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LINEAR DITERPENES FROM THE CARIBBEAN SPONGE *MYRMEKIODERMA STYX*

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ABSTRACT.—Four new oxygenated diterpenes **1–4** were isolated from the Caribbean sponge *Myrmekioderma styx*. The structures of these compounds were deduced by ms, uv, ir, ^{1}H- and ^{13}C-nmr, ^{1}H-^{1}H COSY, XHCORR, and COLOC experiments. Compounds **1–4** are lethal to brine shrimp (*Artemia salina*).

Diterpenes are the most abundant non-steroidal secondary metabolites isolated from the marine environment. They are widely distributed among seaweeds and coelenterates of the order Alcyonaceae (soft corals and sea pens), while their occurrence is relatively limited among Porifera (1,2). In this phylum they are not uniformly distributed but are most commonly found in species belonging to the orders Dictyoceratida and Dendroceratida (3).

Structurally, the diterpenes from sponges possess polycarbocyclic skeletons, which sometimes are very degraded with loss of one or two carbon atoms to give nor or bis-nor derivatives. Simple linear diterpenoids, biogenetically closer to geranylgeraniol, have been found in just two species, which belong to the genera *Didiscus* (4) and *Halicondria* (5).

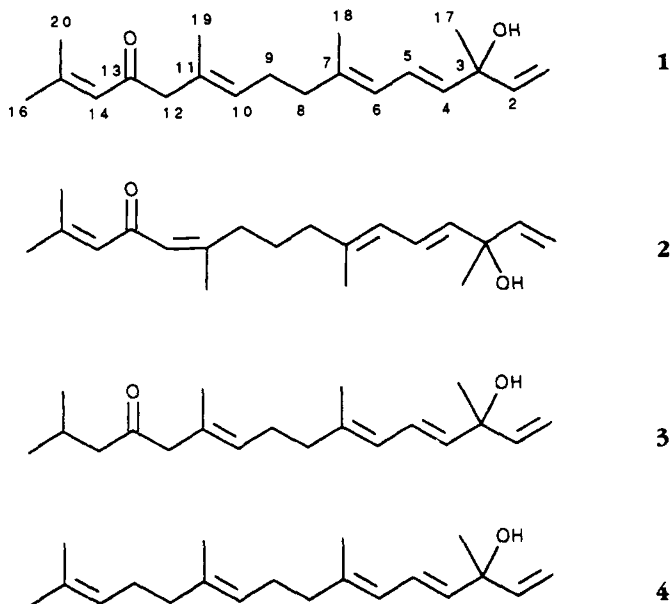
In the course of our continuing studies for bioactive constituents of marine organisms, we have been investigating the sponge *Myrmekioderma styx* De Laubenfels (family Halichondridae, order Halichondrida), a yellowish massive sponge of crumbly consistency, collected at a depth of 18 m along the coast of San Salvador Island (Bahamas).

We report here that this organism also elaborates oxygenated linear diterpenes, which showed cytotoxic activity. Interestingly, Van Soest *et al.* (6) have recently suggested assigning the genera *Didiscus* and *Myrmekioderma* into the same family (Halicondridae), based on interpretation of their characters that suggest a close phylogenetic relationship. This assumption now seems to be chemotaxonomically corroborated, since species of the genera produce quite similar diterpenoid metabolites. These similarities, however, refer to the comparison of *M. styx* with two sponges identified at generic level: a *Didiscus* from Belize (4) and a *Halicondria* from Oahu (5). Pomponi *et al.* (7), on the contrary, who examined a wide spectrum of halicondrid species, on the basis of tlc patterns of the extracts, did not find similar affinities comparing *M. styx* with *Didiscus oxeata* specimens coming from Belize and Venezuela. Therefore the affinities between the two genera, which undoubtedly exist, should be chemically verified at species level in the future.

RESULTS AND DISCUSSION

Freshly collected specimens of *M. styx* were stored frozen and subsequently extracted with MeOH-toluene (3:1). The EtOAc-soluble material from the extract was chromatographed on Si gel and subsequently purified by hplc (SiO_2) leading to pure compounds **1–4**, whose structures were deduced from spectroscopic data.

