double bond. More importantly, the large coupling constant between the olefinic protons in **3** ($^3J_{2,3}$ = 15.5 Hz) markedly decreased in the ^{1}H NMR spectrum of **5** ($^3J_{2,3}$ = 11.5 Hz), indicating a (*Z*) geometry of the double bond. This conclusion was supported by the diagnostic NOE correlation between 2-H and 3-H in the NOESY spectra. The presence of a secondary alcohol at C-4′ in **5** allowed the determination of its absolute stereochemistry by using a modified Mosher's method.[46–48] The (*S*)- and (*R*)-MTPA esters of *cis*-dihydropyrenophorin (**5**) were prepared by treatment at room temperature with (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride in dry pyridine, respectively. Significant $\Delta\delta$ values ($\Delta\delta = \delta_{(S)\text{-MTPA ester}} - \delta_{(R)\text{-MTPA ester}}$) for the protons near to the chiral center C-3 were observed as shown in Figure 2 (a). According to Mosher's rule, the absolute configuration at C-4′ was determined as (*S*), the same as those in its analogues **1**–**4**.



Scheme 1. Chemical interconversion of pyrenophorin derivatives: (a) $Cl_3C_6H_2COCl$, *i*Pr_2NEt, DMAP, pyridine, PhMe, room temp., 20 h, 60%.

Table 3. NMR spectroscopic data for compounds **5** and **6**.

No.	*cis*-Dihydropyrenophorin (**5**)		No.	Tetrahydropyrenophorin (**6**)	
	δ_H, m, *J* [Hz]	δ_C, m[a]		δ_H, m, *J* [Hz]	δ_C, m[a]
1		164.4, s	1		172.1, s
2	6.03, d, 11.5	124.6, d	2a	2.39, ddd, 16.8, 6.5, 4.0	28.6, t
			2b	2.74, ddd, 16.8, 12.0, 4.5	
3	6.45, d, 11.5	142.9, d	3a	2.44, ddd, 17.0, 6.5, 4.5	37.3, t
			3b	2.86, ddd, 17.0, 12.0, 4.0	
4		203.4, s	4		207.5, s
5a	2.44, dt, 17.5, 7.0	37.5, t	5a	2.51, ddd, 18.0, 11.5, 3.5	39.1, t
5b	2.92, dt, 17.5, 7.0		5b	2.62, ddd, 18.0, 12.0, 3.0	
6a	1.78, m	28.9, t	6a	1.77, m	28.8, t
6b	2.18, m		6b	2.16, m	
7	5.09, m	69.6, d	7	4.91, m	71.7, d
8	1.31, d, 6.5	19.7, q	8	1.28, d, 6.0	20.3, q
1′		165.8, s	1′		165.6, s
2′	6.01, d, 15.0	122.3, d	2′	5.89, dd, 15.5, 0.7	122.6, d
3′	6.83, dd, 15.0, 4.5	147.8, d	3′	6.77, dd, 15.5, 6.5	149.0, d
4′	4.50, m	70.1, d	4′	4.28, m	71.2, d
5a′	1.83, m	29.9, t	5a′	1.77, m	30.1, t
5b′	1.88, m		5b′	1.82, m	
6a′	1.59, m	28.9, t	6a′	1.59, m	29.7, t
6b′	1.73, m		6b′	1.68, m	
7′	4.90, m	71.2, d	7′	4.91, m	70.1, d
8′	1.23, d, 6.5	18.9, q	8′	1.22, d, 6.5	18.7, q

[a] By DEPT sequence.

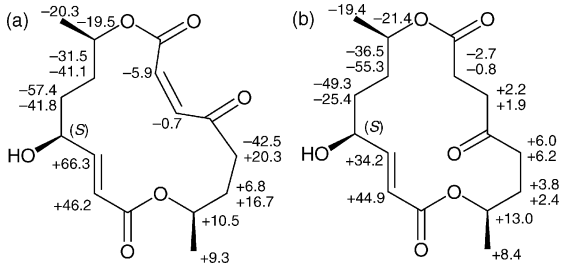


Figure 2. $\Delta\delta$ ($\delta_{(S)} - \delta_{(R)}$) values [Hz] for the MTPA esters of **5** (a) and **6** (b).

Based on the formation of the pyrenophorol family in the same fungus, the related negative optical rotations of pyrenophorol ($[\alpha]_D^{20}$ = –13.3[7] and –14.9[4]) with values measured for our derivatives (see Exp. Sect.), and the consistent chemical correlations, we assume identical absolute configuration of the derivatives presented in Scheme 1. The structure of **5** was thus determined as a double-bond isomer of **3**, and is assigned as (–)-(7*R*,4′*S*,7′*R*)-**5**.

