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Trihydroxylated linear diterpenes from the brown alga *Bifurcaria bifurcata*

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Abstract

Two novel polar diterpenes were isolated from the brown alga *Bifurcaria bifurcata* collected off the Atlantic coast of Morocco, and their structures established by spectral methods. Both compounds are trihydroxylated acyclic diterpenes derived from 12-hydroxygeranylgeraniol. They were tested in vitro for their cytotoxicity and proved to be active against the NSCLC-N6 cell line. Their absolute configuration at the C-12 position has been determined with a modified Mosher's method [J. Am. Chem. Soc. 113 (1991) 4092] and that of the 12-hydroxygeranylgeraniol (bifurcadiol) has been revised.

Keywords: *Bifurcaria bifurcata*; Cystoseiraceae; Brown alga; Trihydroxylated linear diterpenes; 12-Hydroxygeranylgeraniol derivatives; Cytotoxicity; Chemotaxonomy

1. Introduction

The algal family Cystoseiraceae (order Fucales, class Phaeophyceae) is typified by the name-bringing genus *Cystoseira*, which includes some 66 currently recognised species (Guiry, unpubl. data). By contrast, *Bifurcaria* is a relative small genus of the Cystoseiraceae. It currently includes three species: *Bifurcaria bifurcata* (Velley) R. Ross, distributed on Atlantic coasts from Morocco (southern limit) to north-western Ireland (northern limit); *Bifurcaria brassicaeformis* (Kützing) Barten confined to the western (Stegenga et al., 1997) and Indian ocean coasts of South Africa (Silva et al., 1996); and *Bifurcaria galapagensis* (Piccone and Grünow) Womersley (Womersley, 1964). Previous studies of the secondary metabolites from lipid extracts of these three species have firstly shown that *B. bifurcata* contains a rich array of acyclic diterpenes (Biard et al., 1980; Combaut and Piovetti, 1983; Culioli et al., 1999a,b, 2000, 2001; Daoudi et al., 2001; Hougaard et al., 1991; Semmak et al., 1988; Valls et al., 1993a,b, 1995). By contrast, *B. galapagensis* shows the presence of a meroditerpene (bifurcarenone) as the main constituent of the diterpene composition of its lipid extract (Sun et al., 1980; Mori and Uno, 1989), and *B. brassicaeformis* shows the total absence of diterpenes in its lipid extract (Daoudi et al., 2001).

In the course of our chemotaxonomic studies on *B. bifurcata* (Combaut and Piovetti, 1983; Valls et al., 1986, 1993a,b, 1995; Semmak et al., 1988; Culioli et al., 1999a,b, 2000, 2001), including the geographical variation of its diterpenoid composition (Valls et al., 1993a,b, 1995) we investigated the moderately polar fraction of the lipid extract obtained from specimens of the alga collected in different locations off the Moroccan, Spanish, French and Irish Atlantic coasts. The acyclic diterpenes obtained were mono- and dioxygenated geranylgeraniol

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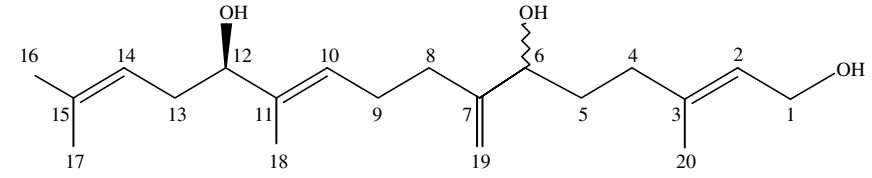
derivatives in which the second oxygenated function was located at C-12 or C-13 depending on the place where the alga was collected (Valls et al., 1993a,b, 1995). We have now started a study of the polar fraction of the lipid extracts of the alga to isolate trioxygenated acyclic diterpenes and verify if our previous chemotaxonomic data on this species (Valls and Piovetti, 1995; Valls et al., 1993a,b, 1995) are applicable to these compounds. As a first result of this study, we describe the isolation and structure elucidation of two novel trihydroxylated linear diterpenes (**1**, **2**) derived from 12-hydroxygeranylgeraniol (**3**), a previously reported acyclic diterpene which was named bifurcadiol by its discoverers (Valls et al., 1986). They were isolated from the polar fraction of the lipid extract obtained from a specimen of *B. bifurcata* collected at Oualidia (Morocco). As bifurcadiol (**3**) was known to have a potent cytotoxic activity against human cell lines (Zee et al., 1999), compounds **1** and **2** were, in addition, tested in vitro for their cytotoxicity against a cell line system NSCLC-N6.