Compound **1** was the major metabolite isolated from the sponge, comprising 2% of the organic extract; it was isolated as uv-absorbing (λ max 240 nm) and optically active



($[\alpha]^{25}$D $+13.4°$) oil. The molecular formula $C_{20}H_{30}O_2$ was determined for this metabolite by hrms analysis ($[M]^+$ *m/z* 302.2176) and by interpretation of the ^{13}C-nmr spectrum. This molecular composition demanded the presence of 6 unsaturations, which could be satisfied by 1 carbonyl function and 5 olefinic double bonds, as suggested by the ^{13}C-nmr spectrum, which contained eleven low field signals at δ 199.23 (C=O), 155.55 (C), 143.86 (CH), 138.70 (C), 136.35 (CH), 129.72 (C), 128.56 (CH), 124.17 (CH), 124.08 (CH), 122.60 (CH), and 111.76 (CH_2). The frequency of the C=O stretching vibration (1684 cm^{-1}) in the ir spectrum and the chemical shift of the pertinent carbon (δ 199.23) in the ^{13}C-nmr spectrum implied that the carbonyl function belongs to an α,β-unsaturated ketone. The ir band at 3420 cm^{-1} and the intense ion at *m/z* 284.2157 $[M-H_2O]^+$ in the mass spectrum suggested that the functional group incorporating the second oxygen atom of **1** was a hydroxyl group, which must be linked to a trisubstituted carbon atom as indicated by the presence of a quaternary carbon resonance at δ 73.04.

Consideration of the characteristics of the ^{1}H-nmr spectrum of **1** and ^{1}H-^{1}H connectivities observed in ^{1}H-^{1}H decoupling experiments and in the COSY spectrum showed that all the protons in **1** belong to four isolated spin systems. With the help of XHCORR data (all the proton-bearing carbons and their protons were precisely matched), the four units were confidently identified as the part structures A (C-1/C-2), B (C-4/C-12, C-18, C-19), C (C-14/C-16, C-20), and D (C-17). A long range carbon-proton correlation COLOC experiment (see Table 1) allowed the partial structures to be combined through the unprotonated C-3 and C-13 carbon atoms. The carbonyl carbon was coupled to the protons at δ 3.00 (H_2-12) and δ 6.08 (H-14), allowing partial structure C to be connected to substructure B through C-13. The C-3 quaternary carbon atom at δ 73.04 and the C-2 and C-4 olefinic methine carbon atoms (δ 143.86 and 136.85, respectively) were observed to be coupled to the H_3-17 methyl protons resonating at δ 1.37. Consequently the gross structure of **1** was formulated as 3-hydroxy-3,7,11,15-tetramethyl-1,4,6,10,14-hexadecapentaen-13-one. The mass spectrum, which showed the ions reported in the Experimental, fully agrees with the proposed structure.

Further consideration of ^{1}H- and the ^{13}C-nmr spectra allowed the stereochemistry

TABLE 1. COLOC Correlation Data of Compounds **1**, **2**, and **3**.

Carbon	Compound		
	1	**2**	**3**
C-1			
C-2	H_3-17	H_3-17	H_3-17
C-3	H_3-17	H_3-17	H_3-17
C-4	H-6, H_3-17	H_3-17	H_3-17
C-5			
C-6	H_3-18	H-4, H_3-18	
C-7	H_3-18	H_3-18	H_3-18
C-8	H_3-18	H_3-18	
C-9		H_2-10	
C-10	H_2-12, H_3-19	H-12, H_3-19	H_2-12, H_3-19
C-11	H_2-12, H_3-19	H_2-10, H_3-19	H_2-12, H_3-19
C-12	H_3-19	H_3-19	
C-13	H_2-12, H-14	H-12	H_2-12
C-14	H_3-16	H_3-16, H_3-20	
C-15	H_3-16	H_3-16, H_3-20	
C-16	H-14, H_3-20	H-14, H_3-20	
C-17	H-4		
C-18	H-6	H-6	
C-19	H-12		
C-20	H-14, H_3-16	H-14, H_3-16	

of the double bonds to be determined. The high field chemical shifts of C-18 and C-19 (δ 16.49 and 16.18, respectively) in the ^{13}C-nmr spectrum (indicative of the shielding effect of a cis alkenyl group) evidenced the *E* configuration of the Δ^6 and Δ^{10} double bonds (8). Finally, the stereochemistry of the Δ^4 double bond was assigned as *E* on the basis of the large coupling constant (14.7 Hz) between H-4 and H-5 observed in the ^{1}H-nmr spectrum.

