Two New Cleistanthane Diterpenes and a New Isocoumarine from Cultures of the Basidiomycete *Albatrellus confluens*

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Two new cleistanthane-type diterpenes, 3α,5α,8β-trihydroxycleistanth-13(17),15-dien-18-oic acid (1) and 8β-hydroxy-18-norcleistanth-4(5),13(17),15-trien-3-one (2), a new isocoumarine, 3*R*-(2*R*-hydroxypropyl)-8-hydroxyl-7-methyl-3,4-dihydroisocoumarine (3), along with three known aurovertins, aurovertins B (4), C (5) and E (6), four known polyesters, orbuticin (7), BK223A (8), BK223B (9) and 15G256α-2-me (10), and a known isocoumarine, 3*R*-6-hydroxymellein (11), were isolated from cultures of the basidiomycete *Albatrellus confluens*. The structures of these compounds were established on the basis of spectroscopic and chemical methods.

Key words *Albatrellus confluens*; cleistanthane; isocoumarine; aurovertin; polyester

Albatrellus confluens (ALB. & SCHWEIN.) KOTL. & POUZAR is a highly productive fungus, which produce a large variety of structurally diverse secondary metabolites, such as albaconol,[1)] grifolin and its derivatives,[1,2)] pyrazine-derivatives from the fruiting bodies,[1,3)] and aurovertins from its cultures.[4)] Most of these compounds isolated from this fungus are biologically active, and our initial investigations have reported the activities of albaconol on human and rat vanilloid receptor 1 (VR1).[5)] During our more recent studies on *A. confluens*, the immunosuppressive and anti-inflammatory activities of albaconol,[6,7)] and the antitumor activity of grifolin[8,9)] have been discovered. As part of our search for naturally occurring bioactive substances from higher fungi in China,[10,11)] we investigated the constituents of the cultures of *A. confluens* once more and isolated a series structurally diverse compounds, including two new cleistanthane-type diterpenes (**1**, **2**), a new isocoumarine (**3**), along with three known aurovertins (**4**—**6**), four known polyesters (**7**—**10**) and a known isocoumarine (**11**).

Results and Discussion

Compound **1** was obtained as amorphous powder and was assigned a molecular formula of $C_{20}H_{30}O_5$ by negative HR-electrospray ionization (ESI)-MS (*m*/*z* $[M-H]^-$ 349.2028, Calcd for $C_{20}H_{29}O_5$: 349.2014). The IR spectrum showed absorptions at 3425 and 1639 cm^{-1}, revealing the presence of hydroxyl and a carbonyl groups. The ^{13}C and distortionless enhancement by polarization transfer (DEPT) NMR spectra (Table 2) exhibited 20 carbons, including a carboxyl carbon (δ_C 183.5, C-18), two terminal double bonds (δ_C 150.5, s, C-13; 137.9, d, C-15; 117.6, t, C-16; 109.8, t, C-17), three oxygen-bearing carbons (δ_C 75.3, d, C-3; 81.4, s, C-5; 74.8, s, C-8) and two methyls (δ 20.9, 18.2). The presence of two terminal double bonds was also confirmed from the ^1H-NMR spectrum, which showed signals at δ_H 5.93 (ddd, J=17.6, 9.9, 9.9 Hz, H-15), 5.14 (dd, J=9.9, 2.2 Hz, H-16a) and 4.98 (dd, J=17.6, 2.2 Hz, H-16b) due to the vinyl group, and at δ_H 4.79 (d, J=1.6 Hz, H-17a) and 4.57 (d, J=1.6 Hz, H-17b) for the exomethylene. So far, three degrees of unsaturation have been assigned. The other three can be accommodated by assuming the presence of a tricyclic carbon skeleton. The NMR data of **1** were similar to those of zythiostromic acid B (**12**) and assigned tentatively as a cleistanthane-type diterpene.[12)] The distinct difference between them was that a methine (δ_C 41.4) in **12** was replaced by an oxygenated quaternary carbon (δ_C 74.8) in **1**, which suggested that **1** was derived from **12** by oxidation. This assignment was supported by heteronuclear multiple bond coherence (HMBC) correlations from H-6, H-7, H-9, H-14 and H-15 to C-8 (Fig. 2). In the ^1H-NMR spectrum, the signal for

