An unusual fatty acid and its glyceride from the marine fungus *Microsphaeropis olivacea*[1]

Chao-Mei Yu, Jonathan M. Curtis, Jeffrey L.C. Wright, Stephen W. Ayer, and Ziba R. Fathi-Afshar

Abstract: Two new metabolites (**1** and **2**) were isolated from the marine fungus *Microsphaeropsis olivacea*. The structures were elucidated, through analysis of the spectroscopic data, as an unusual methyl-branched unsaturated fatty acid, 10-methyl-9*Z*-octadecenoic acid (**1**), and the glyceride (**2**). The locations of the double bond and the methyl branching were determined from the electron impact (EI) mass spectrum of the picolinyl derivative of **1** and from MS/MS data on the hydrogenation products of **1** and **2**.

Key words: *Microsphaeropsis olivacea*, marine fungus, methyl-branched unsaturated fatty acid, picolinyl ester.

Résumé : On a isolé deux nouveaux métabolites (**1** et **2**) à partir du champignon marin *Microsphaeropsis olivacea*. Faisant appel à des données spectroscopiques, on a établi que les structures sont associées à un acide gras insaturé portant un méthyle comme chaîne latérale, l'acide 10-méthyloctadéc-9*Z*-ènoïque (**1**) et de son glycéride (**2**). Les positions des doubles liaisons et de la ramification méthyle ont été déterminées par spectrométrie de masse par impact électronique sur le dérivé pinacolyle du composé **1** et par des données de SM/SM sur les produits d'hydrogénation des composés **1** et **2**.

Mots clés : *Microsphaeropsis olivacea*, champignons marins, acide gras insaturé portant une ramification méthyle.

[Traduit par la rédaction]

Introduction

Terrestrial microorganisms have long been a traditional source of biologically active metabolites but, after extensive studies, it is increasingly difficult to discover new bioactive agents from this source. In contrast, marine microorganisms are attracting more attention as sources of novel bioactive compounds. These ocean-life forms likely evolved different physiological and biosynthetic capabilities to survive in the unique marine environment at increased salinity and pressure, variable temperatures, and different nutrient compositions. Such capabilities may result in the production of metabolites not found previously in terrestrial microorganisms (1).

Our search for new pharmaceutical-lead compounds from marine microbes led us to investigate the metabolites of the marine fungus *Microsphaeropsis olivacea* (strain SF-10), isolated from a Florida sponge, and to the discovery of a new methyl-branched and unsaturated fatty acid (**1**) and its monoglyceride (**2**) (Fig. 1). In this paper, we describe the production, isolation and structure elucidation of **1** and **2** through their chemical and spectroscopic analyses.

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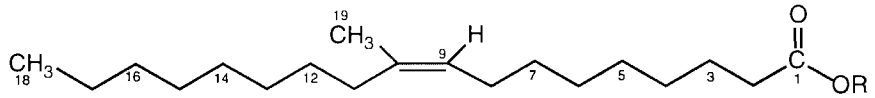
Results and discussion

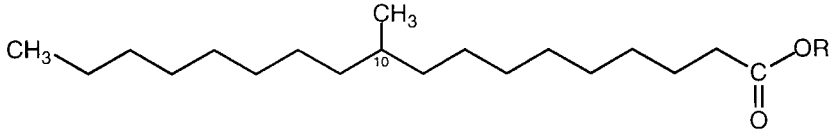
Metabolite **1**, a colorless oily compound, was isolated from the EtOAc extract of the culture filtrate of *M. olivacea*. The molecular formula of $C_{19}H_{36}O_2$ was determined from the high-resolution EI mass spectrum. The ^{1}H NMR spectrum indicated that this compound is an unsaturated linear fatty acid, but the unusual structural feature of an olefinic methyl branch (δ_H 1.65, d, 1.2 Hz) encouraged us to complete the structural elucidation. The multiplicity of the individual carbons was obtained by ^{13}C NMR data (Table 1) and DEPT experiments, indicating 1 terminal methyl group, 1 olefinic methyl group, a trisubstituted double bond (thereby implying the branching position of the olefinic methyl group), 14 methylene groups, and a carboxyl carbon (δ_C 178.11) that was supported by a characteristic infrared absorption at 1733 cm^{-1}. The HMQC experiment showed connectivities between the carbons and protons with the exception of C-4–C-7 and C-13–C-17. This is due to the extensive overlaps of the methylene proton resonances in the range δ 1.29–1.31, which correspond to 18 protons.