Three further metabolites, closely related to **1**, were also isolated from the *M. styx* extracts. The structures of these compounds were assigned by spectral analysis and by analogy to the data for **1**.

Compound **2** $[\alpha]^{25}$D +14.2; λ max 243 nm; ν max 1667 cm^{-1} (α,β- α′,β′-unsaturated ketone) and 3420 cm^{-1} (OH) possesses the molecular formula $C_{20}H_{30}O_2$, determined on the basis of ^{13}C nmr and hrms, $[M]^+$ *m/z* 302.2269, indicating that **2** is an isomer of **1**, which it resembles in the mass fragmentation (see Experimental). Additional analogies between the two products were revealed by a comparison of their ^{1}H-nmr spectra, which were quite similar, but showed significant differences in the chemical shifts and multiplicities of a few key protons. The broad singlet and the vinylic triplet resonating in the proton spectrum of **1** at δ 3.00 (H_2-12) and 5.18 (H-10), respectively, were replaced in the spectrum of **2** by a singlet at δ 6.01 and a triplet at δ 2.57. Furthermore, a significant downfield shift (from δ 1.59 in **1** to δ 1.85 in **2**) for the methyl singlet assigned in **1** to H_3-19 was observed. These dissimilarities appeared to be consistent with a change of the double bond location from C-10 in **1** to C-11 in **2**. A comparison of ^{13}C-nmr data of **2** and those of the corresponding carbon atoms of **1** was used to verify that **2** represented the correct structure. The observed chemical shift dissimilarities (Table 2) afforded an excellent confirmation that a double-bond isomerization differentiated the two compounds.

The stereochemistry of the Δ^{11} double bond in **2** was assumed to be *Z* due to the observed chemical shift value in the ^{1}H-nmr spectrum of the H_3-19 signal (δ 1.85), which agrees with reported resonances of methyl groups trans to a carbonyl group while

TABLE 2. Nmr Spectral Data of Compounds **1**, **2**, **3**, and **4**.[a]

Position	Compound 1		Compound 2		Compound 3		Compound 4
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)
1 cis	5.04 (d, 10.6)	111.76 (CH_2)	5.05 (d, 10.6)	111.95 (CH_2)	5.06 (d, 10.6)	112.02 (CH_2)	5.06[b]
1 trans	5.22 (d, 17.3)		5.24 (d, 17.3)		5.24 (d, 17.3)		5.24 (d, 17.3)
2	5.99 (dd, 10.6, 17.3)	143.86 (CH)	5.95 (dd, 10.6, 17.3)	143.95 (CH)	5.97 (d, 10.6, 17.3)	143.93 (CH)	5.97 (dd, 10.6, 17.3)
3		73.04 (C)		73.26 (C)		73.29 (C)	
4	5.68 (d, 15.2)	136.35 (CH)	5.65 (d, 15.3)	136.14 (CH)	5.67 (d, 15.3)	136.44 (CH)	5.67 (d, 15.3)
5	6.43 (dd, 10.6, 15.2)	124.17 (CH)	6.50 (dd, 10.8, 15.3)	124.51 (CH)	6.44 (dd, 15.3, 10.8)	124.41 (CH)	6.44 (dd, 15.3, 10.8)
6	5.81 (d, 10.6)	124.08 (CH)	5.83 (d, 10.8)	124.04 (CH)	5.82 (d, 10.8)	124.21 (CH)	5.81 (d, 10.8)
7		138.70 (C)		139.52 (C)		139.01 (C)	
8	2.08[b]	39.37 (CH_2)	2.10[b]	39.88 (CH_2)	2.09[b]	39.50 (CH_2)	2.08[b]
9	2.15[b]	26.36 (CH_2)	1.59[b]	26.31 (CH_2)	2.17[b]	26.45 (CH_2)	2.12[b]
10	5.18[b]	128.46 (CH)	2.57 (d, 7.8)	33.26 (CH_2)	5.23[b]	129.02 (CH)	5.08[b]
11		129.72 (C)		158.01 (C)		129.29 (C)	
12	3.00 (s)	55.16 (CH_2)	6.02 (s)	126.36 (CH)	2.99 (s)	54.40 (CH_2)	1.98 (t, 7.0)
13		199.23 (C)		191.04 (C)		209.50 (C)	2.05[b]
14	6.08 (s)	122.60 (CH)	6.03 (s)	126.22 (CH)	2.27 (d, 6.9)	50.53 (CH_2)	5.08[b]
15		155.55 (C)		154.10 (C)	2.11[b]	24.41 (CH)	
16	1.84 (s)	27.56 (Me)	1.86 (s)	27.68 (Me)	0.88 (d, 6.6)	22.55 (Me)	1.68 (s)
17	1.37 (s)	27.87 (Me)	1.38 (s)	28.05 (Me)	1.39 (s)	28.09 (Me)	1.39 (s)
18	1.74 (s)	16.49 (Me)	1.76 (s)	16.59 (Me)	1.75 (s)	16.66 (Me)	1.75 (s)
19	1.59 (s)	16.18 (Me)	1.85 (s)	25.37 (Me)	1.59 (s)	16.40 (Me)	1.59 (s)
20	2.10 (s)	20.51 (Me)	2.14 (s)	20.51 (Me)	0.88 (d, 6.6)	22.55 (Me)	1.60 (s)