Compound **6**, an optically active colorless oil, had the molecular formula $C_{16}H_{24}O_6$ as deduced from HRESIMS, and thus possessing two mass units more than **3** or **5**. Again, the ^{1}H and ^{13}C NMR spectra of **6** (Table 3) showed similarity to those of **3**, except that the signals for the double bond of the unsaturated ketone (δ_C = 131.0, 139.7 ppm; δ_H = 6.59, 6.99 ppm) in **3** were replaced by signals for a pair of methylene groups (δ_C = 28.6, 37.3 ppm; δ_H = 2.39, 2.74, and 2.44, 2.86 ppm) in **6**. In addition, both the signals for the ketone and ester carbonyl atoms were shifted downfield (δ_C = 164.4, 200.2 ppm in **3**; δ_C = 172.1, 207.5 ppm in **6**) due to the disappearance of the conjugated system. Thus, compound **6** was identified as the 2,3-dihydro analogue of dihydropyrenophorin (**3**). A modified Mosher's method[46–48] confirmed the (*S*) configurtion at C-4′ [Figure 2 (b)]. The structure was thus determined as (–)-(7*R*,4′*S*,7′*R*)-**6**.

The polar compound **7** was isolated as an optically active colorless oil, with the molecular formula of $C_{16}H_{24}O_7$, as deduced from HRESIMS. The IR spectrum of **7** was reminiscent of that of dihydropyrenophorin (**3**), showing functional absorption bands for hydroxy groups (3396 cm^{-1}) and an α,β-unsaturated ester group (1722, 1279, 1058 cm^{-1}). The similarity was also observed in the ^{1}H and ^{13}C NMR spectra of **7** (Table 4) with some differences in the signals for C-1, C-7′ and C-8′. In particular, the signal for 7′-H in the ^{1}H NMR was shifted upfield in **7** (δ_H = 3.77 ppm) with respect to that in **3** (δ_H = 5.20 ppm), displaying a characteristic signal for a secondary alcohol proton instead of an ester proton. In addition, the analysis of the HMBC spectrum of **7** revealed a long-range correlation from 7-H to C-1′, but no correlation from 7′-H to C-1 in contrast to the situation in **3**. These facts, in conjunction with the marked increase in the polarity of **7**, suggested an open-chain derivative of **3** by cleavage of the C-1–C-7′ lactone. Treatment of **7** with diazomethane gave the methyl ester **12** (Scheme 1), which further validated the presence of a carboxylic acid group in **7**. Interestingly, a simultaneous 1,3-dipolar cycloaddition was observed,[49,50] and the diazomethane was also trapped by the highly activated, conjugated double bond to form a pyrazole ring system.[51] The presence of two nitrogen atoms was proven by the molecular formula $C_{18}H_{28}N_2O_7$ as deduced from HREIMS and further supported by a positive reaction with the Dragendoff reagent, and a complete ^{1}H and ^{13}C NMR assignment based on 2D NMR spectra. In particular, the HMBC correlations from NH to C-2, C-3, C-9, from 9-H_2 to C-1, C-2, C-3, and from 2-H to C-1 and C-3, suggested the pyrazole ring substruc-

Table 4. NMR spectroscopic data for compounds **7** and **8**.

No.	*seco*-Dihydropyrenophorin (**7**)		7′-Acetyl-*seco*-dihydropyrenophorin (**8**)	
	δ_H, m, *J* [Hz]	δ_C, m[a]	δ_H, m, *J* [Hz]	δ_C, m[a]
1		167.5, s		167.7, s
2	6.59, d, 16.0	131.5, d	6.60, d, 16.0	132.0, d
3	6.95, d, 16.0	139.4, d	6.95, d, 16.0	139.2, d
4		199.5, s		199.6, s
5a	2.65, t, 7.2	37.1, t	2.65, t, 7.5	37.1, t
5b	2.65, t, 7.2		2.65, t, 7.5	
6a	1.88, m	29.8, t	1.88, m	29.8, t
6b	1.93, m		1.92, m	
7	4.95, m	70.3, d	4.95, m	70.4, d
8	1.22, d, 6.0	19.9, q	1.23, d, 6.0	19.9, q[b]
1′		166.4, s		166.3, s
2′	5.93, dd, 16.0, 1.5	119.9, d	5.92, dd, 15.5, 1.5	120.2, d
3′	6.85, dd, 16.0, 4.7	150.8, d	6.83, dd, 15.5, 5.0	150.6, d
4′	4.23, m	70.8, d	4.22, m	70.2, d
5a′	1.55, m	32.9, t	1.55, m	32.0, t
5b′	1.68, m		1.55, m	
6a′	1.51, m	35.0, t	1.53, m	31.4, t
6b′	1.60, m		1.68, m	
7′	3.77, m	67.7, d	4.87, m	70.8, d
8′	1.15, d, 6.0	23.2, q	1.18, d, 6.5	19.8, q[b]
OAc			1.98, s	21.2, q
				171.3, s

[a] By DEPT sequence. [b] Signals maybe interchangeble.

ture. Not unexpectedly, the diazomethane adduct was a mixture of two isomers as evident from the appearance of signal pairs in the NMR spectra. The structure and absolute configuration of the acid **7** was unambiguously confirmed by cyclization to **3** using a modified Yamaguchi procedure [Scheme 1 (a)].[52,53]

