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CYTOTOXIC HATERUMADIENONE CONGENERS FROM THE OKINAWAN MARINE SPONGE *DYSIDEA* SP.

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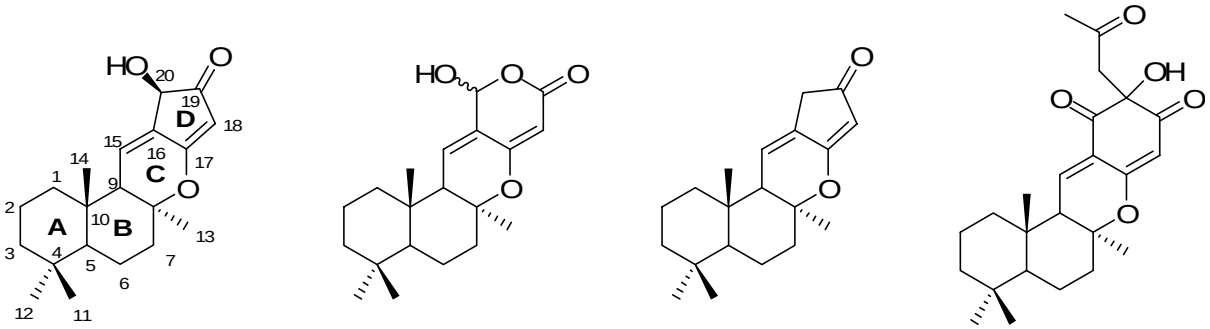
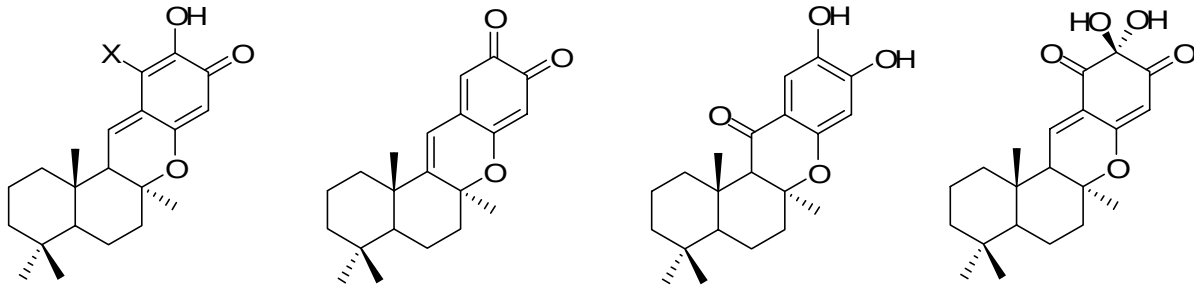
Abstract - 20-Hydroxyhaterumadienone (**1**) and haterumadienelactol (**2**), new haterumadienone (**3**) congeners, were isolated from the sponge *Dysidea* sp. together with puupehenone (**6**) and three known metabolites (**7**-**9**) related to **6.** The absolute stereochemistry of **1** and the relative stereochemistry of **2** were determined by spectroscopic and chemical analyses. The metabolites (**1** and **2**) inhibited the division of fertilized sea urchin eggs.

INTRODUCTION

Marine sponges are a rich source of structurally unique and biologically active terpenoids. Puupehenone (**6**), which was first isolated in 1979 from a Hawaiian sponge,[1] and its derivatives are an important group of marine metabolites because they display a wide range of properties including cytotoxic, antiviral, antitumor, antifungal, antimalarial, antituberculosis and immunomodulatory activities.[1-10] As part of our continuing chemical studies on Okinawan marine organisms, we examined the constituents of the sponge *Dysidea* sp., whose extract strongly inhibited the cell division of fertilized sea urchin eggs. In our recent report, we described the isolation and structure elucidation of haterumadienone (**3**) and the artificial acetone adducts (**4** and **5**) from the toxic extract of the sponge.[10] Further bioassay- and ^{1}H NMR-guided fractionation of the same extract led to the isolation of 20-hydroxyhaterumadienone (**1**),



