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Absolute stereochemistry and conformational analysis of achaetolide isolated from *Ophiobolus* sp.

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ABSTRACT

Achaetolide, as reported by Bodo et al. in 1983, was isolated from a fermentation broth of *Ophiobolus* sp. We established the absolute stereochemistry of achaetolide to be 3*S*,6*R*,7*S*,9*R* by way of relative stereochemical assignment with ^{1}H NMR analyses employing the corresponding acetonide, determination of C3 and C9 stereochemistries by an extended Mosher method.

1. Introduction

Polyhydrolxylated 10-membered macrolides, such as aspinolide B,[1] decarestrictines A–D,[2–5] microcarpalide,[6] pinolidoxin,[7] and herbarmins I–II,[8] have been isolated from fungal sources. Since some of these exhibit potent biological activities (cholesterol biosynthesis inhibition activity, antimicrofilament activity, and phytotoxicity, and so on), this class of lactones has received special attention over recent years.[9] They have also attracted synthetic chemists in relation to the development of effective methodologies constructing medium-sized rings.[10–15] Within this family, the stereochemistry of achaetolide (**1**), originally isolated from a culture broth of *Achaetomium cristalliferum* by Bodo et al., remains unknown. We succeeded in establishing the absolute stereochemistry of **1** by way of relative stereochemical assignment with ^{1}H NMR analyses employing the corresponding acetonide, determination of C3 and C9 stereochemistries by extended Mosher method,[16] and confirmation of C3 position by a combination of conversion into methyl L-malate with chiral GC analysis. In this paper we would also like to discuss about conformational isomers.

2. Results and discussion

Achaetolide (**1**) was obtained as oil by conventional extraction/chromatography operations from a culture broth of *Ophiobolus* sp. isolated from dead stem of *Achillea alpina* ssp. *pulchra*. The ESIMS spectrum provided a protonated molecular ion signal at m/z=301.2033, thus establishing its molecular formula ($C_{16}H_{28}O_5$). In the ^{1}H NMR spectra in $CDCl_3$, most resonances of the sample thus obtained consisted of pairs of signals with a 9:1 ratio, despite the HPLC chromatograms suggesting single compound. Since the ratio in the ^{1}H NMR spectra varied by solvents (2:1 in CD_3OD, 5:3 in acetone-d_6), we concluded that the sample was pure but involved conformational isomers in the ^{1}H NMR spectra. Further analysis involving 2D NMR spectra suggested a 10-membered macrolide structure (Fig. 1), which had been reported by Bodo et al. as achaetolide, a transpiration enhancer of cut barley leaves.[17] The ^{1}H NMR data for the major conformer in $CDCl_3$ accorded well with those in the literature, although there is no mention about the presence of conformational isomers in the ^{1}H NMR spectra. Since the relative and absolute stereochemistry of **1** has not been discussed, we established it as described below.

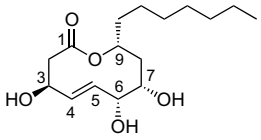

Figure 1. Structure of achaetolide (**1**).

It was found that a sole conformer was detected in the ^{1}H NMR spectra when the 6-*O*-7-*O*-isopropylidene derivative **2** was measured in $CDCl_3/C_6D_6$ (1:1). Isopropylidene **2** was obtained in 72% yield by treating **1** with $HClO_4$ in acetone (Scheme 1). This finding allows us to discuss the relative stereochemistry of the macrolide moiety based on the ^{1}H NMR spectra. Although the coupling constant between H6 and H7 (6.1 Hz) did not provide crucial information about the 1,3-dioxolane ring, a remarkable NOE between these protons was observed to reveal a cis-relationship on the dioxolane ring (Fig. 2). Large coupling constants for $J_{H7-H\alpha8}$ (10.2 Hz) and $J_{H\alpha8-H9}$ (9.7 Hz) indicated pseudo-*anti* orientations

