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Structure Elucidation of Fungal Sespendole, an Inhibitor of Lipid Droplet Synthesis in Macrophages

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Abstract A new fungal metabolite named sespendole was isolated as an inhibitor of lipid droplet synthesis in mouse macrophages from the culture broth of the fungal strain *Pseudobotrytis terrestris* FKA-25. The structure and stereochemistry of sespendole were elucidated by spectroscopic studies including various NMR spectral analyses, exciton chirality experiments and the modified Mosher method. Sespendole was found to possess a new indolosesquiterpene skeleton modified with two isoprenes.

Keywords sespendole, indolosesquiterpene, fungal metabolites, structure elucidation

Introduction

Sespendole (**1**, Fig. 1) produced by *Pseudobotrytis terrestris* FKA-25 [1] is a potent inhibitor of lipid droplet synthesis in macrophages. The fermentation, isolation and biological activities of **1** were described previously [2].

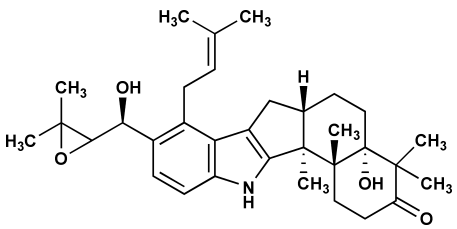

Fig. 1 Structure of sespendole (**1**).

In this report, we describe the structure elucidation of **1** and show that **1** possesses an indolosesquiterpene core with two additional isoprenyl side chains. A number of indoloditerpenes have been reported from fungi [3], such as paspalicine [4], paxilline [5, 6], janthitrems [7, 8], lolitrems [9] and penitrems [10～12] and terpendoles [13～15]. To our knowledge, **1** is the first microbial metabolite having an indolosesquiterpene core. The biosynthesis of **1** was reported previously [16].

Results and Discussion

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 1. Compound **1** is a colorless amorphous solid, and is soluble in chloroform, methanol, acetone and ethyl acetate. The molecular formula was revealed to be $C_{33}H_{45}NO_4$ by HR-EI-MS (*m/z* 519.3339; calcd. 519.3349). The UV spectrum exhibited characteristic absorption maxima at 239 and 288 nm in methanol, suggesting the presence of an indole moiety in the structure. The IR spectrum showed –OH and/or –NH absorption at 3426 cm^{-1} and carbonyl absorption at 1693 cm^{-1}.

Structure of Sespendole

The ^{1}H and ^{13}C NMR spectra of **1** showed 45 protons and 33 carbons in pyridine-d_5 as shown in Table 2. The carbon signals were classified into 8 methyl, 6 methylene, 6 methine, and 13 quaternary carbons by analysis of the

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Table 1 Physico-chemical properties of **1**

Appearance	Colorless amorphous solid
Molecular weight	519
Molecular formula	$C_{33}H_{45}NO_4$
HREI-MS	
calcd	519.3339 for $C_{33}H_{45}NO_4$
found	519.3349
UV λ_{max} nm (ε) in MeOH	239 (37,900), 288 (8,800)
IR ν_{max} cm^{-1} (KBr)	3426, 2929, 1693, 1456, 1378
Optical Rotation	$[\alpha]_D^{23}$ −18.0° (*c* 0.1, MeOH)
Solubility	
soluble	$CHCl_3$, MeOH, Acetone, EtOAc
insoluble	Hexane, H_2O