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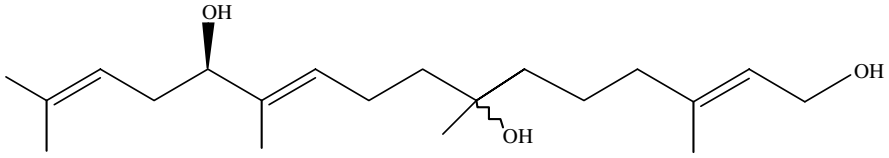

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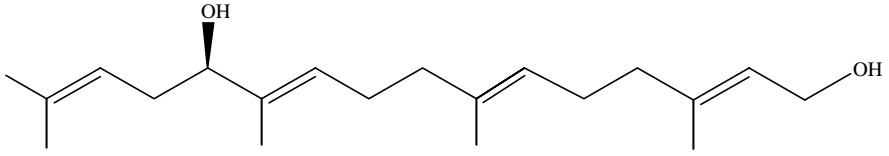

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2. Results and discussion

The lipid extract of shade dried *B. bifurcata* collected at Oualidia, first investigated to study its moderately polar diterpenes (Culioli et al., 2001), was re-investigated with the object of isolating the polar diterpenes as yet undescribed in this extract. Thus, the $CHCl_3$–MeOH extract was partitioned in the mixture MeOH–isooctane (1:1), leading to a MeOH extract which was then dissolved in the mixture MeOH–$CHCl_3$–H_2O (4:3:1). The organic extract obtained from the last extraction was fractionated by liquid chromatography using silica gel. The fractions eluted with EtOAc–isooctane (1:1 to 3:1) contained sterols, geranylgeraniol and mono- and dihydroxylated diterpenes previously described (Culioli et al., 2001). The fractions eluted with EtOAc and EtOAc–MeOH (98:2), respectively, were further purified by HPLC on a C-18 reverse-phase column, eluent MeCN–H_2O (1:1 and/or 2:3). From this separation we obtained two new compounds **1** and **2**.

Diterpene **1**, $C_{20}H_{34}O_3$ (HRMS), was an optically active oil which showed several spectral features in common with geranylgeraniol-derived diterpenes, particularly with 12-hydroxygeranylgeraniol (**3**), usually called bifurcadiol (Valls et al., 1986). It showed a strong hydroxyl absorption ($\nu_{OH} = 3365\ cm^{-1}$) in its IR spectrum. Inspection of the ^1H and ^{13}C NMR data (Tables 1 and 2) compared with those of bifurcadiol (**3**) showed that the first and the last two isoprenic units of both molecules (C-1 to C-4 with C-20 and C-9 to C-18) were similar. In particular, a secondary hydroxyl group lo-

Table 1
^{13}C NMR spectral data of compounds **1**, **2** and **3** (TMS as int. standard)[a]

C	**1**		**2**		**3**	
	$CDCl_3$ (100 MHz)	DEPT	$CDCl_3$ (100 MHz)	DEPT	$CDCl_3$ (90 MHz)[b]	DEPT
1	59.2	CH_2	59.2	CH_2	59.2	CH_2
2	123.7	CH	123.6	CH	123.5	CH
3	139.1	C	139.4	C	139.1	C
4	35.5	CH_2	39.8	CH_2	39.4	CH_2
5	33.3	CH_2	21.9	CH_2	26.7	CH_2
6	74.9	CH	41.4	CH_2	124.0	CH
7	151.3	C	72.8	C	134.9	C
8	30.9	CH_2	41.2	CH_2	39.2	CH_2
9	26.1	CH_2	22.1	CH_2	26.0	CH_2
10	125.6	CH	126.1	CH	125.8	CH
11	137.1	C	136.9	C	136.7	C
12	77.1	CH	77.1	CH	77.1	CH
13	34.1	CH_2	34.2	CH_2	34.2	CH_2
14	120.1	CH	120.1	CH	120.2	CH
15	134.6	C	134.7	C	134.2	C
16	25.8	CH_3	25.9	CH_3	25.8	CH_3
17	17.9	CH_3	18.0	CH_3	17.9	CH_3
18	11.7	CH_3	11.7	CH_3	11.6	CH_3
19	109.9	CH_2	26.7	CH_3	15.9	CH_3
20	16.2	CH_3	16.1	CH_3	16.2	CH_3

[a] Multiplicities were obtained with DEPT sequences.
[b] ^{13}C NMR data of Valls et al., 1986 (for comparison).