1 **2** **3** **4** and **5**



6: X = H **7:** X = Cl **8** **9** **10**

haterumadienelactol (**2**) along with the known (+)-puupehenone (**6**),[1,3,8,12] chloropuupehenone (**7**),[3] puupehedione (**8**),[3] and 15-oxopuupehenol (**9**).[4] The relative structures of the **A**-, **B**- and **C**-ring moieties in **1** and **2** were established by NOE experiments and coupling constant analysis. The relative stereochemistry of **1** at C20 was deduced from a series of NOEDS spectral analyses of the (*S*)- and (*R*)-MTPA esters and the absolute stereochemistry of C20 was determined using the modified Mosher's method.[11] The absolute stereochemistry of the **A**-, **B**- and **C**-ring moieties of **2** was tentatively deduced to be as depicted in **2** based on an assumption regarding the biosynthetic relationship between **1**-**9**. The known compounds were identified by comparison of their spectral data with values in the literature. Puupehenone (**6**) was the major constituent of the sponge. In this report, we have described the isolation, structure elucidation and biological activity of **1** and **2**, along with detailed experimental procedures for **3**, **4** and **5**.

RESULTS AND DISCUSSION

The yellow encrusting sponge (750 g) was collected by hand from the coast of Hateruma Island, Okinawa, and stored at –15 °C before being extracted with acetone. The acetone extract was initially partitioned between ethyl acetate and water. The cytotoxic EtOAc-soluble material was triturated with hexanes and filtered. The hexane-insoluble part was fractionated by silica-gel column chromatography with a gradient from hexanes to EtOAc to MeOH, followed by ODS HPLC (MeOH) to furnish 20-hydroxyhaterumadienone (**1**, 0.0008% of wet weight), haterumadienelactol (**2**, 0.00066%) and acetone

Table 1. NMR data for **1**, **2** and **3**.

	1[a]		**2**[a]		**3**[b]	
C no.	^{13}C	^{1}H (Hz)	^{13}C	^{1}H (Hz)	^{13}C	^{1}H (Hz)
1	39.5	1.10 dt (4.0, 12.5)	39.8, 39.7[c]	1.14 m, 1.63 m	39.4	0.67 dt (5.5, 12.5)
		1.72 brd (12.5)				1.26 m
2	18.0	1.40 m	18.0	1.40 m	18.3	1.25 m
3	41.6	1.16 dt (4.0, 12.5)	41.6, 41.5[c]	1.15 m, 1.39 m	41.8	1.02 dt (4.0, 13.5)
		1.42 m				1.31 m
4	33.1		33.1		33.1	
5	54.1	0.94 dd (2.5, 12.5)	53.8	0.92 m	53.7	0.56 dd (2.0, 11.5)
6	18.3	1.54 m	18.2	1.55 m	18.4	1.25 m, 1.36 m
7	39.2	1.65 dt (5.0, 14.5)	38.8, 38.9[c]	1.55 m, 2.16 m	39.4	1.15 dt (5.0, 14.0)
		2.22 td (3.0, 14.5)				1.96 td (3.0, 14.0)
8	81.6		78.4, 78.5[c]		80.1	
9	53.6	1.98 d (6.5)	54.8, 54.6[c]	1.96 d (7.0)	53.8	1.31 d (6.5)
				1.98 d (7.0)[c]		
10	40.1		40.1, 40.0[c]		39.8	
11	21.9	0.82 s	21.8	0.83 s	22.0	0.74 s
12	33.6	0.89 s	33.6, 33.1[c]	0.89 s	33.6	0.81 s
13	28.9	1.26 s	28.3, 28.2[c]	1.24 s, 1.18 s[c]	28.5	0.94 s
14	14.3	0.78 s	14.6, 14.5[c]	0.78 s, 0.81 s[c]	14.5	0.68 s
15	123.3	6.26 d (6.5)	133.3, 132.6[c]	6.41 d (6.0)	121.2	5.24 d (6.5)
				6.55 d (6.0)[c]		
16	135.1		128.2		132.9	
17	177.8		161.5, 162.4[c]		177.9	
18	105.0	5.34 brs	93.8, 94.3[c]	5.28 s, 5.25 s[c]	110.1	5.65 s
19	202.1		166.6		199.2	
20	70.8	4.60 s	94.1, 93.6[c]	6.05 s, 6.15 s[c]	37.8	2.78 d (20.0)
						2.82 d (20.0)