DEPT spectra. The connectivity of proton and carbon atoms was established according to the ^{13}C-^{1}H HMQC spectra. As shown by bold lines in Fig. 2, five partial structures composed of I (–CH_2–CH_2–), II (–CH_2–CH_2–CH–CH_2–), III (–CH_2–CH=), IV (–CH–CH–) and V (–CH=CH–) were deduced from the ^{1}H-^{1}H COSY spectra. The ^{13}C-^{1}H long-range couplings of ^{2}J and ^{3}J in the HMBC spectra (Fig. 2) proved the presence of the following linkages. 1) The cross peaks from H18 (δ 7.59) to C16 (δ 131.6) and C20 (δ 141.1), and from H19 (δ 7.48) to C15 (δ 126.5) and C17 (δ 130.2) suggested the presence of a 1,2,3,4-tetrasubstituted benzene ring that is shown in the partial structure V. The coupling constant (8.5 Hz) observed between H18 and H19 supported the possibility that they are in the *ortho* position of the benzene ring. The long-range couplings from NH (δ 11.46) to C2 (δ 154.5), C14 (δ 116.6), C15 and C20 showed that a pyrole ring is attached to the benzene ring, thus revealing the presence of a 2,3,4,5-tetrasubstituted indole moiety. The presence of an indole moiety was also supported by the UV spectrum (absorption maxima at 239 and 288 nm) and the fragment ion peak (*m*/*z* 113) in EI-MS (Fig. 3). 2) The long-range couplings from $H_3$28 (δ 1.83) and $H_3$29 (δ 1.65) to C26 (δ 126.5) and from $H_2$25 (δ 4.29, 4.07) to C27 (δ 131.2) suggested the presence of a 2-isopentenyl residue as shown in the partial structure III, which was supported by the fragment ion peak (*m*/*z* 69) in EI-MS (Fig. 3). Furthermore, long-range couplings were observed from $H_2$25 to C15 and C17 and from H26 to C16, suggesting that the 2-isopentenyl residue is connected to C16 of the indole moiety. 3) The long-range couplings from $H_3$33 (δ 1.39) and $H_3$34 (δ 1.37) to C31 (δ 69.5) and C32 (δ 58.7), from H30 (δ 5.32) to C32 and from the OH30 (δ 7.18) to C31 suggested the presence of an isopentanyl unit as designated in the partial structure IV. Furthermore, the presence of an epoxy group at the C31, 32-positions was inferred from the molecular formula, the chemical shifts of the ^{1}H and ^{13}C NMR signals of these positions and the relatively large $^{1}J_{CH}$ value for C31 ($^{1}J_{C\text{-}31,H\text{-}31}$=167 Hz). Long-range couplings were observed from OH30 to C17, from H30 to C16 and C18, from H18 to C30 and from H31 to C17, suggesting that the isopentanyl unit is connected to C17 of the indole moiety. 4) The long-range couplings from $H_2$13 (δ 2.76, 3.07) to C2 and C3 (δ 53.5), from H12 (δ 2.98) to C14 and C2, and from $H_3$21 (δ 1.75) to C2, C3 and C12 suggested that the cyclopentene ring A sharing the partial structure II is attached to the indole moiety. 5) The long-range couplings from $H_2$11 (δ 2.20, 1.70) to C3 and C9 (δ 80.9), from H10 (δ 1.98, 1.94), H12 and $H_3$21 to C4 (δ 43.9), from $H_3$22 (δ 1.44) to C3 and C9, from OH9 (δ 5.93) to C4 and C9 and from $H_3$21 to C4 suggested that the cyclohexane ring B is attached to ring A. 6) The long-range couplings from $H_2$5 (δ 3.14, 2.10) to C7 (δ 216.6), C9 and C22, from $H_2$6 (δ 2.82, 2.73) to C4 and C8 (δ 55.4) and from $H_3$23 (δ 1.42) and $H_3$24 (δ 1.31) to C7 and C9 suggested that the dimethyl cyclohexanone ring C in the partial structure I is attached to ring B to form the decalin substructure. Based on all the data taken together, the structure of **1** was concluded to be as shown in Fig. 1. The fragment ion peaks of *m*/*z* 448, 418, 236 and 71 observed in EI-MS (Fig. 3) also supported the structure.

Stereochemistry of Sespendole

Compound **1** has six chiral carbons in the structure. The relative stereochemistry was elucidated as follows. First, the configurations at C3, C4, C9 and C12 of the decalin moiety (C3 through C12) were deduced from NOE experiments in pyridine-d_5 as shown in Fig. 4. The NOEs were observed between $H_3$21 and OH9/H_a11/H_b13, and between $H_3$22 and H_b10/H12, suggesting that they are all oriented to axial on a chair conformation of ring B. The NOEs were observed between H_a5 and OH9, between H_a6 and $H_3$23, and between H_b6 and $H_3$22, but not between H_b10 and $H_3$24, suggesting that the relative stereochemistry of the decalin moiety is 3*S**, 4*R**, 9*S** and 12*S** (Fig. 4) and that the ring C has a twist-boat conformation. Therefore, the geometries at C4 and C9 were determined to be *trans*.