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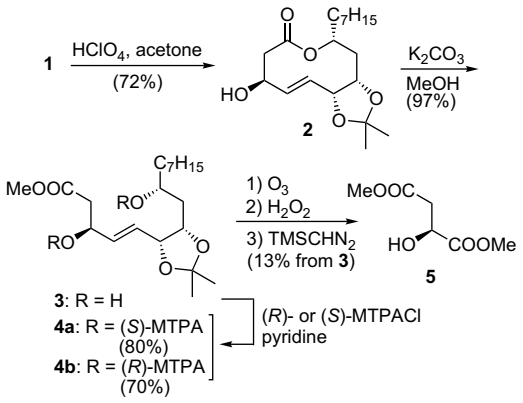

Scheme 1.

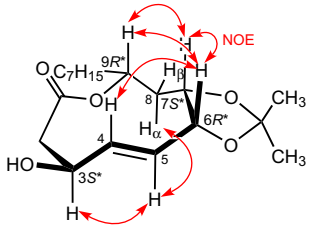

Figure 2. Stereochemistry of the C3–C9 moiety and characteristic NOEs observed in $CDCl_3/C_6D_6$ (1:1).

for H7/H_α8 and H_α8/H9. Interestingly the H_β8 appeared as a doublet (J=15.8 Hz) coupled only with H_α8, which suggests perpendicular relationships for H7/H_β8 and H_β8/H9. The NOESY spectrum provided correlation peaks H5/H_α8, H6/H7, H6/H9, and H7/H9, confirming the stereochemical relationship for the C6–C9 moiety. The coupling constants for H3/H4 and H5/H6 (8.3 and 9.0 Hz, respectively) and strong NOESY signals H3/H5 and H4/H6 disclosed the C3–C6 plane. Combining these results revealed a conformation as depicted in Figure 2 and the relative stereochemistry of C3–C9 moiety to be 3S^*,6R^*,7S^*,9R^*.

However, conformation around C1–C3 did not seem to be fixed because the coupling constants for H_α2/H3 and H_β2/H3 were both 8.0 Hz. A conformational search of the *model A* with semiempirical AM1[18] found six stable conformers within 2.0 kcal/mol from the global minimum conformation. As shown in Figure 3, all of these conformers took a similar conformation for C3–C9 and satisfied the coupling constants and NOEs discussed above. These conformers results in conformational change of C3OH group and O–C1–C2 moiety. These conformers might be interconverted on the NMR time scale.

We next investigated the absolute stereochemistry of **1**. It was found that basic treatment of **2** in methanol gave acyclic diol **3** in 97% yield. After **3** was converted to bis-(*S*)- and (*R*)-MTPA esters[19] (**4a**: 80%, **4b**: 70%, respectively) with the corresponding MTPACl, the $\Delta\delta$ values ($\delta_S-\delta_R$, ppm) in the ^{1}H NMR spectra ($CDCl_3$) were obtained as shown in Figure 4, to which applied to the empirical rule by Kusumi et al.[16] to suggest the chiralities for C3 and C9 positions as 3*S*,9*R*. Taking the above discussions into account, our studies disclosed the absolute stereochemistry of **1** to be 3*S*,6*R*,7*S*,9*R*.

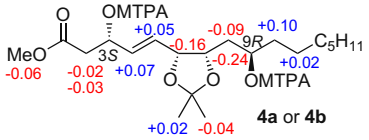

Figure 4. $\Delta\delta$ values ($\delta_S-\delta_R$) for the MTPA esters **4a** and **4b** in ppm.