In the ^{1}H–^{1}H COSY spectrum, the resonance at δ 2.26 was assigned to the methylene protons H_2-2 adjacent to the carboxyl group, based on chemical shift considerations and supported by the long-range C/H correlation with C-1 in the HMBC experiment. This methylene group was also coupled with H_2-3 (δ 1.59), which in turn was coupled with other methylene groups (δ 1.29–1.31) indicating the connectivity of CH_2-2/CH_2-3/other CH_2 groups. In the HMBC experiment the methylene protons at H_2-2 and H_2-3 were observed to have $^3J_{CH}$ correlation with two methylene carbons C-4 (δ 30.29) and C-5 (δ 30.37), respectively, which established the partial

Fig. 1. The structures of metabolites (**1**, **2**) and their derivatives (**3**, **4**, **5**).



1, R = H; **2**, R = $-\overset{1'}{C}H_2\overset{2'}{C}H(OH)\overset{3'}{C}H_2OH$; **3**, R = —$CH_2$—(N)



4, R = H; **5**, R = $-CH_2CH(OH)CH_2OH$

Table 1. ^1H and ^{13}C NMR data (CD_3OD) for metabolites **1** and **2**.

	1		**2**	
Position	$\delta^{13}C$ (ppm)	δ^1H (ppm)	$\delta^{13}C$ (ppm)	δ^1H (ppm)
1	178.11	—	175.50	—
2	35.18	2.26 (t)	34.94	2.34 (t)
3	26.19	1.59 (m)	26.01	1.61 (m)
4	30.29[a]	1.29–1.31 (m)	30.22[a]	1.29–1.31 (m)
5	30.37[a]	1.29–1.31 (m)	30.35[a]	1.29–1.31 (m)
6	30.46[b]	1.29–1.31 (m)	30.45[c]	1.29–1.31 (m)
7	31.16[a]	1.29–1.31 (m)	31.17[a]	1.29–1.31 (m)
8	28.78	1.97 (m)	28.79	1.97 (m)
9	126.34	5.11 (t)	126.34	5.11 (m)
10	136.36	—	136.37	—
11	32.60	2.01 (t)	32.60	2.01 (t)
12	29.10	1.37 (m)	29.09	1.37 (m)
13	30.29[b]	1.29–1.31 (m)	30.28[c]	1.29–1.31 (m)
14	30.61[b]	1.29–1.31 (m)	30.60[c]	1.29–1.31 (m)
15	30.66[b]	1.29–1.31 (m)	30.66[c]	1.29–1.31 (m)
16	33.08[a]	1.29–1.31 (m)	33.07[a]	1.29–1.31 (m)
17	23.75[a]	1.29–1.31 (m)	23.75[a]	1.29–1.31 (m)
18	14.45	0.89 (t)	14.45	0.90 (t)
19	23.64	1.65 (d)	23.63	1.65 (d)
1′	—	—	66.48	4.05 (dd), 4.13 (dd)
2′	—	—	71.15	3.80 (m)
3′	—	—	64.07	3.54 (dd), 3.55 (dd)