Compound **8** was isolated as an optically active, colorless oil with the molecular formula $C_{18}H_{26}O_8$, as deduced from HRESIMS. The ^1H and ^{13}C NMR spectra of **8** (Table 4) were closely related to those of **7**, except for the presence of an additional acetoxy group [δ_H = 1.98 (s) ppm; δ_C = 171.3 (s), 21.2 (q) ppm]. The location of the acetoxy group at C-7 was assured by the downfield shift of the 7′-H signal (δ_H = 4.87 ppm in **8**; δ_H = 3.77 ppm in **7**), and compound **8** was determined as 7′-acetyl-*seco*-dihydropyrenophorin. The long-range correlation from 7′-H to the acetyl carbonyl atom (δ_C = 171.3 ppm) in the HMBC spectrum further confirmed the proposed structure.

Compound **9** was obtained as an optically active colorless oil. Its molecular formula $C_{16}H_{26}O_6$ was established by HRESIMS, indicating four double-bond equivalents. The IR spectrum of **9** showed the presence of a lactone carbonyl functionality (1774 cm^{-1}), hydroxy groups (3603 cm^{-1}), and an α,β-unsaturated ester group (1721, 1282, 1193 cm^{-1}). Comparison of the ^1H and ^{13}C NMR spectra of **9** (Table 5) with those of **7** revealed identical chemical shifts for the signals from C-7 to C-7′, presenting a characteristic (*E*) double bond [δ_H = 6.60 (dd, *J* = 15.7, 1.2 Hz), 6.91 (dd, *J* = 15.7, 4.7 Hz) ppm; δ_C = 120.1 (d), 150.5 (d) ppm] and a conjugated ester group (δ_C = 166.4 ppm), which accounted for two double-bond equivalents. The remaining two double-bond equivalents were attributed to a lactone ring in the structure, confirmed by diagnostic long-range correlations from C-1 to 2-H_2, 3-H_2, and 4-H, as deduced from the HMBC spectrum. This compound was assigned as *seco*-dihydropyrenophorin-1,4-lactone (**9**); the configuration at C-4 was not determined.

Table 5. NMR spectroscopic data for compound **9**.

No.	δ_H, m, *J* [Hz]	δ_C, m[a]	No.	δ_H, m, *J* [Hz]	δ_C, m[a]
1		177.2, s	1′		166.4, s
2a	2.52, dd, 9.5, 6.5	28.8, t	2′	6.60, dd, 15.7, 1.2	120.1, d
2b	2.52, dd, 9.5, 6.5				
3a	1.83, m	27.9, t	3′	6.91, dd, 15.7, 4.7	150.5, d
3b	2.32, dt, 13.5, 6.5				
4	4.49, m	80.4, d	4′	4.28, m	70.9, d
5a	1.64, m	31.3, t[b]	5a′	1.65, m	33.2, t
5b	1.78, m		5b′	1.80, m	
6a	1.64, m	31.6, t[b]	6a′	1.55, m	35.1, t
6b	1.78, m		6b′	1.65, m	
7	5.01, m	70.1, d	7′	3.81, m	67.8, d
8	1.24, d, 6.5	20.0, q	8′	1.19, d, 7.0	23.5, q

[a] By DEPT sequence. [b] Signals maybe interchangeable.

Bioactivity

The isolated compounds **1**–**12** were tested in an agar diffusion assay for their antibacterial, antifungal and algicidal properties and compared with a set of controls (penicillin, nystatin, actidione, and tetracycline; Table 6). Compounds **2**–**6**, **8**, and **11** inhibited all four test organisms, whereas compounds **1**, **10**, and **12** inhibited *Microbotryum violaceum* and the alga *Chlorella fusca*. Compounds **7** and **9** were antifungal against *Microbotryum violaceum* and antibacterial against *Escherichia coli* and *Bacillus megaterium*.

Table 6. Agar diffusion assays for antibacterial, antifungal, and antialgal activities.[a,b]

Compound	*Escherichia coli*	*Bacillus megaterium*	*Microbotryum violaceum*	*Chlorella fusca*
1	0	11	7	10
2	7	9	7	9
3	9	10	10	11
4	9	8	7	9
5	10	10	7	10
6	12	12	10	12
7	8	11	10	0
8	10	6	9	10
9	10	6	7	0
10	0	10	9	9
11	10	11	23	13
12	0	11	7	10
Penicillin	18	14	0	0
Tetracycline	18	18	10	0
Nystatin		0	0	20
Actidione		0	35	50
Acetone		0	0	0

[a] 50 μL of substance dissolved in acetone (1 mg/mL) were applied to a filter disc and sprayed with a suspension of the respective test organism. [b] Radii of the zones of inhibition are given in mm.