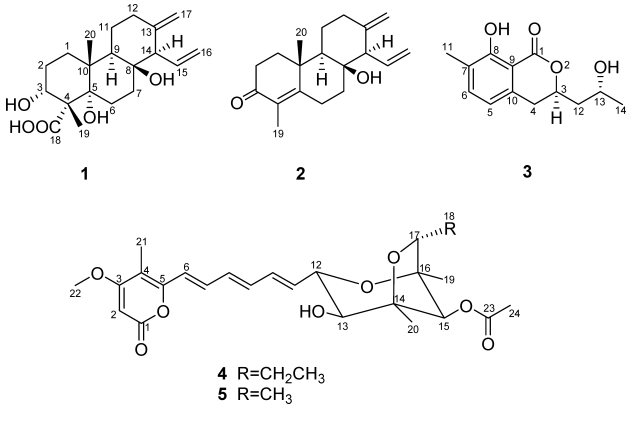

Fig. 1. Structures of Compounds **1**—**5**

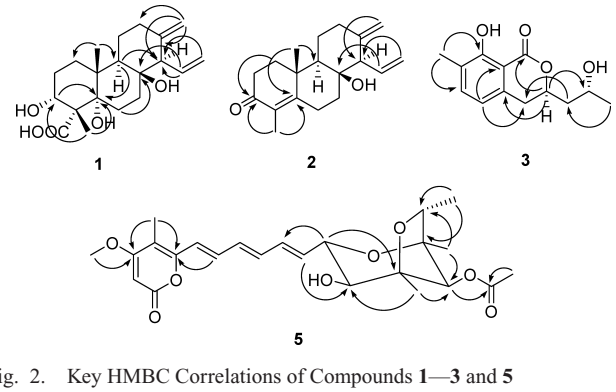

Fig. 2. Key HMBC Correlations of Compounds **1**—**3** and **5**

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Table 1. ^{1}H-NMR Data of Compounds **1**, **3** in CD_3OD and **2**, **5** in $CDCl_3$ at 500 MHz

No.	1	2	3	5
1	1.66 (m)	2.01 (m)		
	1.27 (m)	1.64 (m)		
2	2.50 (m)	2.44 (m)		5.48 (s)
	1.58 (m)	2.36 (m)		
3	4.17 (dd, 2.7, 2.7)		4.75 (m)	
4			3.03 (dd, 16.6, 3.6)	
			2.93 (dd, 16.6, 10.9)	
5			6.68 (d, 7.8)	
6	2.65 (m)	2.63 (m)	7.31 (d, 7.8)	6.32 (d, 15.3)
	1.67 (m)	2.48 (m)		
7	1.70 (m)	2.01 (m)		7.15 (dd, 15.3, 11.0)
	1.52 (m)	1.33 (m)		
8			6.40[a)]	
9	1.92 (dd, 12.6, 3.8)	1.36 (m)		6.40[a)]
10				6.40[a)]
11	1.61 (m)	1.82 (m)	2.20 (s)	5.91 (dd, 14.0, 6.1)
	1.54 (m)	1.51 (m)		
12	2.42 (ddd, 13.2, 3.3, 3.3)	2.49 (m)	2.05 (ddd, 14.0, 7.3, 7.3)	4.17[a)]
	2.07 (ddd, 13.2, 13.2, 4.4)	2.06 (m)	1.80 (ddd, 14.0, 6.7, 5.2)	
13			4.01 (m)	3.27 (d, 8.5)
14	2.51 (d, 9.9)	2.54 (d, 9.4)	1.24 (d, 6.2)	
15	5.93 (ddd, 17.6, 9.9, 9.9)	5.92 (ddd, 17.1, 10.3, 9.4)	4.79 (s)	
16	5.14 (dd, 9.9, 2.2)	5.23 (dd, 10.3, 2.1)		
	4.98 (dd, 17.6, 2.2)	5.05 (dd, 17.1, 2.1)		
17	4.79 (d, 1.6)	4.99 (d, 1.3)		4.17[a)]
	4.57 (d, 1.6)	4.75 (d, 1.3)		
18				1.29 (d, 6.7)
19	1.34 (s)	1.79 (s)		1.14 (s)
20	1.12 (s)	1.28 (s)		1.25 (s)
21				1.94 (s)
22				3.81 (s)
24				2.14 (s)

a) Overlapped signals.