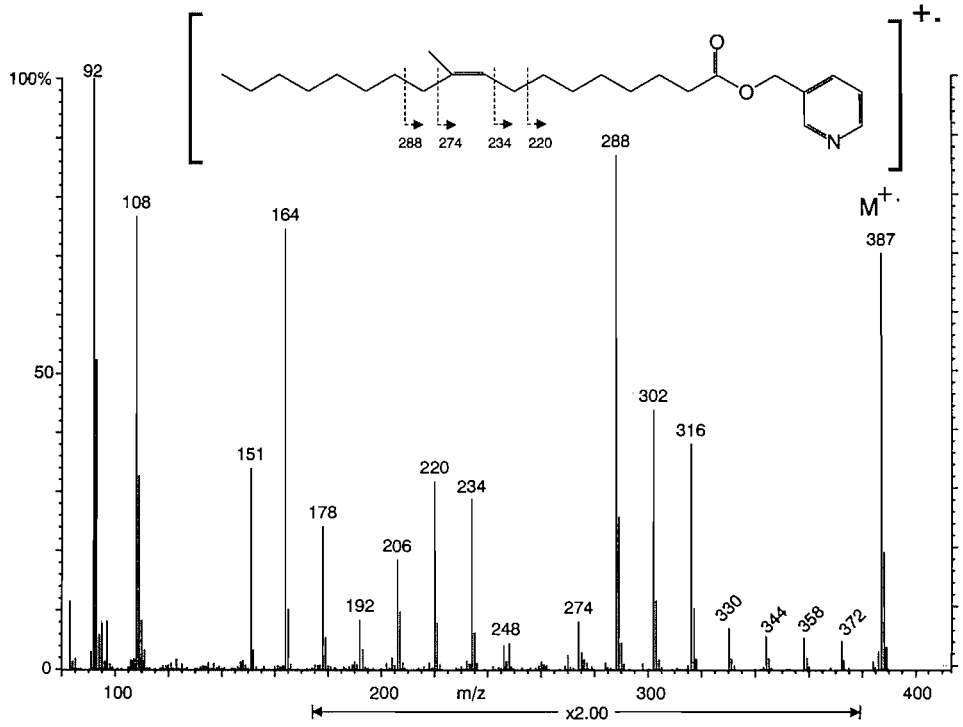
[b,c]Assignments with the same superscript may be interchanged.
[a]Assigned from the HMBC spectrum.

structure -$CH_2CH_2CH_2CH_2COOH$ and the assignments of C-4 and C-5.

From further interpretation of the ^1H–^1H COSY data, it was found that an olefinic methine proton H-9 (δ 5.11) showed adjacent coupling with H_2-8 (δ 1.97) and allylic couplings with the olefinic methyl group H_3-19 (δ 1.65) as well as H_2-11 (δ 2.01). Each of H_2-8 and H_2-11 was further coupled with other methylene groups of the long hydrocarbon chain at δ 1.29–1.31. The HMBC between C-7/H_2-8, C-10/H_2-8, C-9/H_2-11, C-9/H_3-19, C-10/H_2-11, C19/H_2-11, and C-12/H_2-11 helped in the assignment of C-7, and provided further support for the observed ^1H–^1H COSY correlations and determined the partial structure -$CH_2CH_2C(CH_3)$=$CHCH_2CH_2$-.

The final partial structure of $CH_3CH_2CH_2$- was identified from the HMBC from both C-16 (δ 33.08) and C-17 (δ 23.75) to the terminal methyl group H_3-18 (δ 0.89). Therefore, the combined NMR data led to a structural formula of **1** as $CH_3CH_2CH_2(CH_2)_nCH_2CH_2(CH_3)C$=$CHCH_2CH_2(CH_2)_mC$ $H_2CH_2CH_2CH_2COOH$ where n and m could not be determined from the NMR data alone.

Fig. 2. The low-resolution EI mass spectrum of **3** from GC/MS analysis.