Conclusion

The discovery of an array of pyrenophorol derivatives, including the minor constituents, demonstrates the productivity of the fungus and is a beautiful example of chemical diversity, extending the pyrenophorol family. This chemical diversity may be due to reduced enzyme specificity or to non-enzymatic side reactions. In particular, the co-occurrence of the open-chain acids as putative biosynthetic precursors is noteworthy. They have rarely been isolated together with macrodiolides, perhaps due to their polar nature. The stereochemical investigation and in particular the chemical interconversion showed that the absolute configuration of all the pyrenophorol members seems to be uniform. They have (*R*) configuration at C-7 and C-7′, and (*S*) configuration at C-4 and/or C-4′, regardless of the oxidation state, double-bond geometry, or cyclization mode. Obviously, compounds **7**–**9** are structurally related to pyrenophorol (**1**), (–)-dihydropyrenophorin (**3**) and the other new analogues **2** and **4**–**6**. The diversity and interconversion of the metabolites starting from the same skeleton is created by just a few simple chemical transformations: Reduction of carbonyl groups, oxidation of hydroxy groups, hydrogenation of double bonds, lactonization of hydroxy acid to lactone of different ring size, and finally simple acetylation of hydroxy groups. The acetylation on the secondary alcohol at C-7′ in **8** and lactonization between C-1 and C-4 in **9** may have prevented further macrolactoniztion of these structures. The isolation of twelve different derivatives by isolation or chemical transformations and comparison of their data enabled the complete assignment of all signals in the ^{1}H and ^{13}C NMR spectra of the pyrenophorol family that have not previously been completely reported.[20–24,36–42]

Experimental Section

General Experimental Procedures: For microbiological methods and culture conditions, see refs.[54–56] Commercial silica gel (Merck, 0.040–0.063 mm) was used for column chromatography. Precoated silica gel plates (Merck, G60 F-254 or G50 UV-254) were used for analytical and preparative thin-layer chromatography (TLC), respectively. NMR spectra were recorded at 293 K with a Bruker Avance 500 spectrometer. Chemical shifts are reported in parts per million (δ) by using $CDCl_3$ as an internal standard with coupling constants (J) in Hz. ^{1}H and ^{13}C NMR assignments were supported by ^{1}H-^{1}H COSY, HMQC, HMBC, NOESY, and NOEDIFF experiments. Optical rotations were measured with a Perkin–Elmer 241 MC polarimeter at the sodium D line. IR spectra were recorded with a Nicolet-510P spectrophotometer, peaks are reported in cm^{-1}. UV spectra were recorded with a UV-2101PC spectrophotometer, peaks are reported in nm. Mass spectra and high-resolution mass spectra were recorded with MAT 8200 and Micromass LCT mass spectrometers.

Culture, Extraction and Isolation: The endophytic fungus *Phoma* sp., internal strain No. 8874, was isolated following surface sterilisation from the leaves of *Lycium intricatum*, a succulent woody shrub from Gomera, and was cultivated on 12 L of 5% w/v biomalt solid agar media at room temperature for 28 d.[54,55] The culture media were then extracted with ethyl acetate to afford 7.8 g of a residue after removal of the solvent under reduced pressure. The extract was subjected to column chromatography (CC) on silica gel, eluted with a gradient of dichloromethane in 2-propanol (95:5, 90:10, 80:20, 60:40, 40:60) to give 14 subfractions. Fractions 3, 8, and 11 gave pure **4** (7.2 mg), **1** (552.0 mg), and **7** (119.0 mg), respectively, by simple Sephadex LH-20 CCs, eluted with $CHCl_2$/MeOH (30:1 for **4**, 10:1 for **1**, and 3:1 for **7**). Fraction 5 was split by a Sephadex LH-20 CC ($CHCl_2$/MeOH, 10:1) followed by silica gel CC (400–600 mesh, petroleum ether/acetone, 5:1) to afford **3** (24.4 mg) and **5** (1.0 mg). Fraction 6 was subjected to a Sephadex LH-20 CC ($CHCl_2$/MeOH, 10:1) and was then fractionated by silica gel CC (400–600 mesh, petroleum ether/acetone, 4:1) to yield **2** (10.0 mg) and **6** (2.1 mg). Finally, a Sephadex LH-20 CC ($CHCl_2$/MeOH, 3:1) on fraction 9 gave pure **8** (16.3 mg) and crude **9**, which was purified by a subsequent silica gel CC (400–600 mesh, petroleum ether/acetone, 4:3) to afford the pure product (**9**, 6.7 mg).

Data for Pyrenophorol (1): Colorless crystals, m.p. 130–133 °C. $[\alpha]_D^{20}$ = –10.2 (c = 1.04, $CHCl_3$). ^{1}H and ^{13}C NMR spectroscopic data: see Table 1. HRESIMS: m/z = 313.16456 (calcd. 313.16511 for $C_{16}H_{25}O_6$).

Data for 4-Acetylpyrenophorol (2): Colorless oil, $[\alpha]_D^{20}$ = –23.9 (*c* = 2.44, $CHCl_3$). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3603, 2934, 2874, 1717, 1652, 1466, 1370, 1244, 1134, 1028 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 2. HRESIMS: *m*/*z* = 377.15757 (calcd. 377.15762 for $C_{18}H_{26}O_7Na$).