Next, the absolute configuration of the *trans*-decalin moiety was resolved using optical rotation and circular dichroism experiments [17]. Compound **1** exhibited a negative Cotton effect at around 295 nm in the CD spectrum due to the C7 carbonyl group, as shown in Fig. 5A. The relevance of this Cotton effect to the issue of absolute configuration is explainable in terms of the twist-boat cyclohexanone [18]. When viewed along the oxygen-

Table 2 ^{1}H and ^{13}C NMR chemical shifts of **1**

Position	^{13}C chemical shift (ppm)[a]		^{1}H chemical shift (ppm)[b]
1		NH	11.46 (1H, br.s)
2	154.5 s		
3	53.5 s		
4	43.9 s		
5	29.2 t	Ha	3.14 (1H, ddd, *J*=8.0, 9.0, 13.0 Hz)
		Hb	2.10 (1H, ddd, *J*=3.6, 7.7, 13.0 Hz)
6	35.1 t	Ha	2.82 (1H, ddd, *J*=3.6, 8.0, 15.4 Hz)
		Hb	2.73 (1H, ddd, *J*=7.7, 9.0, 15.4 Hz)
7	216.6 s		
8	55.4 s		
9	80.9 s		
		OH	5.93 (1H, s)
10	31.6 t	Ha	1.98 (1H, ddd, *J*=5.0, 10.2, 13.0 Hz)
		Hb	1.94 (1H, ddd, *J*=6.1, 9.0, 13.0 Hz)
11	21.9 t	Ha	2.20 (1H, dddd, *J*=5.0, 6.1, 13.1, 16.8 Hz)
		Hb	1.70 (1H, dddd, *J*=2.6, 9.0, 10.2, 16.8 Hz)
12	50.1 d		2.98 (1H, dddd, *J*=2.6, 5.8, 10.0, 13.1 Hz)
13	30.0 t	Ha	2.76 (1H, dd, *J*=10.0, 12.5 Hz)
		Hb	3.07 (1H, dd, *J*=5.8, 12.5 Hz)
14	116.6 s		
15	126.5 s		
16	131.6 s		
17	130.2 s		
18	120.5 d		7.59 (1H, d, *J*=8.5 Hz)
19	110.6 d		7.48 (1H, d, *J*=8.5 Hz)
20	141.1 s		
21	17.6 q		1.75 (3H, s)
22	22.6 q		1.44 (3H, s)
23	23.5 q		1.42 (3H, s)
24	24.6 q		1.31 (3H, s)
25	29.6 t	Ha	4.29 (1H, dd, *J*=6.5, 15.5 Hz)
		Hb	4.07 (1H, br d, *J*=15.5 Hz)
26	126.5 d		5.56 (1H, dt, *J*=1.0, 6.5 Hz)
27	131.2 s		
28	18.4 q		1.83 (3H, br s)
29	25.7 q		1.65 (3H, br s)
30	71.2 d		5.32 (1H, dd, *J*=3.6, 8.0 Hz)
		OH	7.18 (1H, d, *J*=3.6 Hz)
31	69.5 d		3.88 (1H, d, *J*=8.0 Hz)
32	58.7 s		
33	19.9 q		1.39 (3H, s)
34	25.3 q		1.37 (3H, s)

NMR experiments were performed on a Varian Unity 400 spectrometer. [a] Chemical shifts are shown with reference to C_5D_5N as 149.9 ppm. [b] Chemical shifts are shown with reference to C_5D_5N as 8.71 ppm.

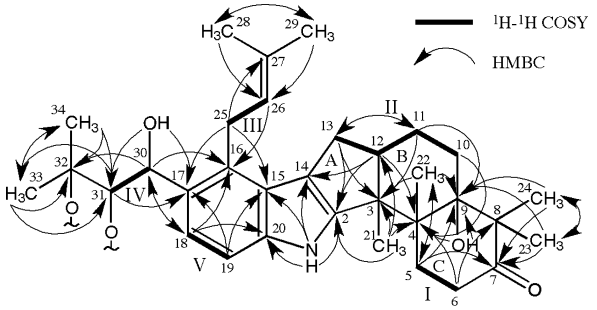


Fig. 2 ^{1}H-^{1}H COSY and HMBC experiments for **1**.