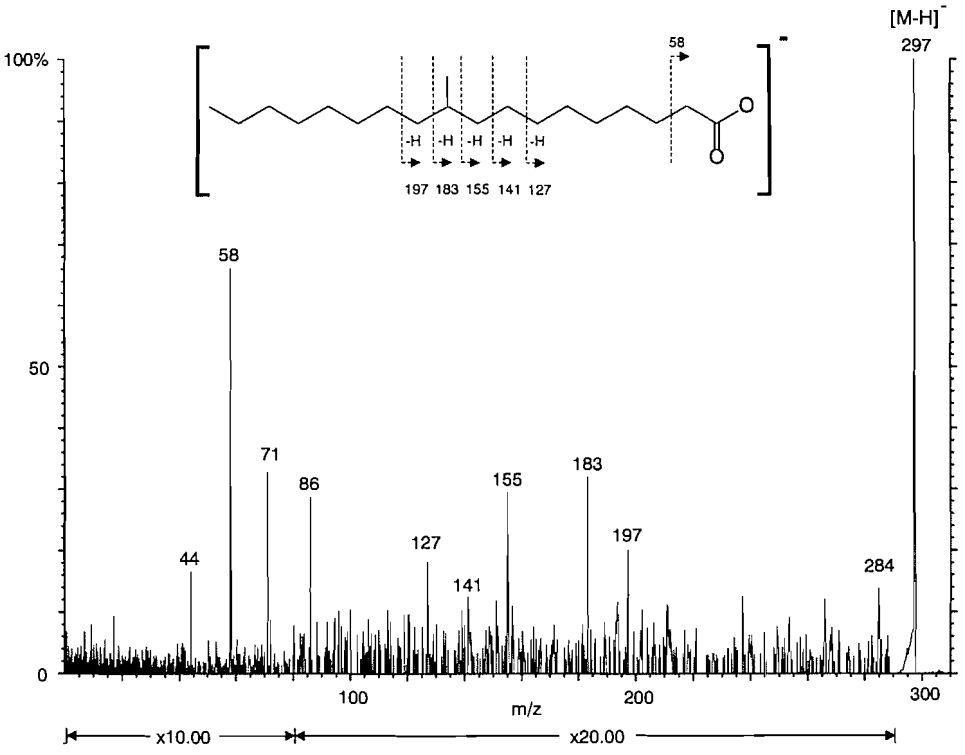


The major difficulties usually encountered in the structure elucidation of long-chain fatty acids by NMR involve the determination of the positions of methyl branching and double bonds. However, location of the double bonds and methyl branching in long-chain fatty acids can be achieved via EIMS analysis of derivatives that strongly localize the charge, for example, on a pyridine ring (2). In this case, the 3-picolinyl ester (**3**) of **1** was prepared (3), and GC/MS analysis of **3** showed the expected molecular ion at *m/z* 387 (Fig. 2). It was anticipated that a diagnostic difference of 40 mass units, corresponding to the trisubstituted double bond -(CH_3)C=CH-, would be found between adjacent ions in the series of charge-remote fragmentations (2). In general, the fragment ions corresponding to vinylic cleavages are of relatively low intensity whereas those due to cleavage at the allylic positions are more intense (2). Furthermore, an enhancement in fragment ion intensity for cleavage at the allylic position on the distal side of the double bond is expected (4). This behavior is clearly seen for **3** in Fig. 2 where the difference of 40 mass units between *m/z* 234 and 274 establishes the location of the double bond. In addition, the enhanced intensity of the peak at *m/z* 288, due to fragmentation at the allylic position, confirms that the weaker peak at *m/z* 274 is indeed due to fragmentation adjacent to the double bond.

Thus, the GC/MS analysis of the 3-picolinyl ester of **1** unambiguously assigned the position of the double bond as Δ^9. However, the data cannot distinguish between a branching position for the methyl group at either C-9 or C-10. To establish this, it was necessary to reduce **1** to the methyl-branched saturated derivative **4**. As previously reported (5), under appropriate conditions for collision-induced decomposition, molecular carboxylate anions of such saturated fatty acids undergo charge-site-remote fragmentations (in an analogous manner to picolinyl ester derivatives of fatty acids in the positive ion mode), resulting in a series of fragment ions characteristic of the alkyl chain. Thus, from the electrospray MS/MS spectrum (Fig. 3) of the $[M - H]^-$ ion of **4** at *m/z* 297, the position of methyl branching was determined by a mass difference of 28 (-$CH(CH_3)$-) rather than 14 (-CH_2-) between the adjacent fragment ions *m/z* 183 and *m/z* 155, proving that the methyl branch is at C-10.

Metabolite **2** was isolated from the CH_2Cl_2 extract from mycelium of *M. olivacea* as a colorless oily compound. The ^1H and ^{13}C NMR data (Table 1) showed signals almost identical with those of **1** except for the additional characteristic signals of a glycerol moiety (H_2-1′: 4.05 (dd), 4.13 (dd); H-2′: 3.80 (m) and H_2-3′: 3.54 (dd), 3.55 (dd)). This suggested that **2** was the glyceride ester of **1**. Further support for this hypothesis was provided by the high-resolution EI mass spectrum, which indicated the expected molecular formula of $C_{22}H_{42}O_4$ plus accurate mass measurement for the fragment ion at *m/z* 278 measured as the formula $C_{19}H_{34}O$ corresponding to the loss of a glycerol molecule. Analysis of **2** by electrospray mass spectrometry in the positive ion mode gave major peaks at *m/z* 371 (MH^+), 393 ($[M + Na]^+$), and 353 ($[MH - H_2O]^+$). The MS/MS spectrum of the MH^+ ion (Fig. 4*a*) shows a major fragment ion at *m/z* 279, once again due to loss of a glycerol moiety, plus a minor fragment at *m/z* 297, which is the protonated fatty acid. The position of the substituted double bond is recognized as Δ^9, the same as that of **1**, from the MS/MS spectrum of the $[M + Na]^+$ ion (Fig. 4*b*). The observed series of charge-remote fragment ions from *m/z* 378 down to *m/z* 294 are due to losses of increasingly large neutral fragments incorporating varying numbers of methylene groups, and the 68 mass unit difference between *m/z* 294 and 226 indicated cleavage at the allylic positions. Interestingly, there is a change in