H-3 at δ_H 4.17 showed a double doublet peak (J=2.7, 2.7 Hz) indicative of eq–ax and eq–eq coupling interaction, suggesting the α-axial orientation of the hydroxyl group at C-3. The β-orientation of H_3-19 and the α-orientation of H-14 was deduced from the rotating frame Overhauser enhancement spectroscopy (ROESY) correlations of H_3-19 with H-3β, H-14 with H-9α, and the α-axial orientation of the hydroxyl group at C-5 and β-axial orientation of the hydroxyl group at C-8 were apparent from its chair conformation. Therefore, compound **1** was elucidated as 3α,5α,8β-trihydroxycleistanth-13(17),15-dien-18-oic acid.

Compound **2**, obtained as amorphous powder, had the molecular formula of $C_{19}H_{26}O_2$ based on positive HR-FAB-MS (m/z $[M-H]^-$ 287.2021, Calcd for $C_{19}H_{27}O_2$: 287.2011). The IR spectrum displayed absorption at 3447 cm^{-1} assigned to hydroxyl group, and at 1641 cm^{-1} for an α,β-unsaturated carbonyl group. This was confirmed by the ^{13}C-NMR signals at δ_C 72.9 (s), 198.8 (s), 128.0 (s), 163.6 (s). The remaining ^{1}H- and ^{13}C-NMR data (Tables 1, 2) of **2** were closely similar to those of **1**, suggested that **2** was a norcleistanthane diterpenoid. On biogenetic considerations, the methine at C-3 bearing a hydroxyl group in terpenoids was often oxidized to a carbonyl group, which was supported by HMBC correlations from H-1, H-2 and H-19 to C-3 (Fig. 2) in **2**. Additionally, other significant HMBC correlations were also observed: from H-19 to C-4 (δ_C 128.0) and C-5 (δ_C 163.6), from H-20 to C-5 (δ_C 163.6). In the ROESY experiment, H-14 showed correlation with H-9α and no correlation with H_3-

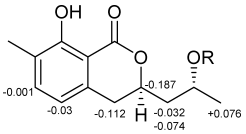
Table 2. ^{13}C-NMR Data of Compounds **1**, **3** in CD_3OD and **2**, **5** in $CDCl_3$ at 125 MHz

No.	1	2	3	5
1	28.8 t	35.9 t	171.9 s	163.6 s
2	27.6 t	33.3 t		88.8 d
3	75.3 d	198.8 s	79.3 d	170.5 s
4	52.4 s	128.0 s	33.4 t	108.0 s
5	81.4 s	163.6 s	118.6 d	154.2 s
6	26.8 t	23.9 t	138.1 d	119.5 d
7	33.8 t	37.1 t	125.9 s	135.6 d
8	74.8 s	72.9 s	161.4 s	132.1 d
9	49.7 s	54.8 d	108.7 s	137.0 d
10	43.3 s	39.4 s	138.5 s	131.6 d
11	23.3 t	23.3 t	15.4 q	134.1 d
12	37.4 t	35.7 t	44.6 t	77.8 d
13	150.5 s	148.1 s	64.8 d	76.2 d
14	61.9 d	59.7 d	23.6 q	83.7 s
15	137.9 d	134.6 d		80.1 d
16	117.6 t	118.6 t		82.7 s
17	109.8 t	111.5 t		79.5 d
18	183.5 s			11.8 q
19	20.9 q	11.1 q		16.0 q
20	18.2 q	18.9 q		14.9 q
21				8.8 q
22				56.1 q
23				169.8 s
24				20.7 q

20β, which revealed H-14 was α-oriented. In the same way with **1**, the β-axial orientation of the hydroxyl group at C-8 was suggested. Consequently, compound **2** was elucidated as 8β-hydroxy-18-norcleistanth-4(5),13(17),15-trien-3-one.