The stereochemistry of C3 was further confirmed by chiral GC method. Ozonolysis of **3** followed by oxidative work-up gave malic acid monomethyl ester, which was purified after conversion into dimethyl malate **5** (13% from **3**) by $TMSCHN_2$.[20] The chiral GC conditions (Rt-βDEXm™ [0.25 mm ID×30 m], at 100–200 °C for 15 min) clearly separated the racemic dimethyl malate, with sufficient reproducibility, to show a pair of peaks at t_R=9.20 and 9.25 min. The equipped MS detector provided identical mass profiles to confirm these GC peaks.[21] On the other hand, **3** yielded a single peak at t_R=9.25 min. Since authentic (*S*)-dimethyl malate showed identical retention time (t_R=9.25 min) as **3**, the absolute chemistry of C3 position could be confirmed to be *S*-form, which consisted with that suggested by the extended Mosher method.

Finally, conformation of **1** is discussed. As mentioned, **1** was observed as a mixture of conformers in the ^{1}H NMR spectra. Although it was difficult to evaluate hydrogen bondings in solvents by modeling calculations, the conformation search calculations with HF-631G* provided two characteristic conformers of *model B* within 2.0 kcal/mol from that with lowest steric energy as shown in Figure 5. These were conformational isomers caused by flipping the C3–C6 plane.[22] Figure 6 shows an olefinic proton region in the ^{1}H NMR spectrum of **1** in $CDCl_3$ disclosing that **1** consists predominantly of two conformers with a 9:1 ratio. The signal profiles differ between the major and minor conformers, which indicate that the circumstances around the C4=C5 double bonds for these conformers are dissimilar to each other. These discussions are consistent with those based on the molecular modeling calculations.

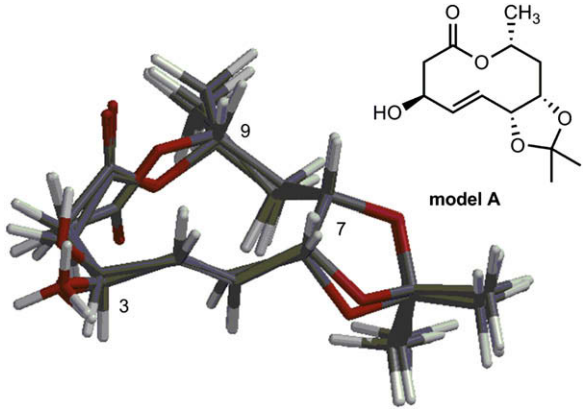

Figure 3. Six stable conformers of model A within 2.0 kcal/mol obtained by conformational search with AM1.

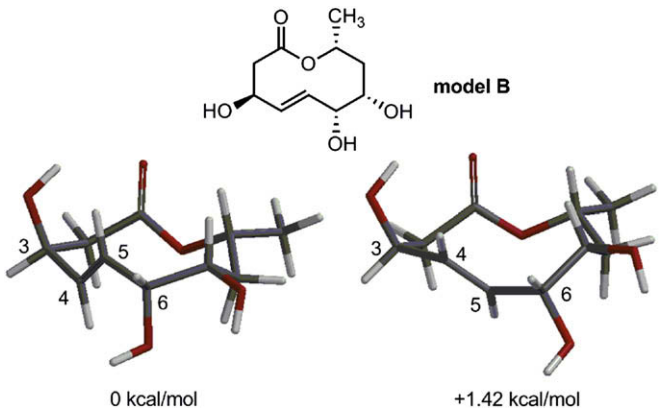

Figure 5. Two characteristic stable conformers of model B within 2.0 kcal/mol obtained by conformational search with HF-631G*.