[a]Assignment based on DEPT, COSY, HETCOSY and COLOC experiments.
[b]Submerged by other signals.

a cis relationship would require a lower field signal (9). Further supporting evidence for this conclusion was obtained by the ^{13}C-nmr resonance of C-19 (δ 25.37) (8).

A further related diterpenoid, **3**, $[\alpha]^{25}$D +12.3; λ max 241 nm, has a molecular formula of $C_{20}H_{32}O_2$ determined by hrms, $[M]^+$ *m/z* 304.2419, and ^{13}C-nmr data (Table 2). The ir spectrum showed a band in the OH region (3420 cm^{-1}) and a carbonyl absorption at 1708 cm^{-1}. A proton-proton COSY nmr experiment and comparison of overall ^{1}H- and ^{13}C-nmr data revealed similarities between **3** and **1**. However, significant changes in both ^{1}H- and ^{13}C-nmr signals, associated with the head isoprene unit, were observed. In the ^{1}H-nmr spectrum of **3** the methyls H_3-16 and H_3-20 resonate in the upfield region at δ 0.89 as a 6H doublet, coupled with a methine signal of δ 2.13 (H-15), which is in turn coupled with a 2H sharp doublet resonating at δ 2.26 (H_2-14). The above spectral features, together with the ir absorption band at 1708 cm^{-1}, attributable to a nonconjugated ketone, and consideration of the molecular formula clearly indicated that **3** was the 14,15-dihydro derivative of **1**. Support for the proposed structure **3** was gained by the fragmentation in the mass spectrum (see Experimental) and by the interpretation of the ^{13}C-nmr spectrum, whose resonances were assigned as reported in Table 2 with the aid of one-bond and long-range carbon-proton correlation experiments (see Table 1).

Finally the less polar compound **4**, isolated in very low yields as an optically active oil, $[\alpha]^{25}$D +13.1°, has the molecular formula $C_{20}H_{32}O$ from the hrms. The presence of an OH group was indicated from the ir absorption at 3420 cm^{-1}, while the uv band at λ max 241 nm provided evidence for a conjugated double bond. Because the limited availability of the compound, structure **4** was based on the interpretation of the 500 MHz ^{1}H-nmr spectrum. It was very similar to that of **1**, differing from the latter only in the resonances of the head isoprene unit, where the C-13 carbonyl is replaced by a methylene group. This was deduced from the analysis of the ^{1}H-^{1}H COSY spectrum which clearly delineated a large spin system accounting for the whole molecule apart from the tail end (C-1/C-3 and C-17). The proton resonances of the C-12/C-16, C-20 unit, in particular, were assigned as follows. The broad triplet resonating at δ 1.98 (2H, H_2-12, J=7 Hz) exhibited long range coupling to the H-10 and vicinal coupling to the H_2-13 multiplet at δ 2.08, that is in turn coupled to the vinylic H-14 resonating at δ 5.11, allylically coupled to H_3-16 and H_3-20. Compounds **1–3** showed significant toxicity toward brine shrimp (*Artemia salina*) (10). The determined LC_{50} were 154 μg/ml for **1**, 163 μg/ml for **2**, and 3 μg/ml for **3**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All ^{1}H and ^{13}C nmr were performed in $CDCl_3$ on a Bruker AMX-500 spectrometer. ^{1}H- and ^{13}C-nmr chemical shifts are referenced to the solvent signal (δ 7.26 and 77, respectively). Methyl, methylene, and methyne carbons were distinguished by a DEPT experiment. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined with an XHCORR pulse sequence optimized for $^1J_{CH}$ of 135 Hz. Two- and three-bond ^{1}H-^{13}C connectivities were determined by a COLOC experiment, optimized for $^{2,3}J_{CH}$ of 8 Hz. Ms spectra were recorded on a Kratos MS80 mass spectrometer at 70 eV. Uv spectra were performed on a Beckman DU70 spectrometer in EtOH solution. Ft-ir spectra were recorded on a Bruker IFS-48 spectrophotometer with $CHCl_3$. Optical rotations were measured on a Perkin-Elmer 192 polarimeter with *n*-hexane, using a 10-cm microcell.