Fig. 3. The negative ion electrospray MS/MS spectrum of the $[M - H]^-$ ion of **4**.



the dominant fragmentation mode, from simple radical cleavage giving rise to the series from *m/z* 294 to 378 to one involving loss of a hydrogen to give the fragment ions at *m/z* 225, 197, 183, and 169. The position of the methyl branch in **2** was verified by the preparation of the hydrogenation product (**5**) (Fig. 1) and subsequent MS analysis. This gave an $[M - H]^-$ ion at *m/z* 371 in the negative ion electrospray mass spectrum. The MS/MS fragmentation of this ion exhibited only one fragment ion at *m/z* 297, which is the stable carboxylate anion formed by loss of the glyceride moiety. The MS/MS spectrum of this fragment ion at *m/z* 297 (which was present in the electrospray mass spectrum of **5**) shows fragmentation identical to that of **4** (Fig. 3), confirming that the methyl group is located at C-10.

The stereochemistry of the double bond in **1** and **2** was established as *Z* configuration from the NOESY spectrum, which showed the through-space correlations between H_3-19 and H-9, H_3-19 and H_2-12, and between H-9 and H_2-7. Based on these combined data, the structures of 10-methyl-9*Z*-octadecenoic acid and 2,3-dihydroxypropyl-10-methyl-9*Z*-octadecenate were assigned to **1** and **2** respectively (Fig. 1).

Different fatty acids possessing an olefinic methyl branch, such as 7-methyl-7-hexadecenoic acid and 7-methyl-6*E*-hexadecenoic acid, have been reported from whale oil (6) and sponge (7), respectively. To our knowledge, **1** and **2** are the first examples of fungal fatty acids having a methyl branch on a *cis*- double bond. Branched methyls can be introduced in fatty acids through the addition of C_1 units, such as *S*-adenosyl methionine, or by introduction of an isoprenoid unit. This fatty acid probably arose through the former biosynthetic pathway.