Data for Dihydropyrenophorin (3): Colorless oil, $[\alpha]_D^{20}$ = –10.7 (*c* = 1.00, $CHCl_3$). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3537, 2934, 2853, 1717, 1652, 1455, 1355, 1289, 1174, 1023 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 6.99 (d, *J* = 15.5 Hz, 1 H, 3-H), 6.98 (dd, *J* = 15.7, 3.7 Hz, 1 H, 3′-H), 6.59 (d, *J* = 15.5 Hz, 1 H, 2-H), 5.99 (dd, *J* = 15.5, 2.0 Hz, 1 H, 2′-H), 5.20 (m, 1 H, 7′-H), 5.00 (m, 1 H, 7-H), 4.39 (m, 1 H, 4′-H), 2.70 (ddd, *J* = 13.0, 8.0, 4.0 Hz, 1 H, 5b-H), 2.56 (ddd, *J* = 13.0, 8.5, 4.5 Hz, 1 H, 5a-H), 2.07 (m, 2 H, 6-H_2), 2.01 (m, 1 H, 5′b-H), 1.88 (m, 1 H, 5′a-H), 1.75 (m, 2 H, 6′-H_2), 1.32 (d, *J* = 6.5 Hz, 3 H, 8′-H_3), 1.26 (d, *J* = 6.5 Hz, 3 H, 8-H_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 200.2 (s, C-4), 165.5 (s, C-1′), 164.4 (s, C-1), 150.3 (s, C-3′), 139.7 (s, C-3), 131.0 (s, C-2), 121.4 (s, C-2′), 71.1 (d, C-7′), 70.7 (d, C-7), 69.7 (d, C-4′), 37.2 (t, C-5), 32.4 (t, C-6), 30.3 (t, C-5′), 28.4 (t, C-6′), 19.6 (q, C-8), 17.7 (q, C-8′) ppm. HREIMS: *m*/*z* = 310.14149 (calcd. 310.14164 for $C_{16}H_{22}O_6$).

Data for 4′-Acetyldihydropyrenophorin (4): Colorless oil, $[\alpha]_D^{20}$ = –27.9 (*c* = 0.72, $CHCl_3$). IR: ($CHCl_3$) $\tilde{\nu}_{max}$ = 3044, 2939, 2866, 1727, 1682, 1450, 1375, 1289, 1184, 1134 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 2. HREIMS: *m*/*z* = 352.15291 (calcd. 352.15218 for $C_{18}H_{24}O_7$).

Data for *cis*-Dihydropyrenophorin (5): Colorless oil, $[\alpha]_D^{20}$ = –12.8 (*c* = 0.10, $CHCl_3$). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3609, 2925, 2862, 1719, 1606, 1464, 1380, 1279, 1179, 1025 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 3. HREIMS: *m*/*z* = 310.14180 (calcd. 310.14164 for $C_{16}H_{22}O_6$).

Data for Tetrahydropyrenophorin (6): Colorless oil, $[\alpha]_D^{20}$ = –11.0 (*c* = 0.21, $CHCl_3$). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3632, 2934, 2858, 1723, 1602, 1461, 1370, 1285, 1189, 1014 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 3. HRESIMS: *m*/*z* = 335.14650 (calcd. 335.14706 for $C_{16}H_{24}O_6Na$).

Data for *seco*-Dihydropyrenophorin (7): Colorless oil, $[\alpha]_D^{20}$ = –12.0 (*c* = 2.35, MeOH). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3396, 2979, 2929, 1722, 1279, 1183, 1058 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 4. HRESIMS: *m*/*z* = 351.14142 (calcd. 351.14197 for $C_{16}H_{24}O_7Na$).

Data for 7′-Acetyl-*seco*-dihydropyrenophorin (8): Colorless oil, $[\alpha]_D^{20}$ = –4.4 (*c* = 0.79, MeOH). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3507, 2938, 2863, 1727, 1380, 1264, 1179, 1048 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 4. HRESIMS: *m*/*z* = 393.15199 (calcd. 393.15254 for $C_{18}H_{26}O_8Na$).

Data for *seco*-Dihydropyrenophorin-1,4-lactone (9): Colorless oil, $[\alpha]_D^{20}$ = –12.1 (*c* = 0.67, MeOH). IR ($CHCl_3$): $\tilde{\nu}_{max}$ = 3603, 2943, 2863, 1774, 1721, 1663, 1462, 1282, 1193, 1018 cm^{-1}. ^1H and ^{13}C NMR spectroscopic data: see Table 5. HRESIMS: *m*/*z* = 337.16266 (calcd. 337.16271 for $C_{16}H_{26}O_6Na$).