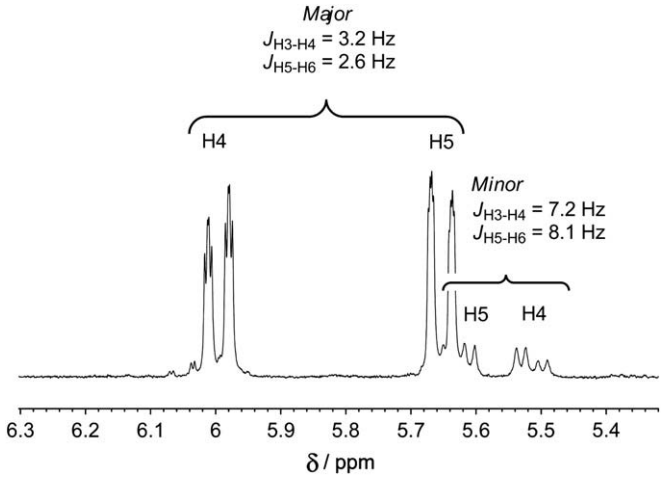

Figure 6. Olefinic proton region in the ^{1}H NMR spectrum of **1** in $CDCl_3$ (Small signals due to the third isomer were also seen at 6.05 ppm.).

We succeeded in revealing the absolute stereochemistry of achaetolide (**1**) by converting it into the isopropylidene **2**. We primarily revealed that **1** exists as a mixture of conformers in the ^{1}H NMR spectra. Thus far, we have found no potent biological activities; further biological investigations of **1** are presently under way in our laboratories.

3. Experimental

3.1. General

The ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were recorded in $CDCl_3$ (δ_H 7.24 ppm), CD_3OD (δ_H 3.30 ppm), acetone-d_6 (δ_H 2.04 ppm), or $CDCl_3/C_6D_6$ (1:1) (δ_H 7.16 ppm for C_6HD_5, δ_C 128.0 ppm for C_6D_6) using a JEOL JNM-ECA500 spectrometer. The EI-GC/MS spectrum was measured with a Shimadzu GC–MS-QP2010 spectrometer using an Rt-βDEXm™ (Shimadzu GLC Inc.) chiral capillary column (0.25 mm ID×30 m). The HRESI/MS spectrum was obtained by a HITACHI NanoFrontier LD spectrometer. Measurements of IR spectra were performed with a HORIBA FT-720 spectrometer. The optically rotation value were measured using a HORIBA SEP-700 spectrometer. Chemicals used in these experiments were obtained from Wako Pure Chemical Industries Ltd. and Nacalai Tesque Inc.