[a] Recorded at 500MHz ^{1}H NMR and 125 MHz ^{13}CNMR in $CDCl_3$. [b] Recorded at 500MHz ^{1}H NMR and 125 MHz ^{13}C NMR in C_6D_6.
[c] Values of a minor compound at equilibrium.

adducts (**4** and **5**) as a mixture [**4**/**5** (10:9)] together with the known puupehedione (**8**, 0.00067%) and 15-oxopuupehenol (**9**, 0.0004%). This mixture was then purified by repeated ODS HPLC with 30% H_2O/MeOH to give pure **4** (0.00033%) and **5** (0.0004%). Silica gel chromatography (hexanes/EtOAc gradient) of the hexane-soluble part and subsequent ODS chromatography (10% H_2O/MeOH) yielded haterumadienone (**3**, 0.0019%), puupehenone (**6**, 0.05%) and chloropuupehenone (**7**, 0.00067%).

Analysis of **1** by ^{13}C NMR (Table 1) and HRESIMS [*m/z* 339.1947$(M+Na)^+$, calcd for $C_{20}H_{28}O_3$ Na, 339.1936] gave a formula of $C_{20}H_{28}O_3$, which accounted for seven degrees of unsaturation. The ^{1}H NMR signals in the aliphatic region were almost identical to those of puupehenone (**6**). Compound (**1**) showed one less carbon than **6**. Detailed analysis of the UV [λ_{max} 284 (ε 20000) nm], IR (ν_{max} 3314, 2926, 1660, 1594, 1419 and 1164 cm^{-1}) and NMR data (Table 1) indicated the presence of a conjugated ketone (δ_C 202.1), a conjugated diene [δ_C 123.3 (d), 135.1 (s), δ_H 6.26 (d, *J*=6.5 Hz) and δ_C 177.8 (s), 105.0 (d), δ_H 5.34 (d, brs)], an oxygenated quarternary sp^3 carbon (δ_C 81.6), an isolated oxygenated methine (δ_C 70.8, δ_H 4.60) and four singlet methyls (δ_C 21.9, 33.6, 28.9, 14.3 ; δ_H 0.82, 0.89, 1.26, 0.78). Of the four methyls, two were part of a *gem*-dimethyl group as indicated by HMBC correlations of $H_3$11/C12,

$H_3$12/C11, $H_3$11/C4 and $H_3$12/C4, one could be attached to the oxygenated quarternary sp^3 carbon at δ_C 80.1 based on an HMBC correlation between $H_3$13 and C8, and another was located at a bridge-head position based on an HMBC correlation of $H_3$14 to C10. The major spin systems (**a**, **b** and **c**) were revealed to be as shown in Figure 1 based on ^{1}H-^{1}H COSY and HMBC data. The partial structures (**a**, **b** and **c**) and other fragments (C11-C4-C12, C10-C14, C13-C8 and C16-C18) were connected using HMBC correlations to give the planar structure of the **A**-, **B**- and **C**-ring moieties (Figure 1). Since this and the presence of a ketone accounted for six of the seven degrees of unsaturation, **1** is tetracyclic. At this point in the structure determination, three fragments (an oxygenated methine, a ketone and an sp^2 methine) were identified, but not assembled. Therefore, the one remaining degree of unsaturation corresponds to the presence of a cyclopentenone ring, which was confirmed by HMBC correlations of H18/C16, H18/C19, H18/C20, H20/C15, H20/C16, H20/C17 and H20/C19, with a hydroxyl group clearly positioned at C-20 (Figure 1). The relative stereochemistry of **1** was established based on NOE data. The NOEs observed between Me14/Me11, Me14/H15, and Me13 /H9 revealed a *trans-anti-cis* fusion of **A**/**B**/**C** rings, which was the same as in puupehenone (**6**) (Figure 2). The relative stereochemistry of **1** at

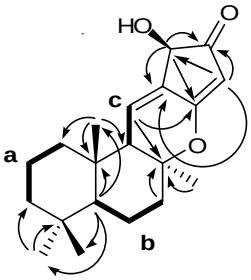


Figure 1. Partial structures (**a**,**b** and **c**) of 20-hydroxy-haterumadienone (**1**) based on COSY (bold line) and some important HMBC correlations (arrows).