Medium-pressure liquid chromatography (mplc) was performed on a Buchi 861 apparatus using an SiO_2 (230–400 mesh) column. Hplc was performed on a Varian 880-PU apparatus with an RI-4 refractive index detector.

EXTRACTION AND ISOLATION OF DITERPENES **1–4**.—The yellow-orange sponge *M. styx* was collected (depth 18 m) in the summer of 1990 along the coast of San Salvador Island, Bahamas, and was stored frozen at −18°. Reference specimens are deposited at the Dipartimento di Chimica delle Sostanze Naturali dell' Università di Napoli.

The sponge (100 g dry wt after extraction) was homogenized and extracted with MeOH-toluene (3:1) (3×600 ml). The combined MeOH/toluene solutions, after filtration, were concentrated in vacuo to an aqueous suspension which was partitioned with EtOAc (4×250 ml). The organic phase, after evaporation of EtOAc, afforded 7 g of an orange brown oil that showed activity on *A. salina*. This residue was chromatographed by mplc on a Si gel column (Merck, 110 g) using a stepwise gradient from 0 to 100% EtOAc in *n*-hexane, and the fractions of similar composition, as determined by tlc analysis, were pooled and bioassayed.

The fractions eluting at 10% EtOAc, which exhibited cytotoxic activity, were purified by hplc using a Hibar Si 60 LiChrospher 5 μm (4×250 mm) column [*n*-hexane–THF (85:15)] to afford **4** (0.8 mg). Combined fractions eluting at 20% EtOAc, which exhibited cytotoxic activity, afforded a mixture of **1**, **2**, and **3**. This mixture was separated by hplc using a Hibar Si 60 Lichrospher 10 μm (10×250 mm) column [*n*-hexane–THF (80:20)] to obtain **1** (700 mg), **2** (170 mg), and **3** (67 mg).

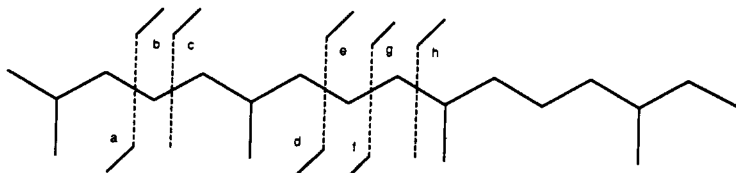


FIGURE 1. Skeleton fragmentations in the mass spectrum of the compounds **1–4**.

(4E, 6E, 10E)-3-Hydroxy-3,7,11,15-tetramethyl-1,4,6,10,14-hexadecapentaen-13-one [**1**].—$[\alpha]^{25}D$ +13.4° (c=0.091, *n*-hexane); uv λ max (EtOH) 240 nm (ε 18615); ir ν max ($CHCl_3$) 3420, 1686 cm^{-1}; ^1H nmr ($CDCl_3$) see Table 2; ^{13}C nmr ($CDCl_3$) see Table 2; hrms *m/z* (assignment, rel int, for skeleton fragmentations see Figure 1) $[M]^+$ 302.2176 $C_{20}H_{30}O_2$ requires 302.2238, 0.37), $[M-H_2O]^+$ 284.2157 ($C_{20}H_{28}O$, 3.18), $[b-H_2O]^+$ 201.1657 ($C_{15}H_{21}$, 2.78), $[c-H_2O]^+$ 187.1437 ($C_{14}H_{19}$, 1.03), [g and/or f]$^+$ 151.1039 ($C_{10}H_{15}O$, 1.66), $[h]^+$ 137.0960 ($C_9H_{13}O$, 0.97), $[g-H_2O]^+$ 133.1022 ($C_{10}H_{13}$, 1.34), $[h-H_2O]^+$ 119.0865 (C_9H_{11}, 3.54), $[a]^+$ 83.0563 (C_5H_7O, 100).