Synthesis of Pyrenophorin (10) by MnO_2 Oxidation of Pyrenophorol (1): To a stirred solution of **1** (10.0 mg, 0.032 mmol) in dry CH_2Cl_2 (2.0 mL) was added active MnO_2 (13.9 mg, 0.16 mmol). The resulting suspension was stirred at room temperature for 50 h, the MnO_2 was filtered off and repeatedly washed with Et_2O (5 mL) to afford **10**[20–23,36–42] (9.4 mg, 90%) as colorless crystals. M.p. 175–176 °C (ref.[42] 177–178 °C). $[\alpha]_D^{20}$ = –40.3 (*c* = 1.3, $CHCl_3$) {ref.[42] $[\alpha]_D^{20}$ = –55.9 (*c* = 0.44, EtOH)}. ^1H NMR (500 MHz, $CDCl_3$): δ = 6.93 (d, *J* = 16.0 Hz, 2 H, 3-H, 3′-H), 6.48 (d, *J* = 16.0 Hz, 2 H, 2-H, 2′-H), 5.03 (m, 2 H, 7-H, 7′-H), 2.65 (ddd, *J* = 14.0, 9.0, 3.5 Hz, 2 H, 5b-H, 5′b-H), 2.54 (ddd, *J* = 14.0, 8.0, 3.5 Hz, 2 H, 5a-H, 5′a-H), 2.13 (m, 2 H, 6b-H, 6′b-H), 2.08 (m, 2 H, 6a-H, 6′a-H), 1.28 (d, *J* = 6.5 Hz, 6 H, 8-H_3, 8′-H_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 199.6 (s, C-4, C-4′), 164.9 (s, C-1, C-1′), 139.7 (s, C-3, C-3′), 131.4 (s, C-2, C-2′), 72.2 (d, C-7, C-7′), 37.2 (t, C-5, C-5′), 32.1 (t, C-6, C-6′), 19.6 (q, C-8, C-8′) ppm. HREIMS: *m*/*z* = 308.12626 (calcd. 308.12598 for $C_{16}H_{20}O_6$).

Synthesis of Pyrenophorin (10) by MnO_2 Oxidation of Dihydropyrenophorin (3): The same reaction was performed with dihydropyrenophorin (**3**) (5.0 mg, 0.016 mmol) and active MnO_2 (3.5 mg, 0.040 mmol) in dry CH_2Cl_2 (1.0 mL). Stirring for 40 h afforded identical pyrenophorin (**10**, 4.1 mg, 83%).[20–23,36–42]

Synthesis of 4,4′-Diacetylpyrenophorol (11) by Acetylation of Pyrenophorol (1): To a solution of **1** (9.0 mg) in dry pyridine (0.5 mL) were added 2 drops of Ac_2O. The mixture was kept at room temperature for 16 h to afford, after usual workup, **11**[4] quantitatively (11.4 mg) as colorless oil. $[\alpha]_D^{20}$ = –49.3 (*c* = 1.20, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ = 6.77 (dd, *J* = 15.7, 6.7 Hz, 2 H, 3-H, 3′-H), 5.98 (dd, *J* = 15.7, 0.7 Hz, 2 H, 2-H, 2′-H), 5.22 (m, 2 H, 4-H, 4′-H), 5.09 (m, 2 H, 7-H, 7′-H), 2.08 (s, 6 H, Me of OAc), 1.86 (m, 4 H, 5-H_2, 5′-H_2), 1.74 (m, 2 H, 6b-H, 6′b-H), 1.62 (m, 2 H, 6a-H, 6′a-H), 1.26 (d, *J* = 6.5 Hz, 6 H, 8-H_3, 8′-H_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 169.7 (s, OAc), 164.7 (s, C-1, C-1′), 143.7 (s, C-3, C-3′), 124.1 (s, C-2, C-2′), 72.0 (d, C-4, C-4′), 69.8 (d, C-7, C-7′), 28.8 (t, C-6, C-6′), 27.9 (t, C-5, C-5′), 21.0 (q, OAc), 18.5 (q, C-8, C-8′) ppm.

Synthesis of 4,4′-Diacetylpyrenophorol (11) by Acetylation of 4-Acetylpyrenophorol (2): The same reaction was performed with **2** (2.0 mg) to afford an identical sample of 4,4′-diacetylpyrenophorol (**11**)[4] (2.2 mg, 100%) as a colorless oil.

Acetylation of Dihydropyrenophorin (3): Treatment of **3** (3.0 mg) with Ac_2O according to the above procedure, afforded an acetate in quantitative yield (3.4 mg, 100%), identical with the natural product **4**.

Esterification of *cis*-Dihydropyrenophorin (5) with (*R*)-MTPA Chloride: Treatment of **5** (0.3 mg) with (*R*)-MTPA chloride (5 μL) in dry pyridine (0.5 mL), stirring at room temperature overnight and purification by a mini silica gel column chromatography (300 mesh, petroleum ether/EtOAc, 6:1) afforded the (*S*)-MTPA ester of **5** (0.37 mg, 72%). ^1H NMR (500 MHz, $CDCl_3$): δ = 6.69 (dd, *J* = 16.2, 7.1 Hz, 1 H, 3′-H), 6.44 (d, *J* = 11.7 Hz, 1 H, 3-H), 6.00 (d, *J* = 11.7 Hz, 1 H, 2-H), 5.92 (d, *J* = 16.2 Hz, 1 H, 2′-H), 5.54 (m, 1 H, 4′-H), 5.09 (m, 1 H, 7-H), 4.84 (m, 1 H, 7′-H), 2.89 (dt, *J* = 17.0, 7.0 Hz, 1 H, 5b-H), 2.40 (dt, *J* = 17.0, 6.6 Hz, 1 H, 5a-H), 2.16 (m, 1 H, 6b-H), 1.84 (m, 1 H, 5′b-H), 1.76 (m, 1 H, 5′a-H), 1.74 (m, 1 H, 6a-H), 1.62 (m, 1 H, 6′b-H), 1.57 (m, 1 H, 6′a-H), 1.30 (d, *J* = 6.4 Hz, 3 H, 8-H_3), 1.17 (d, *J* = 6.4 Hz, 3 H, 8′-H_3) ppm.