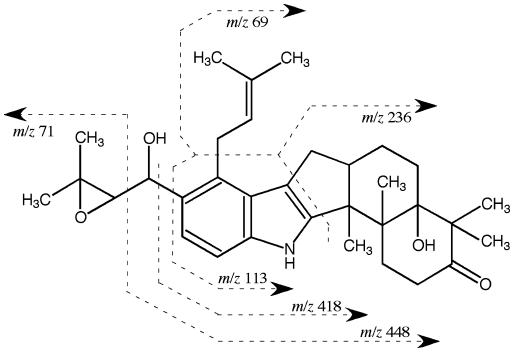
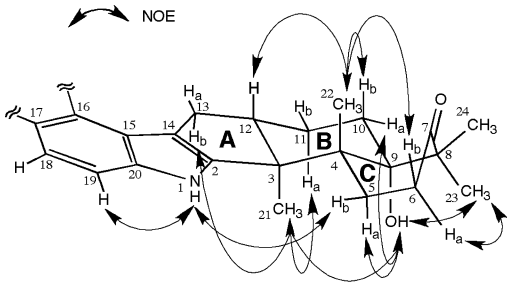


Fig. 3 EI-MS fragmentations of **1**.



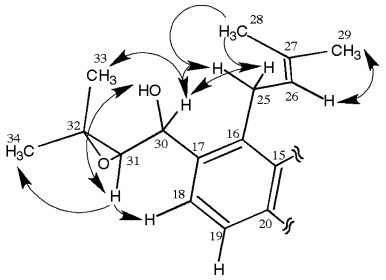


Fig. 4 NOE experiments for **1** measured in pyridine-d_5.

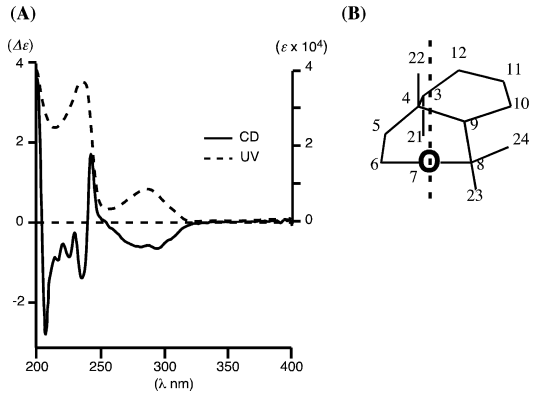


Fig. 5 Optical rotation and circular dichroism experiments for **1**.

(A) CD and UV spectra of **1**. (B) Projection drawing of **1** viewed along the oxygen-carbon axis at the C7 position.

carbon axis at the C7 position, the C7-C4 axis of cyclohexanone (ring C) is located on the left side of the carbonyl group (Fig. 5B). Therefore, the absolute stereochemistry of the *trans*-decalin moiety is presumed to be 3*S*, 4*R*, 9*S* and 12*S*.

These conclusions were supported by the modified Mosher method [19] using the ^{1}H NMR chemical shift differences between the (*R*)- and (*S*)-MTPA esters. Reduction of **1** with $NaBH_4$ gave 7*S**-hydroxy-**1** (**2a**) and 7*R**-hydroxy-**1** (**2b**) in a 2 : 3 ratio, which was supported by NOESY experiments as shown in Fig. 6. Then **2a** was treated with both (*R*)-(−) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride (MTPACl) and the products were subjected to ^{1}H-NMR analysis. As shown in Fig. 7, the $\Delta\delta$ values (δ_S–δ_R) of $H_2$10, $H_2$11, $H_2$12, H21, $H_3$23, $H_3$24 and OH9 were positive, while the $\Delta\delta$ values (δ_S–δ_R) of NH, $H_2$5, $H_2$18, $H_2$19 and $H_2$22 were negative, indicating that the absolute configuration of C7 in **2a** is *S*. Accordingly, the absolute configurations of the four chiral centers in the *trans*-decalin moiety were concluded to be 3*S*, 4*R*, 9*S* and 12*S*, as shown in Fig. 1.