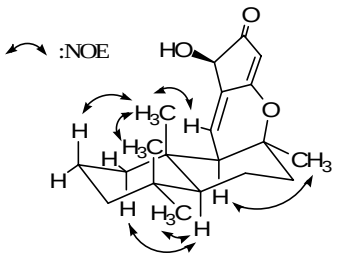
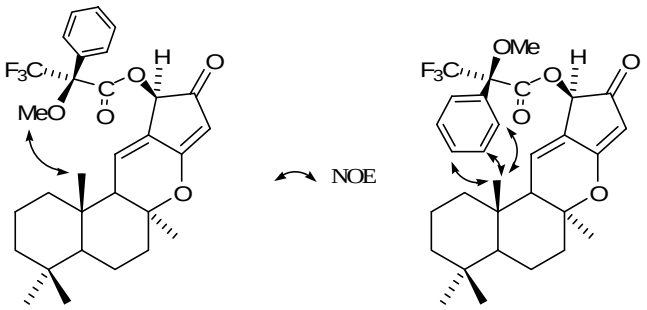


Figure 2. Selected NOE correlations of 20-hydroxyhaterumadienone (**1**).

C20 was deduced from a series of difference NOE experiments of the (*S*)- and (*R*)-MTPA esters, and the absolute stereochemistry of C20 was determined using the modified Mosher's method.[11] Esterification of **1** gave the (*S*)- and (*R*)-MTPA esters (**1a** and **1b**). The enhancement of OMe protons in **1a** (or aromatic protons in **1b**) upon irradiation of the Me14 protons of **1a** (or **1b**) allowed the ester group to be assigned as β because the conformers shown in Figure 3 are accepted as preferable for MTPA esters (the carbinyl proton at C20, the C=O oxygen and the CF_3 group are eclipsed to each other).[12] The ^{1}H NMR signals of the MTPA esters were assigned based on the 2D NMR data, and the $\Delta\delta$ values (δ_S-δ_R, ppm) were then calculated as shown in Figure 4. Thus, the absolute stereochemistry of 20-hydroxyhaterumadienone (**1**) was determined to be 5*S*, 8*S*, 9*R*, 10*S* and 20*R*. The absolute stereochemistry for the **A**-, **B**- and **C**-ring moieties was same as in (+)-puupehenone (**6**).[13]

Compound (**2**) was obtained as an inseparable mixture of two diastereoisomers (10:9). In the ^{1}H or ^{13}C



1a: *S*-MTPA ester **1b**: *R*-MTPA ester

Figure 3. Key NOEs observed for the ester moieties of *S*- and *R*-MTPA esters (**1a** and **1b**).

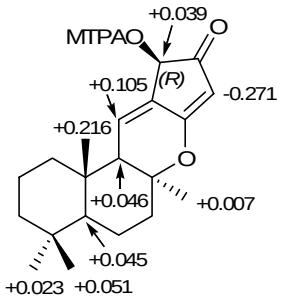


Figure 4. Δδ values (δ*S*-δ*R* ppm) for the MTPA esters (**1a** and **1b**).