Table 2
^{1}H NMR spectral data of compounds **1** **2** and **3** (TMS as int. standard)[a]

H	**1**	**2**	**3**
	$CDCl_3$ (400 MHz)	$CDCl_3$ (400 MHz)	$CDCl_3$ (360 MHz)[b]
1	4.10 *d* (7.0)	4.12 *d* (6.9)	4.09 *d* (6.8)
2	5.39 *t* (7.0)	5.38 *t* (7.0)	5.35 *t* (6.8)
4	1.95–2.15 *m*	1.99 *t* (7.0)	1.99 *m*
5	1.55–1.75 *m*	1.40–1.48 *m*	2.07 *m*
6	4.01 *t* (7.0)	1.38–1.44 *m*	5.07 *t* (6.8)
8	1.95–2.15 *m*	1.45–1.50 *m*	1.99 *m*
9	2.10–2.30 *m*	2.05 *m*	2.07 *m*
10	5.36 *t* (7.0)	5.37 *t* (7.0)	5.32 *t* (6.8)
12	3.94 *t* (6.7)	3.94 *t* (6.7)	3.92 *t* (6.7)
13	2.10–2.30 *m*	2.20–2.28 *m*	2.20 *dd* (6.7; 7.0)
14	5.04 *t* (7.0)	5.05 *t* (7.2)	5.04 *t* (7.0)
16	1.67 *s*	1.69 *s*	1.68 *s*
17	1.59 *s*	1.60 *s*	1.60 *s*
18	1.59 *s*	1.60 *s*	1.57 *s*
19	4.83 *s*–5.01 *s*	1.14 *s*	1.56 *s*
20	1.64 *s*	1.64 *s*	1.63 *s*

[a] Chemical shifts are δ values, coupling constants (J in parentheses) are given in Hz; assignments were confirmed by decoupling and 2D NMR experiments (COSY ^{1}H–^{1}H, HMQC and HMBC).
[b] ^{1}H NMR data of Valls et al., 1986 (for comparison).

calized at C-12 was revealed by: (i) the ^{13}C NMR negative DEPT signal at δ 77.1 (C-12) and its correlated ^{1}H NMR signal at δ 3.94 (t, $J = 6.7$ Hz, H-12); (ii) the correlations between C-13/H-12, C-11/H-12 and C-12/H-13 in the HMBC spectrum. The configuration of the two double bonds at the C-2 and C-10 positions was determined with the NOESY experiment. The spatial correlations between H-2/H-4 and H-1/H-20 on the one hand and between H-10/H-12 and H-9/H-18 on the other hand, clearly showed the E configuration at C-2 and C-10, respectively.

The NMR analysis also showed the presence in the second isoprenic unit of: (i) an olefinic methylene revealed by ^{13}C signals at δ 109.9 and 151.3, and two ^{1}H signals at δ 4.83 (1H, *s*) and δ 5.01 (1H, *s*); (ii) a secondary hydroxyl group with a ^{13}C signal at δ 74.9 and a

^{1}H signal at δ 4.01 (t, $J = 7$ Hz); (iii) two methylene groups with ^{13}C signals at δ 30.9 and δ 33.3.

All these data suggested the structure **1** for this trihydroxylated linear diterpene. This structure was confirmed by means of homonuclear (COSY, NOESY) and heteronuclear (HMQC and HMBC) 2D NMR experiments. In particular, the structure of the second isoprenic unit and its localization in the molecule were precisely determined with the long-range $^2J_{C-H}$, $^3J_{C-H}$ chemical shift correlations, respectively, between C-8/H-19, C-7/H-8, C-6/H-19, C-6/H-5 and C-4/H-5, C-8/H-9. The multiplicities of C-5 (CH_2), C-6 (CH), C-7 (C), C-8 (CH_2) and C-19 (CH_2) were determined with DEPT sequences.