3.2. Isolation of achaetolide (1)

Ophiobolus sp. collected at Rishiri Island (Hokkaido) in 2007 was cultured in a potato/sucrose medium [600 mL, prepared from a potato extract (from 120 g potato), 12 g of sucrose, and water] at 25 °C for 40 days on a rotary shaker (100 rpm). After filtration, the culture broth was extracted with ethyl acetate (600 mL×2) and concentrated in vacuo. The residue was subjected to silica gel column chromatography (hexane/ethyl acetate=1:5) to afford achaetolide (**1**) 42.1 mg. $[\alpha]_D^{23}$ −27 (*c* 0.52, MeOH); IR (film) ν_{max}=3448 (O–H), 3255 (O–H), 2950 (C–H), 2870 (C–H), 1709 (O–C=O) cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz, *a*=0.67, *b*=0.33) δ 0.89 (3H, t, *J*=7.1 Hz, H-16), 1.28 (10H, m), 1.38 (1H×*b*, d, *J*=14.6 Hz, H-8β), 1.41 (1H×*a*, d, *J*=15.3 Hz, H-8α), 1.52 (2H×*a*, m, H-10), 1.55 (1H×*b*, m, H-10β), 1.70 (1H×*b*, m, H-10α), 2.26 (1H×*b*, dd, *J*=8.7, 13.5 Hz, H-2β), 2.37 (1H×*a*, m, H-8β), 2.37 (1H×*b*, m, H-8α), 2.49 (1H×*a*, dd, *J*=3.4, 11.8 Hz, H-2β), 2.52 (1H×*a*, dd, *J*=3.8, 11.8 Hz, H-2α), 2.93 (1H×*b*, dd, *J*=7.6, 13.5 Hz, H-2α), 3.44 (1H×*b*, m, H-7), 3.64 (1H×*a*, d, *J*=9.9 Hz, H-7), 4.28 (1H×*b*, dd, *J*=3.2, 7.3 Hz, H-6), 4.44 (1H×*a*, m, H-6), 4.44 (1H×*b*, m, H-3), 4.68 (1H, m, H-3), 4.72 (1H, dt, *J*=6.4, 8.5 Hz, H-9), 4.92 (1H×*b*, m, H-9), 5.41 (1H×*b*, dd, *J*=8.8, 16.4 Hz, H-4), 5.57 (1H×*b*, dd, *J*=7.3, 16.4 Hz, H-5), 5.69 (1H×*a*, ddd, *J*=1.4, 2.8, 15.8 Hz, H-5), 5.97 (1H×*a*, dd, *J*=3.4, 15.8 Hz, H-4); ^{1}H NMR (acetone-d_6, 500 MHz, *a*=0.63, *b*=0.37) δ 0.86 (3H, t, *J*=7.1 Hz, H-16), 1.27 (10H, m), 1.37 (1H×*b*, d, *J*=15.8 Hz, H-8β), 1.40 (1H×*a*, d, *J*=15.7 Hz, H-8β), 1.49 (2H×*a*, m, H-10), 1.52 (1H×*b*, m, H-10β), 1.66 (1H×*b*, m, H-10α), 2.22 (1H×*b*, dd, *J*=8.8, 13.4 Hz, H-2β), 2.34 (1H×*a*, ddd, *J*=8.6, 9.8, 15.7 Hz, H-8α), 2.34 (1H×*b*, ddd, *J*=5.8, 11.3, 15.8 Hz, H-8α), 2.44 (1H×*a*, dd, *J*=3.5, 11.7 Hz, H-2β), 2.49 (1H×*a*, dd, *J*=3.7, 11.7 Hz, H-2α), 2.93 (1H×*b*, dd, *J*=7.7, 13.4 Hz, H-2α), 3.30 (1H×*b*, br s, –OH), 3.46 (1H×*b*, m, H-7), 3.65 (1H×*a*, br d, *J*=9.8 Hz, H-7), 3.75 (1H×*b*, br s, –OH), 3.77 (1H×*a*, br s, –OH), 3.83 (2H×*a*, br s, –OH), 4.03 (1H×*b*, br s, –OH), 4.25 (1H×*b*, dd, *J*=3.3, 7.5 Hz, H-6), 4.43 (1H×*a*, br s, H-6), 4.45 (1H×*b*, m, H-3),4.66 (1H×*a*, dt, *J*=8.6, 8.4 Hz, H-9), 4.67 (1H×*a*, m, H-3), 4.92 (1H×*b*, ddd, *J*=4.8, 8.7, 11.3 Hz, H-9), 5.41 (1H×*b*, dd, *J*=8.8, 16.4 Hz, H-4), 5.54 (1H×*b*, dd, *J*=7.5, 16.4 Hz, H-5), 5.68 (1H×*a*, dd, *J*=2.7, 15.7 Hz, H-5), 5.99 (1H×*a*, dd, *J*=5.6, 15.7 Hz, H-4); HRESIMS *m*/*z* found 301.2033 $[M+H]^+$, calcd for $C_{16}H_{29}O_5$ 301.2015, found 283.1914 $[M+H-H_2O]^+$, calcd for $C_{15}H_{27}O_4$: 283.1909. The ^{1}H and ^{13}C NMR spectral data in $CDCl_3$ regarding the major conformer accorded well with those reported by Bodo et al.