NMR spectra, some signals were overlapped and others appeared in pairs. The intensity ratio of signals that formed pairs could change, which indicated that two diastereoisomers were at equilibrium in $CDCl_3$ solution. The ^{13}C NMR (Table 1) and HRESIMS [*m/z* 355.1910 $(M+Na)^+$, calcd for $C_{20}H_{28}O_4Na$, 355.1885] data revealed that **2** had a formula of $C_{20}H_{28}O_4$. The ^1H NMR signals in the aliphatic region were almost identical to those of **1** or puupehenone (**6**). The main differences in the chemical shifts between **1** and **2** were seen for C19 (δ_C 202.1 in **1**, δ_C 166.6 in **2**) and the oxygenated methine at C20 [δ_C 70.8 d, δ_H 4.60 s in **1**; δ_C 94.1 d, δ_H 6.05 s in **2**]. A detailed analysis of 1D and 2D NMR data finally gave the structure of **2** with 5-hydroxy pentenolide in ring **D** as an oxidation product of **1** or **3**. Since the NOEs observed for the **A**-, **B**- and **C**-ring moieties in **2** resembled those described above for **1**, both compounds were assumed to possess an identical stereochemistry for the **A**-, **B**- and **C**-ring moieties. Compound (**2**) was an equilibrium mixture of epimers of each other at C20.

The structures of haterumadienone (**3**) and acetone adducts (**4** and **5**) were determined in the same way as for **1**, as described previously.[10]

CONCLUSION

Puupehenone (**6**) and its derivatives are an important group of marine metabolites because they display a wide range of properties including cytotoxic, antiviral, antitumor, antifungal, antimalarial, antituberculosis and immunomodulatory activities.[1-10] We recently isolated haterumadienone (**3**) and

acetone adducts (**4** and **5**) together with puupehenone (**6**) from the Okinawan sponge *Dysidea* sp.[10] In this report, we have described the isolation, structure elucidation and biological activity of 20-Hydroxyhaterumadienone (**1**) and haterumadienelactol (**2**) which are related to haterumadienone (**3**), along with detailed experimental procedures for **3**, **4** and **5**. The metabolites (**1**, **2** and **3**) showed one less carbon of ring **D** than **6**. 20-Hydroxyhaterumadienone (**1**) and haterumadienelactol (**2**) would be formed by the oxidation of haterumadienone (**3**), which might be biosynthesized via benzylic acid rearrangement of the diketone form of puupehenone (**6**) followed by oxidative decarbonylation.[10] Acetone adducts (**4** and **5**) appear to be artifacts derived from the trione hydrate (**10**) by aldol condensation with acetone in the extraction process. These acetone adducts are the most reasonable adducts because they have less electrostatic interactions between two ketonic groups (1, 3-diketones) than other adduct structures (1, 2-diketones).[10]

20-Hydroxyhaterumadienone (**1**), haterumadienelactol (**2**) and haterumadienone (**3**) inhibited the first cleavage of the fertilized sea urchin eggs by 9%, 47% and 40% at 5 ppm, respectively. Interestingly, the artificial acetone adducts (**4** and **5**) showed the potent toxicity comparable to that of puupehenone (**6**).[10] Further chemical and biological studies on these metabolites are in progress in our laboratory.

EXPERIMENTAL

GENERAL ASPECTS

Optical rotations were measured on a JASCO DIP-1000 polarimeter. UV and IR spectra were measured using a JASCO V-550 spectrophotometer and a JASCO FT/IR-300 spectrometer, respectively. The ^{1}H, ^{13}C, and 2D NMR spectra were recorded on a JEOL α-500 spectrometer, and ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks [δ_H 7.24 and δ_C 77.0 in $CDCl_3$]. HRESI mass spectra were determined on a JEOL JMS-LG200 mass spectrometer. Column chromatography was performed on Kieselgel 60 (70-230 mesh, Merck) and HPLC was performed using a COSMOSIL-packed ODS HPLC column (C18, 10 x 250 mm). Analytical TLC was performed using Kieselgel 60 F_{254} DC-fertigplatten (Merck). All solvents used were reagent grade.

ISOLATION.