The second novel compound (**2**) had the molecular formula $C_{20}H_{36}O_3$ (HRMS). It was isolated as an optically active oil. Like **1**, this compound showed several spectral features in common with **3** (Tables 1 and 2), particularly with respect to the first and the last two isoprenic units of both molecules (a strong hydroxyl absorption, $\nu_{OH} = 3391$ cm^{-1}) in its IR spectrum; a secondary hydroxyl group localized at C-12 with signals at δ 77.1 (^{13}C) and δ 3.94 (^{1}H) in its NMR spectra. Also, the E configuration of the two double bonds at the C-2 and C-10 positions was verified with the NOESY experiment by the same spatial correlations as those found for **1**. The main differences with **3** were the presence in the second isoprenic unit of: (i) a tertiary hydroxyl group with a ^{13}C signal at δ 72.8 (quaternary sp^3 carbon); (ii) a methyl group on the latter sp^3 carbon with a ^{1}H signal at δ 1.14 (s, H-19); (iii) three methylene groups with ^{13}C signals at δ 21.9, δ 41.2 and δ 41.4.

These data showed that this compound, like **1**, was a trihydroxylated linear diterpene derived from **3**. The proposed structure **2** was confirmed by means of homonuclear (COSY, NOESY) and heteronuclear (HMQC and HMBC) 2D experiments. In particular, the localization of the methyl group (C-19) and the tertiary alcohol function was precisely determined with the long-range $^2J_{C-H}$, $^3J_{C-H}$ chemical shift correlations between C-6/H-19, C-7/H-19, C-8/H-19, C-8/H-9, C-6/H-5, C-6/H-4, C-7/H-8 and C-5/H-4, with CH_2-multiplicities for C-5, C-6, C-8, and CH_3-multiplicity for C-19 as determined by DEPT.

Two asymmetric centers were present at positions C-6 and C-12 for **1**, C-7 and C-12 for **2**. As compound **3** was known to have a 12*S* configuration established by Horeau's method (Valls et al., 1986), an *S* configuration at C-12 for compounds **1** and **2** could be attributed on the basis of biosynthetic consideration, since these compounds are 12-hydroxygeranylgeraniol derivatives isolated from the same plant material. So, in order to confirm this 12*S* configuration for the three compounds and to determine the absolute configuration at C-6 for **1** and C-7 for **2**, a modified Mosher's method (Othani et al., 1991) was applied to these metabolites. The comparison of the spectra of the two esters for each compound revealed for **1**, **2** and **3** a slight difference of the δ_H due to the diamagnetic effect of the MTPA benzene ring. Calculation of the $\Delta\delta^{SR} = \delta_H(S) - \delta_H(R)$ from ^{1}H NMR spectra of derivatives of each diterpene allowed assignment of a *R* configuration to the secondary alcohol C-12 whereas the configuration at C-6 for **1** and C-7 for **2** remains to be ascertained. For C-12, the signs were positive due to left-sided protons but negative owing to right-sided protons, thus demonstrating the 12*R* configuration based on the modified Mosher's method (Figs. 1–3). This empirical methodology is more accurate than others, such as the original Mosher or the Horeau methods, as it is dependent on many point comparisons (the chemical shifts of many sets of protons of the same type) instead of only one parameter. Thus, even though this result contradicts the configuration at C-12 established previously by

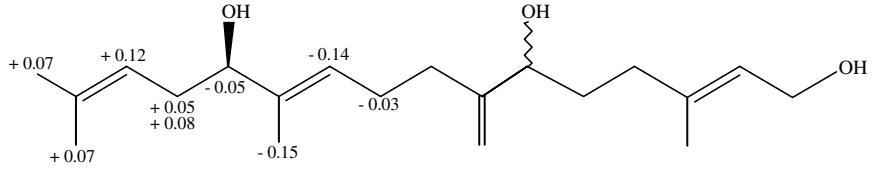

Fig. 1. $\Delta\delta^{SR}$ values for MTPA derivatives **1** and spatial consequences.

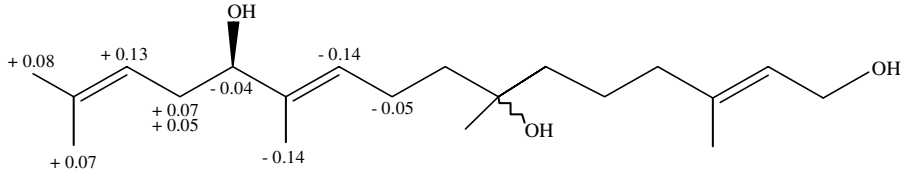

Fig. 2. $\Delta\delta^{SR}$ values for MTPA derivatives **2** and spatial consequences.