The stereochemistries of C30 and C31 could not be determined on the basis of NOE data and the coupling constant between H30 and H31 (8.0 Hz, 130°). Derivatization of OH30, located at the *para*-position to the indole nitrogen, with MTPACl was not successful because of the unfavorable elimination, presumably due to effect of electron-withdrawing residues such as MTPA acid. Therefore, the method using Europium shift reagents in NMR measurement was applied to determine the stereochemistry at C30. Europium tris[3-(heptafluoro-propylhydroxymethylene)-(±)-camphorate] ($Eu(hfc)_3$) [20], which form chelate complexes with hydroxy groups, generally induce a down-field shift of the proton signals located near the hydroxy group. As shown in Fig. 8, the

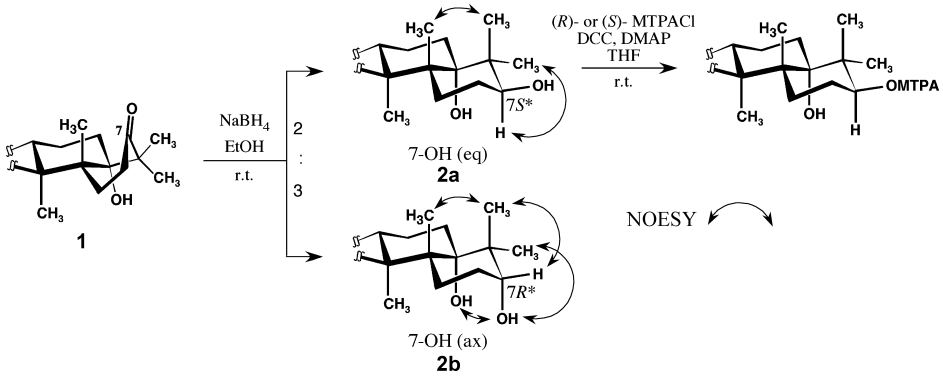


Fig. 6 Preparation of the (*R*)- and (*S*)-MTPA ester derivatives of **2a** and NOESY experiments for **2a** and **2b**.

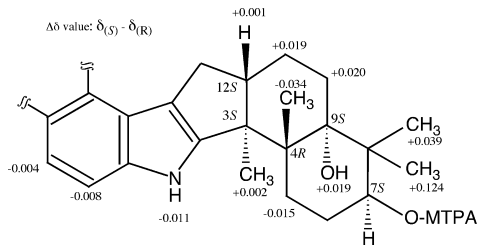


Fig. 7 $\Delta\delta$ values [$\Delta\delta$ (in ppm)$=\delta_S-\delta_R$] obtained for the (*R*)-(−) and (*S*)-(+)-MTPA esters of **2a**.

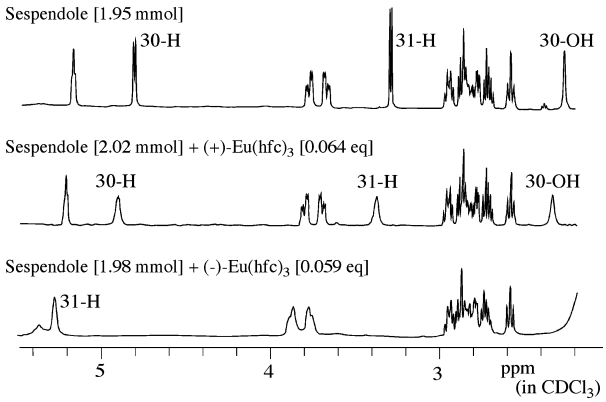


Fig. 8 Stereochemistry of C30 deduced by using chiral shift reagents, Europium tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorate] and Europium tris[3-(heptafluoropropylhydroxymethylene)-(−)-camphorate].

chemical shifts of H30 and H31 in **1** were shifted downfield in the presence of (−)-Eu(hfc)$_3$ (59 meq), whereas (+)-Eu(hfc)$_3$ (64 meq) did not induce a chemical shift change. These data strongly suggested that the absolute stereochemistry of C30 is the *S*-configuration.

Thus, the absolute configurations in **1** except for C31 were determined to be 3*S*, 4*R*, 9*S*, 12*S* and 30*S* (Fig. 1).

Experimental

Spectroscopic Measurements

Various NMR spectra were obtained with JEOL EX-270 (270 MHz), Varian XL-400 (400 MHz) and Inova 600 (600 MHz) spectrometers. Electron impact mass spectrometry (EI-MS) was conducted on a JEOL JMS-AX505H spectrometer. UV-visible and IR spectra were measured with a Beckman DU640 spectrophotometer and a Horiba FT-210 Fourier transform infrared spectrometer, respectively.

Materials

Sespendole was isolated from the culture broth of the fungal strain *Pseudobotrytis terestris* FKA-25 [2]. (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride, (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride, Europium tris[3-(heptafluoro-propylhydroxymethylene)-(+)-camphorate] and Europium tris[3-(heptafluoropropylhydroxymethylene)-(−)-camphorate] were obtained from Sigma (USA).