3.3. Achaetolide C6,C7-*O*-acetonide (2)

To a stirred solution of **1** (5.3 mg, 16 μmol) in acetone (1 mL) was added perchloric acid (1 drop). The reaction mixture was stirred at room temperature for 20 min. After neutralization with satd NH_4Cl_{aq} (10 mL), the reaction mixture was extracted with EtOAc (10 mL×3). The combined EtOAc layer was washed with brine (5 mL), dried with Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/EtOAc=3:1) to give **2** (4.5 mg, 72%) as a colorless oil. $[\alpha]_D^{24}$ −33 (*c* 0.35, $CHCl_3$); IR (film) ν_{max}=3444 (O–H), 3000 (C–H), 2920 (C–H), 2870 (C–H), 1732 (O–C=O) cm^{-1}; ^{1}H NMR ($CDCl_3/C_6D_6$=1:1, 500 MHz) δ 0.81 (3H, t, *J*=6.9 Hz, H-16), 1.08–1.16 (10H, m), 1.20 (3H, s, acetonide–Me), 1.30 (1H, m, H-10β), 1.34 (3H, s, acetonide–Me), 1.44 (1H, d, *J*=15.8 Hz, H-8β), 1.51 (1H, m, H-10α), 2.11 (1H, ddd, *J*=9.7, 10.2, 15.8 Hz, H-8α), 2.16 (1H, dd, *J*=8.2, 13.1 Hz, H-2β), 2.64 (1H, dd, *J*=7.8, 13.1 Hz, H-2α), 3.92 (1H, dd, *J*=6.0, 10.2 Hz, H-7), 4.23 (1H, ddd, *J*=7.8, 8.2, 8.3 Hz, H-3), 4.39 (1H, dd, *J*=6.0, 9.0 Hz, H-6), 4.64 (1H, ddd, *J*=5.5, 9.0, 9.7 Hz, H-9), 5.42 (1H, dd, *J*=8.3, 16.0 Hz, H-4), 5.56 (1H, dd, *J*=9.0, 16.0 Hz, H-5); ^{13}C NMR ($CDCl_3/C_6D_6$=1:1, 125 MHz) δ 14.0 (q, C-16), 22.6 (t, C-15), 25.3 (q, acetonide–Me), 25.6 (t, C-14), 28.0 (q, acetonide–Me), 29.2 (t, C-13), 29.3 (t, C-12), 31.8 (t, C-11), 35.7 (t, C-10), 38.0 (t, C-8), 43.8 (t, C-2), 70.0 (d, C-3), 74.9 (d, C-9), 77.3 (d, C-6), 80.8 (d, C-7), 108.6 (s, acetal), 129.6 (d, C-5), 133.5 (d, C-4), 170.0 (s, C-1); HRESIMS *m*/*z* found 341.2317 $[M+H]^+$, calcd for $C_{19}H_{33}O_6$ 341.2328.

3.4. Methyl (3*S*,6*R*,7*S*,9*R*,*E*)-C6,C7-*O*-isopropilydene-3,6,7,9-tetrahydroxydodec-4-enoate (3)

A solution of **2** (6.6 mg, 18 μmol) in MeOH (2 mL) was stirred with K_2CO_3 (9.8 mg, 97 μmol) at room temperature. After stirring for 9 h, the suspension was poured into satd NH_4Cl_{aq} (3 mL) and extracted with EtOAC (20 mL). The EtOAc layers were combined, washed with brine (3 mL), dried with Na_2SO_4, and concentrated in vacuo. Silica gel column chromatography of the residue (hexane/EtOAc=1:1) gave **3** (7.5 mg, 97%) as a colorless oil. $[\alpha]_D^{23}$ −2.7 (*c* 0.68, MeOH); IR (film) ν_{max}=3448 (O–H), 2945 (C–H), 2880 (C–H), 1739 (O–C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.85 (3H, t, *J*=6.8 Hz, H-16), 1.35 (3H, s, acetonide–Me), 1.30–1.20 (10H, m), 1.39 (1H, ddd, *J*=3.3, 5.3, 14.2 Hz, H-8β), 1.40–1.45 (2H, m, H-10), 1.46 (3H, s, acetonide–Me), 1.60 (1H, ddd, *J*=2.9, 10.0, 14.2 Hz, H-8α), 1.91 (1H, br s, 9-OH), 2.53 (1H, dd, *J*=5.0, 16.2 Hz, H-2β), 2.56 (1H, dd, *J*=5.0,