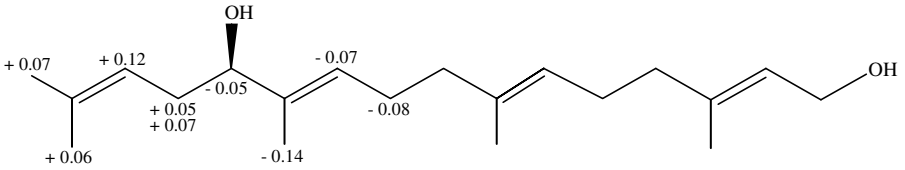

Fig. 3. $\Delta\delta^{SR}$ values for MTPA derivatives **3** and spatial consequences.

Horeau's method, it could be considered more probable. Moreover, it could be noted that Amico et al. (1997) also found a 12*R* configuration in the diterpenic chain of a meroditerpene isolated from an alga belonging to the same family (*Cystoseira adriatica*).

Unfortunately the NMR spectra of the MTPA derivatives did not allow an unambiguously assignment of the configuration of C-6 and C-7 in compounds **1** and **2**, respectively. In fact, these asymmetric carbons were surrounded by methylene groups (H-4, H-5, H-8 and H-9 positions for **1**; H-5, H-6, H-8 and H-9 positions for **2**) with similar chemical shifts (see Table 2). As a consequence, chemical shift differences between the respective diastereoisomers could not be determined exactly avoiding the *R*/*S* assignment according to Mosher.

2.1. Cytotoxic activity

The two novel metabolites **1** and **2** were screened for in vitro cytotoxicity against the NSCLC-N6 cell line (Roussakis et al., 1991) (derived from a human non-small-cell bronchopulmonary carcinoma) as described in Experimental. The concentration of compounds required for 50% cell destruction (IC_{50}) was 12.3 (**1**) and 9.5 (**2**) μg/ml, respectively. These results showed that the novel trihydroxylated linear diterpenes (**1**, **2**) were active and could inhibit in vitro the proliferation of well-differentiated pathologic cells such as NSCLC-N6 by terminal differentiation.

2.2. Chemotaxonomic relationships

Our previous studies of the geographical variations in the diterpenoid composition of *B. bifurcata* (Valls et al., 1993a,b, 1995; Culioli et al., 1999a,b, 2000, 2001) had revealed that the moderately polar fraction of the lipid extract of this species collected off the Moroccan coast, in the Oualidia zone, was clearly distinguishable from the extracts obtained from other zones of collection. In this case, all the di-functionalized oxygenated linear diterpenes were derived from the 12-hydroxygeranylgeraniol (**3**), instead of 13-hydroxygeranylgeraniol like those of all the lipid extracts from the other zones of collection (Culioli et al., 1999a,b). The present study of the polar fraction of the lipid extract obtained from a specimen of the species, collected once again at Oualidia confirms this chemical characteristic: (i) the two novel tri-functionalized oxygenated linear diterpenes isolated (**1** and **2**) are derived from the 12-hydroxygeranylgeraniol (**3**) and (ii) no 13-hydroxygeranylgeraniol derivative has been identified.

3. Experimental

3.1. General

High resolution MS were recorded in EI mode at 70 eV on a Varian MAT 311 spectrometer. NMR experiments were run on a Bruker Avance 400 MHz instrument; standard Bruker pulse programs were used for 2D experiments (chemical shifts are quoted in ppm (δ) relative to TMS and coupling constants are in Hz). Optical rotations were determined using a Perkin–Elmer 341 polarimeter. Final purification of the two new metabolites was achieved by HPLC (Bioteck 525) on a C-18 reverse-phase column (Waters, SymetryShield RP18, 5 μm, 4.6 × 250 mm) with RI monitoring (Varian RI-4).

3.2. Plant material

Bifurcaria bifurcata Ross was collected near Oualidia (32°49′N, 8°57′W), Morocco, in December 1998. A voucher specimen of this species was deposited in the herbarium of Dr. Pellegrini, Laboratoire de Biologie Marine Fondamentale et Appliquée, University of Marseille II, France.