Compound **3** was obtained as amorphous powder. The molecular formula was established as $C_{13}H_{16}O_4$ by positive HR-ESI-MS (*m/z* 259.0945 $[M+Na]^+$, Calcd for $C_{13}H_{16}O_4Na$, 259.0946) in combination with the ^{13}C and DEPT NMR analysis. The ^1H- and ^{13}C-NMR spectra displayed resonances for 13 carbons, including a benzene ring, a carbonyl group, two oxygenated methines, two methylenes and two methyls. The signals at δ_H 7.31, 6.68 (each 1H, d, J=7.8 Hz), δ_C 161.4 (s), 138.5 (s), 138.1 (d), 125.9 (s), 118.6 (d), 108.7 (s) indicated the benzene ring was 1,2,3,4-tetrasubstituted. The HMBC correlations from δ_H 4.75 (H-3) to the carbonyl group (δ_C 171.9, C-1), C-4 and C-10, from δ_H 6.68 (H-5) to C-4, C-9 and C-10 suggested the benzene ring was fused with a δ-lactone at C-9/10 to yield a carbon skeleton of 3,4-dihydroisocoumarine. A methyl (C-11) attached to the benzene ring at C-7 was assigned by HMBC correlations from δ_H 2.20 (3H, s, H-11) to C-6, C-7 and C-8. Subsequently, the following key HMBC correlations (Fig. 2) were observed: from H-12 to C-3, C-4, C-13 and C-14, from H-13 to C-3, C-12 and C-14, and from H-14 to C-12 and C-13, which revealed the linkage of C-3 and C-12. Finally, to fulfill the MS and NMR analysis, C-8 was unambiguously substituted by a hydroxyl group. The optical rotation of 6-hydroxymellein (**11**), also isolated from this fungus, gave $[\alpha]_D^{18}$ −50.0° (c=0.29, CH_3OH), indicating *R*-configuration at C-3.[13] From a biogenetic point of view, the absolute configuration of **3** at C-3 was proposed as the same with **11**. This assignment was supported by the identical coupling constants for H-4 with H-3 in **3** and **11** (δ_H 3.03, dd, J=16.6, 3.6 Hz, H-4a in **3**; 2.93, dd, J=16.6, 10.9 Hz, H-4b in **3**; 2.89, dd, J=16.4, 3.7 Hz, H-4a in **11**; 2.81, dd, J=16.4, 11.0 Hz, H-4b in **11**). In order to determine the stereochemistry of C-13 at the side chain, the modified Mosher method was applied.[14,15] On the basis of the $\Delta\delta$ ($\delta_S-\delta_R$) values (Fig. 3), the absolute configuration at C-13 was determined as 13*R*. Thus, compound **3** was assigned as 3*R*-(2*R*-hydroxypropyl)-8-hydroxyl-7-methyl-3,4-dihydroisocoumarine.

Compound **5** was initially isolated from the fungus *Calcarisporium arbuscula* in 1979 by Beechey and co-workers, but the structure remains uncertain.[16] Compound **5** was isolated as yellow syrup and established as a molecular formula of $C_{24}H_{30}O_8$ by positive HR-ESI-MS (*m/z* 469.1838, Calcd for $C_{24}H_{30}O_8Na$, 469.1838). The IR spectrum indicated the presence of hydroxyl (3431 cm^{-1}) and carbonyl groups (1735, 1708 cm^{-1}). The ^1H- and ^{13}C-NMR spectra of **5** (Tables 1, 2) showed features closely similar to those of aurovertin B (**4**), which suggested that compound **5** was also an aurovertin derivative. Comparison of their NMR data, one up-field methylene was not observed in **5** from its ^{13}C-NMR spectrum, and correspondingly in ^1H-NMR spectrum, a triplet at δ_H 1.03 for a methyl and a multiplet at δ_H 1.64 for a methylene in **4** were absent and replaced by a doublet at δ_H 1.29 for a methyl in **5**. The linkage of C-17 and C-18 was confirmed by HMBC correlations from H-18 to C-16 and C-17. Since the NMR spectroscopic data and optical rotation of **5** showed features similar to aurovertin B, **5** was suggested to have the same absolute configuration as aurovertin B. Finally, the structure of compound **5** was assigned as aurovertin C.



3a R=(*S*)-MTPA
3b R=(*R*)-MTPA

Fig. 3. $\Delta\delta$ ($\delta_S-\delta_R$, in ppm) Obtained for (*S*)- and (*R*)-MTPA Esters of Compound **3**

The structures of the known compounds **4** and **6**—**11** isolated were identified as aurovertin B,[4,17] aurovertin E,[4] orbuticin,[18,19] BK223A,[13,19] BK223B,[13,19] 15G256α-2-me,[19] and 3*R*-6-hydroxymellein,[13] respectively, by comparison of their spectroscopic data with literature values.