16.2 Hz, H-2α), 2.87 (1H, br s, 3-OH), 3.69 (3H, s, ester–Me), 3.77 (1H, m, H-9), 4.42 (1H, ddd, *J*=3.3, 6.5, 10.0 Hz, H-7), 4.55 (1H, dd, *J*=6.5, 7.0 Hz, H-6), 4.56 (1H, m, H-3), 5.71 (1H, ddd, *J*=1.0, 7.0, 15.5 Hz, H-5), 5.76 (1H, dd, *J*=5.0, 15.5 Hz, H-4); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1 (q, C-16), 22.6 (t, C-15), 25.6 (q, acetonide–Me), 25.7 (t, C-14), 28.2 (q, acetonide–Me), 29.2 (t, C-13), 29.6 (t, C-12), 31.8 (t, C-11), 37.4 (t, C-8), 37.8 (t, C-10), 41.0 (t, C-2), 51.9 (q, ester–Me), 68.0 (d, C-12), 69.1 (d, C-9), 75.1 (d, C-7), 78.5 (d, C-6), 108.3 (s, acetal–C), 127.3 (d, C-5), 134.6 (d, C-4), 172.5 (s, C-1); HRESIMS *m*/*z* found: 373.2578 $[M+H]^+$, calcd for $C_{20}H_{37}O_6$ 373.2590.

3.5. Methyl (3*S*,6*R*,7*S*,9*R*,*E*)-C6,C7-*O*-isopropylidene-3,6,7,9-tetrahydroxydodec-4-enoate C2,C9-*O*-bis-(*S*)-MTPA ester (4a)

A solution of **3** (0.5 mg, 1.3 μmol) in pyridine (0.2 mL) was stirred with (*R*)-methoxytrifluorophenylacetyl (MTPA) chloride (5.0 mg) at room temperature for 2 days. After quenching with satd $NaHCO_{3aq}$ (2 mL), the reaction mixture was extracted with EtOAc (5 mL×3). The combined EtOAc layer was washed with satd NH_4Cl_{aq} (2 mL) and brine (2 mL), dried with Na_2SO_4, and concentrated in vacuo. Silica gel column chromatography of the residue (0.3 g, hexane/EtOAc=5:1) gave ester **4a** (0.8 mg, 80%) as a colorless oil. $[\alpha]_D^{16}$ −90 (*c* 0.06, $CHCl_3$); IR (film) ν_{max}=2931 (C–H), 2854 (C–H), 1747 (O–C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.84 (3H, t, *J*=7.2 Hz, H-16), 1.16–1.31 (10H, m), 1.26 (3H, s), 1.43 (3H, s), 1.47–1.63 (4H, m), 2.59 (1H, dd, *J*=4.9, 16.3 Hz, H-2β), 2.75 (1H, dd, *J*=8.9, 16.3 Hz, H-2α), 3.47 (3H, s), 3.55 (3H, s), 3.57 (3H, s), 3.88 (1H, m, H-7), 4.28 (1H, dd, *J*=4.9, 6.5 Hz, H-6), 5.18 (1H, m, H-9), 5.73 (1H, dd, *J*=6.6, 15.6 Hz, H-4), 5.76 (1H, dd, *J*=4.9, 15.6 Hz, H-5), 5.86 (1H, m, H-3), 7.34–7.39 (6H, m), 7.44–7.47 (2H, m), 7.52–7.54 (2H, m); HRESIMS *m*/*z* found 827.3198 $[M+H]^+$, calcd for $C_{40}H_{50}F_6NaO_{10}$ 827.3206.