Preparation of 7*S**-Hydroxy-sespendole (2a) and 7*R**-Hydroxy-sespendole (2b)

To a solution of **1** (5 mg) in EtOH (320 ml) was added 1.0 mg of $NaBH_4$. The reaction mixture was stirred at room temperature for 3 hours. EtOAc (2 ml) and H_2O (2 ml) were added, the organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated to dryness to afford a yellow material, which was purified by preparative silica gel TLC with hexane - EtOAc (1 : 2) as the developing solvent to obtain 7*S**-hydroxy-sespendole (**2a**, 2.1 mg) and 7*R**-hydroxy-sespendole (**2b**, 3.0 mg).

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of 2a

To a solution of **2a** (1 mg) in CH_2Cl_2 (100 μl) was added 1 mg of 4-(dimethylamino)piridine, 2 mg of dicyclohexylcarbodiimide and 2.5 mg of (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride. The reaction mixture was stirred at room temperature for 3 hours. EtOAc (2 ml) and H_2O (2 ml) were added, the organic layer was separated, dried over anhydrous Na_2SO_4, filtered and concentrated to dryness to afford a yellow material, which was purified by preparative silica gel TLC with hexane - EtOAc (1 : 1) as the developing solvent to obtain a colorless powder of the (*R*)-MTPA ester of **2a** (0.9 mg). Similarly, the (*S*)-MTPA ester of **2a** (0.8 mg) was obtained using (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride.

Data of (*R*)-MTPA Ester Derivative of 2

^{1}H NMR (C_5D_5N) δ 11.44 (1H, br s, NH), δ 7.43 (1H, d, J=8.5 Hz, H18), δ 7.38 (1H, d, J=8.5 Hz, H19), δ 6.00 (1H, dd, J=5.3, 11.6 Hz, H7), δ 5.66 (1H, s, OH9), δ 5.49 (1H, br.t, J=5.7 Hz, H26), δ 4.66 (1H, d, J=8.0 Hz, H30), δ 4.10 (2H, br d, J=5.7 Hz, H25), δ 3.71 (1H, d, J=8.0 Hz, H31), δ 3.05 (1H, dd, J=6.0, 15.2 Hz, H13), δ 3.00 (1H, m, H12), δ 2.86 (1H, dt, J=4.4, 13.4 Hz, H5), δ 2.23 (2H, m, H6), δ 2.12 (1H, m, H11), δ 1.94 (1H, m, H5), δ 1.94 (1H, m, H10), δ 1.86 (3H, br s, H28), δ 1.75 (3H, s, H21), δ 1.70 (1H, m, H11), δ 1.67 (3H, br s, H29), δ 1.43 (3H, s, H22), δ 1.28 (3H, s, H33), δ 1.18 (3H, s, H23), δ 1.34 (3H, s, H34), δ 1.10 (3H, s, H24).

Data of (*S*)-MTPA Ester Derivative of 2

^{1}H NMR (C_5D_5N) δ 11.55 (1H, br s, NH), δ 7.43 (1H, d, J=8.5 Hz, H18), δ 7.33 (1H, d, J=8.5 Hz, H19), δ 6.00 (1H, dd, J=5.3, 11.6 Hz, H7), δ 5.68 (1H, s, OH9), δ 5.49 (1H, br t, J=5.7 Hz, H26), δ 4.66 (1H, d, J=7.8 Hz, H30), δ 4.10 (2H, br d, J=5.0 Hz, H25), δ 3.71 (1H, d, J=7.8 Hz, H31), δ 3.05 (1H, dd, J=6.1, 15.7 Hz, H13), δ 3.00 (1H, m, H12), δ 2.85 (1H, dt, J=4.5, 13.4 Hz, H5), δ 2.18 (2H, m, H6), δ 2.25 (1H, m, H11), δ 1.94 (1H, m, H5), δ 1.94 (1H, m, H10), δ 1.86 (3H, br s, H28), δ 1.75 (3H, s, H21), δ 1.71 (1H, m, H11), δ 1.67 (3H, br s, H29), δ 1.40 (3H, s, H22), δ 1.28 (3H, s, H33), δ 1.31 (3H, s, H23), δ 1.36 (3H, s, H34), δ 1.14 (3H, s, H24)

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