Esterification of *cis*-Dihydropyrenophorin (5) with (*S*)-MTPA Chloride: The same reaction of **5** with (*S*)-MTPA chloride afforded the (*R*)-MTPA ester of **5** (0.37 mg, 72%). ^1H NMR (500 MHz, $CDCl_3$): δ = 6.62 (dd, *J* = 15.8, 7.2 Hz, 1 H, 3′-H), 6.44 (d, *J* = 11.9 Hz, 1 H, 3-H), 6.02 (d, *J* = 11.9 Hz, 1 H, 2-H), 5.83 (d, *J* = 15.8 Hz, 1 H, 2′-H), 5.51 (m, 1 H, 4′-H), 5.07 (m, 1 H, 7-H), 4.88 (m, 1 H, 7′-H), 2.85 (dt, *J* = 17.0, 7.4 Hz, 1 H, 5b-H), 2.51 (dt, *J* = 17.0, 6.7 Hz, 1 H, 5a-H), 2.13 (m, 1 H, 6b-H), 1.92 (m, 1 H, 5′b-H), 1.88 (m, 1 H, 5′a-H), 1.73 (m, 1 H, 6a-H), 1.70 (m, 1 H, 6′b-H), 1.64 (m, 1 H, 6′a-H), 1.29 (d, *J* = 6.4 Hz, 3 H, 8-H_3), 1.21 (d, *J* = 6.4 Hz, 3 H, 8′-H_3) ppm.

Esterification of Tetrahydropyrenophorin (6) with (*R*)-MTPA Chloride: Compound **6** (0.5 mg) was treated with (*R*)-MTPA chloride

according to the above procedure to afford the (*S*)-MTPA ester of **6** (0.70 mg, 83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.68 (dd, *J* = 15.8, 7.2 Hz, 1 H, 3′-H), 5.92 (d, *J* = 15.8 Hz, 1 H, 2′-H), 5.52 (m, 1 H, 4′-H), 4.90 (m, 1 H, 7-H), 4.85 (m, 1 H, 7′-H), 2.89 (ddd, *J* = 17.2, 10.9, 2.8 Hz, 1 H, 3b-H), 2.71 (ddd, *J* = 16.0, 10.9, 2.8 Hz, 1 H, 2b-H), 2.65 (ddd, *J* = 18.5, 9.5, 2.8 Hz, 1 H, 5b-H), 2.51 (ddd, *J* = 18.5, 8.4, 2.8 Hz, 1 H, 5a-H), 2.39 (ddd, *J* = 17.2, 5.8, 2.8 Hz, 1 H, 3a-H), 2.33 (ddd, *J* = 16.0, 5.8, 2.8 Hz, 1 H, 2a-H), 2.13 (m, 1 H, 6b-H), 1.85 (m, 1 H, 5′b-H), 1.74 (m, 1 H, 5′a-H), 1.72 (m, 1 H, 6a-H), 1.59 (m, 1 H, 6′b-H), 1.56 (m, 1 H, 6′a-H), 1.28 (d, *J* = 6.2 Hz, 3 H, 8-H_3), 1.17 (d, *J* = 6.4 Hz, 3 H, 8′-H_3) ppm.

Esterification of Tetrahydropyrenophorin (6) with (*S*)-MTPA Chloride: Compound **6** (0.5 mg) was treated with (*S*)-MTPA chloride according to the above procedure to afford the (*R*)-MTPA ester of **6** (0.70 mg, 83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.61 (dd, *J* = 15.7, 7.1 Hz, 1 H, 3′-H), 5.83 (d, *J* = 15.7 Hz, 1 H, 2′-H), 5.51 (m, 1 H, 4′-H), 4.89 (m, 1 H, 7′-H), 4.88 (m, 1 H, 7-H), 2.89 (ddd, *J* = 16.0, 10.8, 3.0 Hz, 1 H, 3b-H), 2.72 (ddd, *J* = 16.0, 10.8, 3.0 Hz, 1 H, 2b-H), 2.64 (ddd, *J* = 18.6, 11.7, 3.0 Hz, 1 H, 5b-H), 2.50 (ddd, *J* = 18.6, 8.6, 3.0 Hz, 1 H, 5a-H), 2.38 (ddd, *J* = 16.0, 6.2, 3.0 Hz, 1 H, 3a-H), 2.34 (ddd, *J* = 16.0, 6.2, 3.0 Hz, 1 H, 2a-H), 2.12 (m, 1 H, 6b-H), 1.90 (m, 1 H, 5′b-H), 1.84 (m, 1 H, 5′a-H), 1.71 (m, 1 H, 6a-H), 1.70 (m, 1 H, 6′b-H), 1.64 (m, 1 H, 6′a-H), 1.26 (d, *J* = 6.2 Hz, 3 H, 8-H_3), 1.21 (d, *J* = 6.4 Hz, 3 H, 8′-H_3) ppm.