Experimental

General Experimental Procedures Optical rotations were measured on a Horiba SEPA-300 polarimeter. UV spectra were measured in Shimadzu UV-2401 PC spectrophotometer. IR spectra were obtained on a Tensor 27 with KBr pellets. NMR spectra were recorded on Bruker AV-400 and Bruker DRX-500 spectrometer. Chemical shifts (δ) were expressed in ppm with reference to the solvent signals. FAB-MS were recorded with a VG Autospec-3000 spectrometer. EI-MS were recorded with a VG Autospec-3000 spectrometer. ESI-MS and HR-ESI-MS were recorded with an API QSTAR Pulsar 1 spectrometer. Preparative HPLC was performed on an Agilent 1100 series with a Zorbax SB-C18 (5 μm, 9.4×150 mm) column. Preparative MPLC was performed on Büchi apparatus equipped with Büchi fraction collector C-660, Büchi pump module C-605 and manager C-615. Silica gel (200—300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), RP-18 gel (40—75 μm, Fuji Silysia Chemical Ltd., Aichi, Japan) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol.

Mushroom Material and Cultivation Conditions The fungus *A. confluens* was collected from Ailao Mountain of Yunnan Province, China, in July 2003, and identified by Prof. Mu Zang, Kunming Institute of Botany. The voucher specimen (HFG0307252) was deposited at the Herbarium of the Kunming Institute of Botany, CAS. Culture medium: potato (peeled), 200 g, glucose, 20 g, KH_2PO_4, 3 g, $MgSO_4$, 1.5 g, citric acid, 0.1 g, and thiamin hydrochloride, 10 mg, in 1 l of deionized H_2O. The pH was adjusted to 6.5 before autoclaving, and the fermentation was carried out on a shaker at 25 °C and 150 rpm for 25 d.

Extraction and Isolation The culture broth (18 l) was extracted three times with EtOAc. The organic layer was evaporated *in vacuo* to give a crude extract (11.4 g), which was applied on silica gel column chromatography (200—300 mesh, 4.5×50 cm) eluted with a $CHCl_3$–MeOH gradient (100 : 0—0 : 100 gradient system) to afford fractions A—G. Both fraction B eluted with $CHCl_3$–MeOH (98 : 2, v/v) and fraction C eluted with $CHCl_3$–MeOH (95 : 5, v/v) were separated by Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1, v/v) column chromatography to obtain fractions B1, B2, C1—C3. Fraction B1 was subjected to preparative MPLC with a reversed-phased C_{18} column (MeCN–H_2O, 40%—70%), followed by Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1, v/v) column chromatography to give subfraction B11 and pure compound **4** (240.3 mg). Subfraction B11 was further purified by preparative HPLC using MeCN–H_2O (from 10%—50%) as mobile phase (flow rate 10 ml/min) to yield **5** (20.0 mg). Fraction B2 was chromatographed on RP-C_{18} MPLC (MeOH–H_2O, 50%—80%) and silica gel column chromatography (pure $CHCl_3$) to afford **2** (2.5 mg). Fraction C1 was separated by RP-C_{18} MPLC (MeOH–H_2O, 30%—50%) and preparative HPLC (MeCN–H_2O, 20%—40%), then by Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1, v/v) column chromatography to give **6** (11.0 mg). Fraction C2 was divided into subfraction C21 and C22 by passage over RP-C_{18} MPLC, eluted with MeOH–H_2O (40%—100%). Subfraction C21 was subjected to preparative HPLC (MeCN–H_2O, 30%—45%) to provide subfraction C211, compounds **1** (5.0 mg) and **3** (14.5 mg). Subfraction C211 was separated by Sephadex LH-20 (MeOH–H_2O, 7 : 3, v/v) column chromatography to give **10** (2.8 mg) and a mixture (360.4 mg) of **8** and **9**. **7** (211.7 mg) was purified by Sephadex LH-20 ($CHCl_3$–MeOH, 1 : 1, v/v) column chromatography

from subfraction C22. **11** (6.9 mg) was obtained from fraction C3 by repeated silica gel column chromatography eluted with $CHCl_3$–Me_2CO (50 : 1, v/v).

3α,5α,8β-Trihydroxycleistanth-13(17),15-dien-18-oic Acid (**1**): Amorphous powder; $[\alpha]_D^{16}$ +40° (c=0.20, CH_3OH); IR (KBr) ν_{max} 3425, 1639, 1562, 1391 cm^{-1}; ^1H- and ^{13}C-NMR data, see Tables 1 and 2; FAB-MS (neg.) m/z: 349 $[M-H]^-$; HR-ESI-MS (neg.) m/z: 349.2028 $[M-H]^-$ (Calcd for $C_{20}H_{29}O_5$, 349.2014).