The yellowish sponge *Dysidea* sp. (750 g, wet weight) was extracted with acetone (1.5 L) twice. After filtration, the extracts were concentrated *in vacuo* to give an acetone extract. The acetone extract was partitioned between H_2O (200 mL) and EtOAc (300 mL x 2). The EtOAc extract (5.1 g) completely inhibited the first cell division of fertilized sea urchin eggs at 20 ppm. The toxic EtOAc-soluble material was triturated with hexanes. The hexane-insoluble part (3.1 g) was first chromatographed on Si gel using hexanes with increasing proportions of EtOAc [hexanes (300 mL) $\rightarrow$ hexanes/EtOAc (5:1, 300 mL $\rightarrow$ 3:1,

300 mL → 1:1, 300mL → 1:3, 300 mL) and then EtOAc with increasing proportions of MeOH [EtOAc (300 mL) → EtOAc/MeOH (9:1, 200 mL → 7:1, 200 mL)] to give 23 fractions. Fractions 13-16 were combined and concentrated under reduced pressure. The resultant residue (0.65 g) was further chromatographed on ODS (33 g) using MeOH (200 mL) and 5% EtOAc in MeOH (100 mL) to give 14 fractions. The fourth fraction (80 mg) was subjected to reversed-phase HPLC on ODS using H_2O-MeOH (1:7) to give 10 fractions. The fourth and fifth fractions (14 mg each) were purified by successive reversed-phase HPLC on ODS using MeOH-H_2O (7:1) to afford acetone adducts (**4**; 2.5 mg and **5**; 3.0 mg). The sixth fraction was subjected to further separation by HPLC on Si60 using hexanes-EtOAc (1:1) to yield **1** (6.1 mg), **2** (5.2 mg), and **9** (3.1 mg). The hexane-soluble part (4.5 g) was first chromatographed on Si gel using hexanes with increasing proportions of EtOAc [hexanes (500 mL) → hexanes/EtOAc (5:1, 500 mL → 3:1, 500 mL → 1:1, 500mL → 1:3, 500 mL) and then EtOAc with increasing proportions of MeOH [EtOAc (300 mL) → EtOAc/MeOH (9:1, 300 mL → 7:1, 300 mL)] to give 10 fractions. The third fraction (2.2 g) was partitioned between hexanes-EtOAc (4:1) (300 mL) and MeOH- H_2O (4:1) (300 mL) and the aqueous layer was concentrated under vacuum. The residual oil (1.2 g) was further chromatographed on Si gel using hexanes-EtOAc (4:1) to give **6** (369 mg) and a mixture of **3**, **7** and **8**. The mixture (150 mg) was subjected to reversed-phase HPLC on ODS using MeOH to give 3 fractions. The first fraction (70 mg) was further purified by reversed-phase HPLC on ODS using MeOH-H_2O (9:1) to afford **6** (6.2 mg), **7** (4.9 mg) and a fraction (25.5 mg) containing **8**. The fraction (25.5 mg) containing **8** was finally purified by reversed-phase HPLC on ODS using MeOH-H_2O (8.5:1.5) to afford **8** (5.3 mg). The fourth fraction (250 mg) from the first column separation of the hexane-soluble part was first subjected to separation by preparative TLC [hexanes-EtOAc (6:1)] to give a fraction that contained **3**. The fraction containing **3** was then purified by reversed-phase HPLC on ODS using MeOH to afford **3** (14.0 mg).

20-Hydroxyhaterumadienone (1) Colorless oil; $[\alpha]_D$ –94° (*c* 0.28 $CHCl_3$); UV (MeOH) λ_{max} 284 (ε 20000) nm; FT IR ν_{max} (film) 3314, 2926, 1660, 1594, 1419 and 1164 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Table 1; HRESIMS *m/z* 339.1947 $(M + Na)^+$ (calcd for $C_{20}H_{28}O_3Na$, 339.1936).

Haterumadienelactol (2): Colorless oil; $[\alpha]_D$ +9.8° (*c* 0.10 $CHCl_3$); UV (MeOH) λ_{max} 288 (ε 18000) nm; FT IR ν_{max} (film) 3355, 2925, 1685, 1560, 1404 and 1186 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Table 1; HRESIMS *m/z* 355.1910 $(M + Na)^+$ (calcd for $C_{20}H_{28}O_4Na$, 355.1885).