3.6. Methyl (3*S*,6*R*,7*S*,9*R*,*E*)-C6,C7-*O*-isopropylidene-3,6,7,9-tetrahydroxydodec-4-enoate C2,C9-*O*-bis-(*R*)-MTPA ester (4b)

In a similar manner as that described for bis-(*S*)-MTPA ester, **3** (0.7 mg, 2 μmol) was treated with (*S*)-MTPA chloride (5.0 mg) to give **4b** (1.1 mg, 70%). $[\alpha]_D^{15}$ −70 (*c* 0.09, $CHCl_3$), IR (film) ν_{max}=2931 (C–H), 2854 (C–H), 1747 (O–C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.84 (3H, t, *J*=7.3 Hz, H-16), 1.14–1.29 (10H, m), 1.30 (3H, s), 1.41 (3H, s), 1.48–1.62 (4H, m), 2.61 (1H, dd, *J*=4.7, 16.4 Hz, H-2β), 2.78 (1H, dd, *J*=9.2, 16.4 Hz, H-2α), 3.48 (3H, s), 3.52 (3H, s), 3.64 (3H, s), 4.12 (1H, m, H-7), 4.44 (1H, dd, *J*=5.6, 5.7 Hz, H-6), 5.20 (1H, m, H-9), 5.66 (1H, dd, *J*=6.8, 15.5 Hz, H-4), 5.71 (1H, dd, *J*=5.6, 15.5 Hz, H-5), 5.85 (1H, ddd, *J*=4.7, 6.8, 9.2 Hz, H-3), 7.31–7.41 (6H, m), 7.43–7.46 (2H, m), 7.51–7.54 (2H, m); HRESIMS *m*/*z* found 827.3220 $[M+H]^+$, calcd for $C_{40}H_{50}F_6NaO_{10}$ 827.3206.

3.7. Chiral GC–MS analysis of dimethyl malate (5) derived from achaetolide

A solution of **3** (3.5 mg, 9.4 μmol) in MeOH (1 mL) was stirred at −78 °C. A steam of ozone was passed through for 20 min. After warming to room temperature, 90% $HCOOH_{aq}$ (1 mL) and 34% H_2O_{2aq} (0.5 mL) were added. The resulting solution was stood overnight and then concentrated in vacuo to give the crude materials, which were diluted again with benzene (700 μL) and MeOH (250 μL). After the addition of trimethylsilyldiazometane (2.0 M in diethyl ether, 50 μL) at room temperature, the resulting solution was stood for 1 h and then concentrated in vacuo. Silica gel column chromatography of the residue (0.3 g, hexane/EtOAc=1:2) gave dimethyl malate (**5**) (0.2 mg, 13% from **3**) as a colorless oil. The methanolic solution of **5** (2 μL, 0.1 mg/mL) was injected into the GC–MS instrument (splitter ratio: 100; column: Rt-βDEXm™ (0.25 mm ID×30 m, Shimadzu GLC LTD.); temperature: 100–200 °C for 15 min, gas flow: 1.33 mL/min). (2*R*)- and (2*S*)-Dimethyl malate (**5**) were eluted from the column after 9.2 and 9.3 min, respectively. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.79 (1H, dd, *J*=5.5, 16.5 Hz), 2.84 (1H, dd, *J*=4.7, 16.5 Hz), 3.15 (1H, br d, *J*=5.1 Hz), 3.70 (3H, s), 3.79 (3H, s), 4.49 (1H, ddd, *J*=4.7, 5.1, 5.5 Hz); EIMS *m*/*z* 103 $[M-CO_2CH_3]^+$.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.07.005.

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21. The MS conditions provided *m*/*z*=103 $[M-(CH_3O)]^+$ but not its molecular ion signal *m*/*z*=162.
22. Molecular modeling calculation provided the expected conformation of transition state, as shown below, through flipping the C3–C6 plane, whose activation energy was 16.2 kcal/mol. It is well-known that two *N*-methyl protons in DMF are observed as two distinct signals in ^{1}H NMR spectrum, resulting from restriction of C–N bond rotation in amide moiety. This energy barrier was estimated to be 19.2 kcal/mol. Therefore, flipping of the C3–C6 plane may account for the observation of conformational mixture in ^{1}H NMR spectrum of **1**.