3.3. Extraction and purification

The shade-dried material collected from Oualidia (1500 g) was ground and extracted with $CHCl_3$–MeOH (1:1) at room temperature. After filtration, the filtrate was evaporated to yield 83 g of crude extract. This extract was partitioned in the mixture MeOH–isooctane (1:1) to give 15.9 g of isooctanic phase (extract A) and 67 g of methanolic phase (extract B). Extract B was then dissolved in the mixture MeOH–$CHCl_3$–H_2O (4:3:1) to yield 51.7 g of organic phase (extract C) and 14.2 g of aqueous phase (extract D). About 20.5 g of extract C were subjected to CC on silica gel with a solvent gradient from isooctane to EtOAc and then from EtOAc to MeOH. The fractions eluted with EtOAc–isooctane

(1:1) contained sterols, geranylgeraniol and monohydroxylated diterpenes previously described (5.6 g). Those eluted with EtOAc–isooctane (3:1) contained bifurcadiol **3** (5.5 g). The two new compounds (**1** and **2**) were eluted with EtOAc and EtOAc–MeOH (98:2), respectively. They were further purified by HPLC on an analytical C-18 reverse-phase column (MeCN–H_2O, 1:1 and/or 2:3) to give **1** (200 mg) and **2** (55 mg).

3.4. Assay for cytotoxic activity

The NSCLC-N6 cell line (Roussakis et al., 1991), derived from a human non-small-cell bronchopulmonary carcinoma (moderatly differentiated, rarely keratinizing, classified as T2N0M0) was used for all experiments. The cells were cultured in RPMI 1640 medium (Intermed, France) supplemented with 5% fetal calf serum to which were added 100 IU penicillin/ml, 100 μg streptomycin/ml and 2 mM glutamine at 37 °C in 5% CO_2 atmosphere. Under these conditions, cell doubling time was 48 h. Cells used in all experiments never exceeded 35 passages.

For cytotoxicity determination, continuous drug exposure experiments were performed in microtiter plates (2×10^5 cells/ml), and the purified compounds **1** and **2** were tested at 10, 5 and 1 μg/ml. Cell growth was estimated by a colorimetric assay according to Mossmann (1983) based on the conservation of tetrazolium dye (MTT) (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetraxolium bromide) (Sigma, France) to a blue–black formazan product, using live mitochondria at 0, 24, 48, 72, 96 and 120 h. The experiment was repeated eight times for each concentration. Growth control was assessed from 16 determinations. Optical density at 570 nm corresponding to solubilized formazan was read for each well on a Titertek Multiskan MKII.

3.5. Compound *1*

Oil; $[\alpha]_D^{25}$ −2.3° (CH_2Cl_2; *c* 2.3); IR ν_{max}^{film} cm^{-1}: 3365, 2969, 2917, 2863, 1442, 1376, 1051, 1007; HRMS: 304.2407 $[M–H_2O]^+$ (calc. for $C_{20}H_{32}O_2$, 304.2402); EIMS (70 eV) *m/z* (rel. int.): 304 $[M–H_2O]^+$(0.3), 286 $[M–2H_2O]^+$(0.3), 253(0.5), 137(9), 136(9), 95(1), 81(53), 69(100), 55(9); ^{13}C and ^1H NMR: Tables 1 and 2.

3.6. Compound *2*

Oil; $[\alpha]_D^{25}$ −5° (CH_2Cl_2; *c* 3.4); IR ν_{max}^{film} cm^{-1}: 3391, 2968, 2934, 2872, 1713, 1667, 1443, 1381, 1150, 1050, 1010; HRMS: 306.2565 $[M–H_2O]^+$ (calc. for $C_{20}H_{34}O_2$, 306.2559); EIMS (70 eV) *m/z* (rel. int.): 306 $[M–H_2O]^+$(0.1), 288 $[M–2H_2O]^+$(0.5), 237(2), 219(2), 201(4), 135(11), 121(8), 109(11), 95(16), 85(2), 81(100), 71(10), 69(25), 67(9), 55(23), 53(6); ^{13}C and ^1H NMR: Tables 1 and 2.

3.7. Preparation of (*R*) and (*S*)-MTPA esters of *1*

To a solution of pure coumpound (4.3 mg) in dry pyridine (50 μl for 1.5 mg) (*R*)-(−)-MTPA-Cl (5 μl for 1.5 mg) was added. The reaction was carried out under Ar atmosphere in a dry test tube fitted with rubber septum. After 4 h under magnetic stirring at room temperature, Et_2O (3 ml) and water (3 ml) were added to the reaction mixture. The organic layer, washed three times with water (3 ml added each time) gave a crude ester which was purified by HPLC on an analytical C-18 reverse-phase column (MeCN–H_2O, 19:1).