8β-Hydroxy-18-norcleistanth-4(5),13(17),15-trien-3-one (**2**): Amorphous powder; $[\alpha]_D^{14}$ +146.7° (c=0.13, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 251 (3.96) nm; IR (KBr) ν_{max} 3447, 1641, 1446, 1334 cm^{-1}; ^1H- and ^{13}C-NMR data, see Tables 1 and 2; EI-MS m/z (%): 286 $[M]^+$ (6), 271 $[M-CH_3]^+$ (100), 268 $[M-H_2O]^+$ (3), 177 (45), 94 (55), 79 (45); HR-FAB-MS (pos.) m/z: 287.2021 $[M+H]^+$ (Calcd for $C_{19}H_{27}O_2$, 287.2011).

3R-(2R-Hydroxypropyl)-8-hydroxyl-7-methyl-3,4-dihydroisocoumarine (**3**): Amorphous powder; $[\alpha]_D^{18}$ −65.5° (c=0.29, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 253 (3.50), 322 (3.27) nm; IR (KBr) ν_{max} 3409, 1659, 1624, 1458, 1427, 806, 735 cm^{-1}; ^1H- and ^{13}C-NMR data, see Tables 1 and 2; FAB-MS (neg.) m/z: 235 $[M-H]^-$; HR-ESI-MS (pos.) m/z: 259.0945 $[M+Na]^+$ (Calcd for $C_{13}H_{16}O_4Na$, 259.0946).

Aurovertin C (**5**): Yellow syrup; $[\alpha]_D^{16}$ −39.8° (c=0.39, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 277 (3.90), 360 (3.91) nm; IR (KBr) ν_{max} 3431, 1735, 1708, 1627, 1406, 1234, 1036 cm^{-1}; ^1H- and ^{13}C-NMR data, see Tables 1 and 2; EI-MS m/z (%): 446 $[M]^+$ (5), 428 $[M-H_2O]^+$ (10), 341 (15), 325 (35), 247 (40), 219 (100), 139 (50); HR-ESI-MS (pos.) m/z: 469.1838 $[M+Na]^+$ (Calcd for $C_{24}H_{30}O_8Na$, 469.1838).

2-Methoxy-2-trifluoromethylphenylacetic Acid (MTPA) Esters of Compound 3 A mixture of **3** (4.6 mg), (*S*)-MTPA (21.5 mg), 4-(dimethylamino)pyridine (DMAP; 5.2 mg), 1,3-dicyclohexylcarbodiimide (DCC; 21.8 mg) dissolved in 5 ml dry CH_2Cl_2 was stirred at room temperature for 24 h. The reaction mixture was filtered, and the concentrated filtrate was chromatographed over a silica gel column (pure $CHCl_3$) to afford (*S*)-MTPA ester of **3** (**3a**, 6.0 mg). In the same manner, (*R*)-MTPA ester of **3** (**3b**, 5.2 mg) was prepared from **3** (3.7 mg) with (*R*)-MTPA (20.6 mg), DCC (20.3 mg), and 4-DMAP (3.8 mg). Results were summarized in Fig. 3.

(*S*)-MTPA Ester **3a**: ^1H-NMR ($CDCl_3$) δ: 1.458 (3H, d, J=6.2 Hz, H-14), 1.950 (1H, m, H-12), 2.258 (1H, m, H-12), 2.249 (3H, s, H-11), 2.747 (2H, m, H-4), 3.569 (3H, s, OCH_3), 4.361 (1H, m, H-3), 5.401 (1H, m, H-13), 6.547 (1H, d, J=7.3 Hz, H-5), 7.280 (1H, d, J=7.3 Hz, H-6), 7.26—7.48 (5H, aromatic protons in MTPA).

(*R*)-MTPA Ester **3b**: ^1H-NMR ($CDCl_3$) δ: 1.382 (3H, d, J=6.0 Hz, H-14), 1.982 (1H, m, H-12), 2.332 (1H, m, H-12), 2.247 (3H, s, H-11), 2.859 (2H, m, H-4), 3.497 (3H, s, OCH_3), 4.548 (1H, m, H-3), 5.406 (1H, m, H-13), 6.577 (1H, d, J=7.3 Hz, H-5), 7.281 (1H, d, J=7.3 Hz, H-6), 7.26—7.50 (5H, aromatic protons in MTPA).

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