Haterumadienone (3): Colorless oil; $[\alpha]_D$ –57° (*c* 0.14 $CHCl_3$); UV (MeOH) λ_{max} 282 (ε 23000) nm; FT IR ν_{max} (film) 2920, 1696, 1573, 1405 and 1175 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Table 1;

HRESIMS *m/z* 323.2017 $(M + Na)^+$ (calcd for $C_{20}H_{28}O_2Na$, 323.1988).

Acetone adduct (4) (polar): Colorless oil; $[\alpha]_D$ –76° (*c* 0,12 $CHCl_3$); FT/IR (film) ν_{max} 3400, 1730, 1260, 1240 cm^{-1}; UV λ_{max} 277 (ε 15000) nm; ^1H and ^{13}C NMR ($CDCl_3$) data were described in the earlier paper;[10] HRESIMS *m/z* 423.2154 $(M + Na)^+$ (calcd for $C_{24}H_{32}O_5Na$, 423.2149).

Acetone adduct (5) (less polar): Colorless oil; $[\alpha]_D$ +183° (*c* 0.16 $CHCl_3$); FT/IR (film) ν_{max} 3400, 1730, 1260, 1240 cm^{-1}; UV λ_{max} 277 (ε 15000) nm; ^1H and ^{13}C NMR ($CDCl_3$) data were described in the earlier paper;[10] HRESIMS *m/z* 423.2207 $(M + Na)^+$ (calcd for $C_{24}H_{32}O_5Na$, 423.2149).

Esterification of 1. To a solution of 20-hydroxyhaterumadienone (**1**, 0.5 mg, 1.6 μmol) in pyridine (0.1 mL) was added (-)-MTPA chloride (24 mg, 96 μmol). The mixture was stirred at rt for 2 h and quenched with methanol (0.2 mL). H_2O (0.2 mL) was then added, and the mixture was extracted with EtOAc (1 ml x 3). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. The residual oil was purified by preparative TLC [R_f=0.2, hexanes-EtOAc (6: 1)] to give the (*S*)-MTPA ester (**1a**, 0.6 mg, 70%). Using the same procedure as described above, (*R*)-MTPA ester (**1b**, 0.6 mg, 70%) was obtained from the reaction between (+)-MTPA chloride (32 mg, 128 μmol) and **1** (0.5 mg, 1.6 μmol) in pyridine (0.1 mL). **1a**: ^1H NMR (500 MHz, $CDCl_3$) δ 7.62 (2H, m), 7.40 (3H, m), 6.13 (1H, d, *J*=6.5), 5.67 (1H, s), 5.46 (1H, s), 3.54 (3H, s), 2.23 (1H, br d, *J*=14.5 Hz), 1.97 (1H, d, *J*=6.5), 1.64 (1H, td, *J*=4.8, 14.5 Hz), 1.51 (1H, m), 1.50 (2H, m), 1.50 (1H, m), 1.42 (1H, m), 1.26 (3H, s), 1.17 (1H, td, *J*=4.3, 13.9 Hz), 1.08 ((1H, dt, *J*=3.5, 13.5 Hz), 0.94 (1H, dd, *J*=4.8, 13.9 Hz), 0.90 (3H, s), 0.83 (3H, s), 0.80 (3H, s); LRESIMS *m/z* 533 $(M + H)^+$ and 555 $(M + Na)^+$. **1b**: ^1H NMR (500 MHz, $CDCl_3$) δ 7.63 (2H, m), 7.38 (3H, m), 6.02 (1H, d, *J*=6.5 Hz), 5.94 (1H, s), 5.42 (1H, s), 3.63 (3H, s), 2.20 (1H, br d, *J*=14.5 Hz), 1.92 (1H, d, *J*=6.5 Hz), 1.61 (1H, td, *J*=4.8, 14.5 Hz), 1.45 (1H, m), 1.43 (1H, m), 1.39 (2H, m), 1.27 (2H, m), 1.26 (3H, s), 1.14 (1H, td, *J*=4.3, 13.8 Hz), 0.97 ((1H, dt, *J*=3.5, 13.5 Hz), 0.91 (1H, m), 0.88 (3H, s), 0.78 (3H, s), 0.58 (3H, s); LRESIMS *m/z* 533 $(M + H)^+$ and 555 $(M + Na)^+$.

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