The (*R*)-MTPA ester of **1** had the ^1H NMR spectral data (400 MHz, $CDCl_3$): δ 5.58 (1H, *t*, *J* = 6.2 Hz, H-10), 5.37 (1H, *m*, H-12), 4.93 (1H, *t*, *J* = 7.2 Hz, H-14), 2.43 (1H, *m*, H-13), 2.24 (1H, *m*, H-13), 2.11 (2H, *m*, H-9), 1.65 (3H, *s*, H-18), 1.64 (3H, *s*, H-16), 1.55 (3H, *s*, H-17).

The (*S*)-MTPA ester of **1** (4.6 mg) was prepared using the same procedure. ^1H NMR spectral data (400 MHz, $CDCl_3$) of (*S*)-MTPA ester of **1**: δ 5.44 (1H, *t*, *J* = 6.2 Hz, H-10), 5.32 (1H, *m*, H-12), 5.05 (1H, *t*, *J* = 7.2 Hz, H-14), 2.51 (1H, *m*, H-13), 2.29 (1H, *m*, H-13), 2.08 (2H, *m*, H-9), 1.71 (3H, *s*, H-16), 1.62 (3H, *s*, H-17), 1.50 (3H, *s*, H-18).

3.8. Preparation of (*R*) and (*S*)-MTPA esters of *2*

About 3.5 and 3.8 mg of compound **2** were reacted, respectively, with (*R*)-(−)- and (*S*)-(+)-MTPA-Cl as described above. The (*R*)-MTPA ester had the ^1H NMR spectral data (400 MHz, $CDCl_3$) for the protons around the asymmetric carbon: δ 5.59 (1H, *t*, *J* = 6.2 Hz, H-10), 5.37 (1H, *m*, H-12), 4.93 (1H, *t*, *J* = 7.2 Hz, H-14), 2.45 (1H, *m*, H-13), 2.24 (1H, *m*, H-13), 2.13 (2H, *m*, H-9), 1.65 (3H, *s*, H-18), 1.64 (3H, *s*, H-16), 1.56 (3H, *s*, H-17). ^1H NMR spectral data (400 MHz, $CDCl_3$) of (*S*)-MTPA ester of **2**: δ 5.46 (1H, *t*, *J* = 6.2 Hz, H-10), 5.33 (1H, *m*, H-12), 5.06 (1H, *t*, *J* = 7.2 Hz, H-14), 2.52 (1H, *m*, H-13), 2.29 (1H, *m*, H-13), 2.08 (2H, *m*, H-9), 1.72 (3H, *s*, H-16), 1.63 (3H, *s*, H-17), 1.51 (3H, *s*, H-18).

3.9. Preparation of (*R*) and (*S*)-MTPA esters of *3*

About 4.9 and 5.6 mg of compound **3** were reacted, respectively, with (*R*)-(−)- and (*S*)-(+)-MTPA-Cl as described above. The (*R*)-MTPA ester had the ^1H NMR spectral data (400 MHz, $CDCl_3$) for the protons around the asymmetric carbon: δ 5.55 (1H, *t*, *J* = 6.2 Hz, H-10), 5.39 (1H, *m*, H-12), 4.94 (1H, *t*, *J* = 7.2 Hz, H-14), 2.46 (1H, *m*, H-13), 2.23 (1H, *m*, H-13), 2.15 (2H, *m*, H-9), 1.64 (3H, *s*, H-16), 1.64 (3H, *s*, H-18), 1.56 (3H, *s*, H-17). ^1H NMR spectral data (400 MHz, $CDCl_3$) of (*S*)-MTPA ester of **3**: δ 5.48 (1H, *t*, *J* = 6.9 Hz, H-10), 5.34 (1H, *m*,

H-12), 5.06 (1H, *t*, $J = 7.3$ Hz, H-14), 2.53 (1H, *m*, H-13), 2.28 (1H, *m*, H-13), 2.07 (2H, *m*, H-9), 1.71 (3H, *s*, H-16), 1.62 (3H, *s*, H-17), 1.50 (3H, *s*, H-18).

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