Experimental

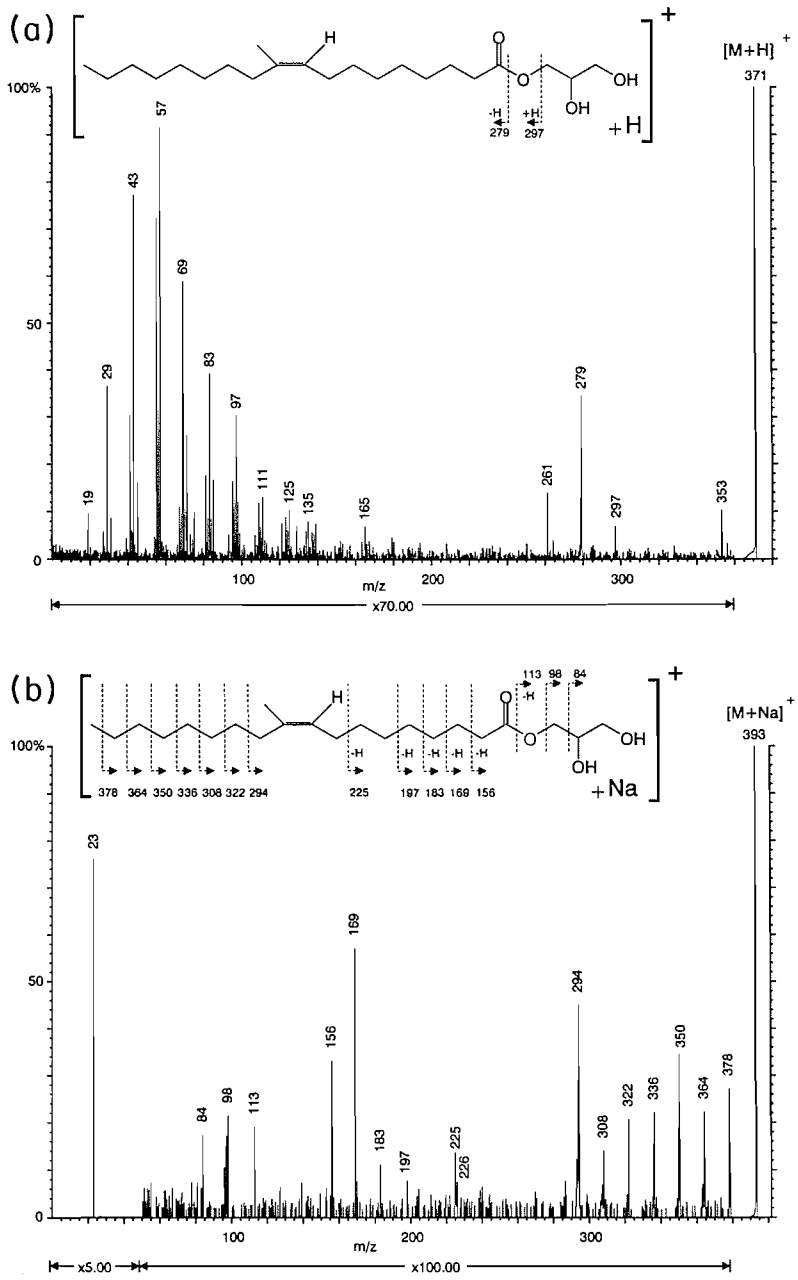
General experimental procedures

NMR spectra were recorded in CD_3OD using a Bruker AMX 500 spectrometer equipped with a Bruker X-32 computer using UXNMR software. Chemical shifts are expressed relative to internal standard (CD_3OD: δ_H 3.30 ppm, δ_C 49.00 ppm). The IR spectrum was measured using a Bomem FT-IR spectrometer (model DA3.02) using a solid film of sample deposited on an AgCl window, and the UV spectrum was obtained using a Hewlett–Packard 1090 HPLC equipped with diode array detector. All EIMS experiments (GC/MS and high-resolution EI) were performed using a VG Organic ZAB-EQ mass spectrometer. GC/MS analysis was carried out on a Hewlett–Packard 5890 Series II GC with a J & W DB225 GC column (30 m × 0.25 mm i.d., 0.25 mm phase thickness). All electrospray and MS/MS experiments were performed using a VG Organic AutoSpec-oaTOF hybrid double-focusing orthogonal acceleration time-of-flight mass spectrometer. MS/MS experiments used a collision energy of 400 eV and Xe collision gas. 3-Pyridylcarbinol was purchased from Aldrich Chemical Company, Milwaukee, Wis.

Fermentation

The producing microorganism identified as *Microsphaeropsis olivacea* was isolated from a marine sponge, *Agelus sp.*, collected off Sombrero Key East, Florida. One loopful of a culture of *M. olivacea* (strain F-10) from an agar slant was used to inoculate five 500-mL Erlenmeyer flasks, each containing media (100 mL) composed of 2.5% glucose and 2.5% pharma-

Fig. 4. The positive ion electrospray MS/MS spectra of **2**: (*a*) $[M + H]^+$, (*b*) $[M + Na]^+$.



media, and incubated on a rotary shaker (210 rpm) for 3 days at 25°C. The broth (5 mL) of this seed culture was inoculated into each of 60 500-mL Erlenmeyer flasks containing production medium (100 mL) composed of 2% molasses, 3% dextrin, 1.5% fish meal, and 1.5% pharmamedia. The fermentation was carried out under the same conditions for 7 days. The culture broth was filtered, respectively, through a cloth filter and a Millipore filter with pore size of 0.8–8.0 μm. The mycelium was lyophilized for the extraction.

Extraction and isolation

*Isolation of **1** from the EtOAc extract of the culture filtrate*
The crude extract (650 mg) was subjected to reversed phase flash column chromatography on Bakerbond® C-18 eluting with an increasing step gradient of methanol in H_2O (60–100%). The fraction eluted with 95% MeOH was rechromatographed on Bakerbond® C-18 eluting with 85% acetonitrile in H_2O. Final purification of **1** was achieved using normal phase flash column chromatography (Merck silica gel 60) and elution with CH_2Cl_2/MeOH (9.3:0.7) to yield 7 mg of **1**.