(4E, 6E, 11Z)-3-Hydroxy-3,7,11,15-tetramethyl-1,4,6,11,14-hexadecapentaen-13-one [**2**].—$[\alpha]^{25}D$ +14.2° (c=0.007, *n*-hexane); uv λ max (EtOH) 243 nm (ε 27170); ir ν max ($CHCl_3$) 3420, 1667 cm^{-1}; ^1H nmr ($CDCl_3$) see Table 2; ^{13}C nmr ($CDCl_3$) see Table 2; hrms *m/z* (assignment, rel int for skeleton fragmentations see Figure 1) $[M]^+$ 302.2269 ($C_{20}H_{30}O_2$ requires 302.2238, 0.44), $[M-H_2O]^+$ 284.2137 ($C_{20}H_{28}O$, 13.26), $[b-H_2O]^+$ 201.1651 ($C_{15}H_{21}$, 10.66), [g and/or f]$^+$ 151.1117 ($C_{10}H_{15}O$, 5.72), $[e-H_2O]^+$ 147.1182 ($C_{11}H_{15}$, 30.14), $[g-H_2O]^+$ 133.1011 ($C_{10}H_{13}$, 5.42), $[a]^+$ 83.0487 (C_5H_7O, 100).

(4E, 6E, 10E)-3-Hydroxy-3,7,11,15-tetramethyl-1,4,6,10-hexadecatetraen-13-one [**3**].—$[\alpha]^{25}D$ +12.3° (c=0.013, *n*-hexane); uv λ max (EtOH) 241 nm (ε 21529); ir ν max ($CHCl_3$) 3420, 1708 cm^{-1}; ^1H nmr ($CDCl_3$) see Table 2; ^{13}C nmr ($CDCl_3$) see Table 2; hrms, *m/z* (assignment, rel int, for skeleton fragmentations see Figure 1) $[M]^+$ 304.2419 ($C_{20}H_{32}O_2$ requires 304.2394, 11.5), $[M-H_2O]^+$ 286.2308 ($C_{20}H_{30}O$, 38.26), $[c]^+$ 205.1595 ($C_{14}H_{21}O$, 12.48), $[b-H_2O]^+$ 201.1648 ($C_{15}H_{21}$, 21.98), $[c-H_2O]^+$ 187.1461 ($C_{14}H_{19}$, 13.84), $[g]^+$ 151.1131 ($C_{10}H_{15}O$, 30.26), $[h]^+$ 137.0950 ($C_9H_{13}O$, 16.42), $[g-H_2O]^+$ 133.1017 ($C_{10}H_{13}$, 100), $[h-H_2O]^+$ 121.1018 (C_9H_{13}, 46.13).

(4E, 6E, 10E)-3-Hydroxy-3,7,11,15-tetramethyl-1,4,6,10-hexadecapentaene [**4**].—$[\alpha]^{25}D$ +13.1° (c=0.001, *n*-hexane); uv λ max (EtOH) 241 nm (ε 21600); ir ν max ($CHCl_3$) 3420 cm^{-1}; ^1H nmr ($CDCl_3$) see Table 2; ^{13}C nmr ($CDCl_3$) see Table 2; hrms *m/z* (assignment, rel int, for skeleton fragmentations see Figure 1) $[M]^+$ 288.2455 ($C_{20}H_{32}O$ requires 288.2445, 2), $[f]^+$ 137.1328 ($C_{10}H_{17}$, 100), $[g]^+$ 133.1004 ($C_{10}H_{13}$, 40), $[d]^+$ 123.1177 (C_9H_{15}, 3), $[e]^+$ 147.1148 ($C_{11}H_{15}$, 35).

BIOLOGICAL ASSAYS.—Brine shrimp (*A. salina*) assays were performed in triplicate DMSO (1% of final volume, using 10 animals suspended in artificial sea water, as reported by Meyer *et al.* (10). For each dose tested, survivor shrimp were counted after 24 h and data were statistically analyzed by the Finney program (11), which yields LC_{50} values with 95% confidence intervals.

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