Methylation of *seco*-Dihydropyrenophorin (7): To a solution of **7** (5.0 mg) in CH_2Cl_2 (1 mL) was added an ethereal solution of CH_2N_2 (1.0 mL). After 2 min at 20 °C, the solvents were evaporated at reduced pressure to afford **12** as a mixture of stereoisomers quantitatively. Most of the NMR signals of **12** resonated in pairs with very small variations in the ^{1}H and ^{13}C NMR spectra for the two stereoisomers. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.91 (dd, *J* = 16.0, 5.0 Hz, 2 H, 3′-H ×2), 6.46/6.43 (s, 2 H, NH ×2), 6.01 (dd, *J* = 16.0, 1.7 Hz, 2 H, 2′-H ×2), 4.99 (m, 2 H, 7-H ×2) 4.34 (m, 2 H, 4′-H ×2), 4.05/4.03 (dd, *J* = 9.5, 2.0 Hz, 2 H, 2-H ×2), 3.92/3.91 (ddd, *J* = 17.5, 9.5, 2.0 Hz, 2 H, 9b-H ×2), 3.87 (m, 4 H, 9a-H ×2, 7′-H ×2), 3.74/3.73 (s, 6 H, OMe ×2), 2.88 (t, *J* = 7.5 Hz, 4 H, 5-H_2 ×2), 1.95 (m, 2 H, 6b-H ×2), 1.92 (m, 2 H, 6a-H ×2), 1.78 (m, 2 H, 5′b-H ×2), 1.66 (m, 2 H, 6′b-H ×2), 1.63 (m, 2 H, 5′a-H ×2), 1.56 (m, 2 H, 6′a-H ×2), 1.26 (d, *J* = 6.0 Hz, 6 H, 8-H_3 ×2), 1.22 (d, *J* = 6.0 Hz, 6 H, 8′-H_3 ×2) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 194.7 (s, C-4), 171.8/171.6 (s, C-1), 166.2/166.1 (s, C-1′), 149.9 (s, C-3′), 147.3 (s, C-3), 120.7/120.6 (d, C-2′), 71.1 (d, C-4′), 70.5 (d, C-7), 68.1 (d, C-7′), 53.9 (t, C-9), 52.7 (q, OMe), 47.3 (d, C-2), 36.0/34.9 (t, C-6′), 33.7 (t, C-5), 33.1 (t, C-5′), 30.3/30.2 (t, C-6), 23.7 (q, C-8′), 20.0/19.9 (q, C-8) ppm. HREIMS: *m*/*z* = 384.18970 (calcd. 384.18963 for $C_{18}H_{28}N_2O_7$).

Yamaguchi Macrolactoniztion of *seco*-Dihydropyrenophorin (7): To a stirred solution of acid **7** (7.0 mg, 0.0213 mmol) in toluene (0.5 mL) were added pyridine (0.05 mL), *i*Pr_2NEt (11.0 µL, 0.064 mmol), $Cl_3C_6H_2COCl$ (5.2 µL, 0.033 mmol), and DMAP (1 mg, 0.008 mmol). The resulting solution was stirred at 20 °C for 20 h. Et_2O (3 mL) and saturated aqueous sodium hydrogen carbonate (3 mL) were then added. The layers were separated, and the aqueous layer was extracted with Et_2O (5×3 mL). The combined organic layers were washed with brine (3×3 mL), dried with Na_2SO_4, and concentrated in vacuo. Flash chromatography (petroleum ether/acetone, 4:1) afforded **3** (4.0 mg, 60%) as a colorless oil.

Agar Diffusion Test for Biological Activity: Compounds **1**–**12** were dissolved in acetone at a concentration of 1 mg/mL; 50 µL of the solution (0.05 µg) were pipetted onto a sterile filter disc (Schleicher & Schuell, 9 mm), which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism.[56] The test organisms were the gram-negative bacterium *Escherichia coli*, the gram positive bacterium *Bacillus megaterium* (both grown on NB medium), the fungus *Microbotryum violaceum*, and the alga *Chlorella fusca* (both grown on MPY medium). Reference substances were penicillin, nystatin, actidione and tetracycline. The radius of zone of inhibition was measured in mm. These microorgansims were chosen because (a) they are non-pathogenic and (b) had in the past proved to be accurate initial test organisms for antibacterial, antifungal and antialgal/herbicidal activities. Commencing at the outer edge of the filter disc, the radius of zone of inhibition was measured in mm.

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