*Isolation of **2** from the CH_2Cl_2 extract of the mycelia*
This extract (6 g) was obtained through partition of the MeOH extract of the mycelia between H_2O and CH_2Cl_2. It was subjected to flash column chromatography on Merck LiChroprep® RP-18 with gradient elution of MeOH in H_2O (25–100%), and then with MeOH and CH_2Cl_2 (1:1). The fraction

eluted by MeOH was fractionated by a flash column on Bakerbond® C-18 using acetonitrile/H_2O gradient (50–100%). The fraction eluted with 80% acetonitrile was further purified through a flash column on Merck silica gel 60 using hexane and EtOAc (2:3). The fraction containing mainly **2** was finally purified by a flash column on Bakerbond® C-18 eluting with 95% MeOH, to obtain 7.4 mg of the pure metabolite **2**.

Metabolite 1: A colorless oil; R_f value 0.16, TLC on Merck silica gel precoated plate in CH_2Cl_2–MeOH (9.5:0.5); UV (MeOH) λ_{max}: 210 nm; IR (film) cm^{-1}: 3448, 2930, 2858, 1733, 1458. HREIMS, $C_{19}H_{36}O_2$ ($M^{+\bullet}$) *m/z*: 296.2703 (+ΔM 1.2 mmu). ^1H and ^{13}C NMR spectral data are given in Table 1.

Picolinyl derivatization of 1: A small quantity of **1** (0.33 mg) in a 1.0 mL Reacti-Vial™ was treated with 175 μL of thionyl chloride at 100°C for 10 min. The reaction solution was blown down to dryness with a N_2 stream, and then treated with 165 μL of 20% 3-pyridylcarbinol in acetonitrile. The mixture was reacted for 1 min at 100°C. The reaction product (**3**) was identified by GC/MS analysis, *m/z* 387 ($M^{+\bullet}$) and fragment ions shown in Fig. 2.

Hydrogenation of 1: 0.25 mg of **1** was hydrogenated in MeOH over a catalytic amount of PtO_2. The hydrogenation product (**4**) was directly analysed by negative ion electrospray MS: *m/z*: 297 ($[M - H]^-$) and fragment ions shown in Fig. 3.

Metabolite 2: A colorless oil; R_f value 0.23, TLC on Merck silica gel precoated plate in CH_2Cl_2–MeOH (9.5:0.5); UV (MeOH) λ_{max}: 210 nm; IR (film) cm^{-1}: 3400, 2925, 2855, 1740, 1458. HREIMS, $C_{22}H_{42}O_4$ ($M^{+\bullet}$) *m/z*: 370.3085 (ΔM –0.2 mmu), $C_{19}H_{34}O$ $[(M - C_3H_8O_3)^{+\bullet}]$ *m/z* 278.2612 (ΔM –0.3 mmu). Positive ion electrospray MS gave *m/z* 371 (MH^+), 393 ($[M + Na]^+$), and 353 ($[MH - H_2O]^+$). The MS/MS spectra of *m/z* 371 and 393 are shown in Fig. 4. ^1H and ^{13}C NMR spectral data are given in Table 1.

Hydrogenation of 2: 0.50 mg of **2** was hydrogenated in MeOH over a catalytic amount of PtO_2. The hydrogenation product (**5**) was directly analysed by negative ion electrospray, MS *m/z* (%): 371 ($[M - H]^-$, 100), 297 (23). The MS/MS spectrum of the fragment ion *m/z* 297 is virtually identical to that given as Fig. 3 for M^- of **4**.

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References

1. W. Fenical and P.R. Jensen. *In* Marine biotechnology. Vol. 2. Pharmaceutical and bioactive natural products. *Edited by* D.H. Attaway and O.R. Zaborsky. Plenum Press, New York. 1993. pp. 419–457.
2. D.J. Harvey. Spectrosc. Int. J. **8**, 211 (1990).
3. D.J. Harvey. Biomed. Mass Spectrom. **9**, 33 (1982).
4. W.W. Christie, E.Y. Brechany, and R.T. Holman. Lipids, **22**, 224 (1987).
5. K.B. Tomer, F.W. Crow, and M.L. Gross. J. Am. Chem. Soc. **105**, 5487 (1983).
6. J.C. Pascal and R.G. Ackman. Lipids, **10**, 478 (1975).
7. N.M. Carballeira and J. Restituyo. J. Nat. Prod. **54**, 315 (1991).