Cytotoxic Substances Produced by a Fungal Strain from a Sponge: Physico-chemical Properties and Structures

TARO AMAGATA, YOSHIHIDE USAMI, KATSUHIKO MINOURA,
TADAYOSHI ITO[†] and ATSUSHI NUMATA*

Osaka University of Pharmaceutical Sciences,
4-20-1 Nasahara, Takatsuki, Osaka 569-11, Japan

[†]Institute for Fermentation, Osaka,
Juso-Honmachi, Yodogawa-ku, Osaka 532, Japan

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Five novel metabolites, trichodenones A~C (**1**~**3**), harzialactone A (**4**) and B (**5**), have been isolated together with known *R*-mevalonolactone (**6**) from the culture broth of a strain of *Trichoderma harzianum* OUPS-N115 originally separated from the sponge *Halichondria okadai*. Their structures have been elucidated by spectral evidence. Among them, **1**~**3** exhibited significant cytotoxicity against cultured P388 cells.

Marine microorganisms are potentially a prolific source of highly bioactive secondary metabolites that might represent useful leads in the development of new pharmaceutical agents. As part of a program designed to discover new antitumour metabolites produced by microorganisms inhabiting the marine environment, we previously reported that antitumour and cytotoxic compounds were produced by microorganisms originally isolated from the marine fishes *Pseudolabrus japonicus*^{1,2)} and *Halichoeres bleekeri*,³⁾ and the marine algae *Enteromorpha intestinalis*^{4~6)} and *Sargassum tortile*,^{7~10)} and their structures were established. In the present study, we examined secondary metabolites from a strain

of *Trichoderma harzianum* OUPS-N115 originally separated from the sponge *Halichondria okadai*, and isolated six novel metabolites, designated trichodenones A~C (**1**~**3**), harzialactone A (**4**) and B (**5**), along with known *R*-mevalonolactone (**6**) (Fig. 1). We describe herein the structure elucidation and cytotoxic activity of these metabolites.

Results and Discussion

A variety of microorganisms were initially isolated from the sponge *Halichondria okadai*, collected in the Tanabe Bay of Japan. Preliminary investigations in a

Fig. 1. Structures of trichodenones and harzialactones.

Table 1-1. Physico-chemical properties of trichodenones.

	Trichodenone A (1)	Trichodenone B (2)	Trichodenone C (3)
Appearance	Colorless oil	Colorless oil	Colorless oil
Mp	—	—	—
[α] _D	+ 56.3° (28°, c 0.75, CHCl ₃)	- 30.4° (28°, c 0.34, CHCl ₃)	- 10.8° (28°, c 1.11, CHCl ₃)
Molecular formula	C ₇ H ₈ O ₂	C ₇ H ₉ O ₃ Cl	C ₇ H ₉ O ₂ Cl
HREI-MS	Calcd. for C ₇ H ₈ O ₂ : 125.0524 Obsd: 125.0524 [M] ⁺	Calcd. for C ₇ H ₁₀ O ₃ Cl: 177.0317 Obsd: 177.0332 [MH] ⁺	Calcd. for C ₇ H ₉ O ₂ Cl: 160.0291 Obsd: 160.0279 [M] ⁺
UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ)	223 (3.74)	227 (3.76)	239 (3.89)
IR $\nu_{\text{max}}^{\text{fil}}$ cm ⁻¹	3419, 1717, 1683	3419, 1721, 1683	3419, 1721, 1683
CD $\lambda_{\text{max}}^{\text{EtOH}}$ nm ($\Delta \epsilon$)	220 (0), 234 (+0.44), 244 (+0.09), 254 (+0.11), 267 (+0.03), 270 (+0.05), 279 (0) (c 4.80 x 10 ⁻⁵ M in EtOH)	213 (0), 233 (+7.84), 270 (0), 315 (-1.99), 360 (0) (c 2.28 x 10 ⁻⁵ M in EtOH)	267 (0), 299 (-0.68), 340 (0) (c 2.88 x 10 ⁻⁵ M in EtOH)
Rf value on TLC	0.366 (CH ₂ Cl ₂ - MeOH, 19:1, silica gel)	0.207 (CH ₂ Cl ₂ - MeOH, 19:1, silica gel)	0.450 (CH ₂ Cl ₂ - MeOH, 19:1, silica gel)
Solubility Soluble:	DMSO, MeOH, CHCl ₃ , hexane, acetone, AcOEt	DMSO, MeOH, CHCl ₃ , hexane, acetone, AcOEt	DMSO, MeOH, CHCl ₃ , hexane, acetone, AcOEt
Insoluble:	H ₂ O	H ₂ O	H ₂ O

^a Measured in a tablet of KBr.

Table 1-2. Physico-chemical properties of harzialactones.

	Harzialactone A (4)	Harzialactone B (5)
Appearance	Colorless powder	Colorless oil
Mp	82 – 84°C	—
[α] _D	+ 33.3° (32°, c 0.3, CHCl ₃)	- 23.5° (32°, c 1.76, CHCl ₃)
Molecular formula	C ₁₁ H ₁₂ O ₃	C ₇ H ₁₀ O ₃
HREI-MS	Calcd. for C ₁₁ H ₁₂ O ₃ : 192.0786 Obsd: 192.0786 [M] ⁺	Calcd. for C ₇ H ₁₀ O ₃ : 142.0629 Obsd: 142.0627 [M] ⁺
UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm (log ϵ)	285 (2.61)	223 (2.66), 278 (2.89)
IR $\nu_{\text{max}}^{\text{fil}}$ cm ⁻¹	3420, 1771, 1600, 1580 ^a	3420, 1733, 1684
CD $\lambda_{\text{max}}^{\text{EtOH}}$ nm ($\Delta \epsilon$)	200 (0), 220 (-0.59), 255 (0) (c 2.82 x 10 ⁻⁴ M in EtOH)	201 (0), 213 (-0.69), 254 (0) (c 3.96 x 10 ⁻⁴ M in EtOH)
Rf value on TLC	0.438 (CH ₂ Cl ₂ - MeOH, 19:1, silica gel)	0.125 (CH ₂ Cl ₂ - MeOH, 19:1, silica gel)
Solubility Soluble:	DMSO, MeOH, CHCl ₃ , hexane, acetone, AcOEt	DMSO, MeOH, CHCl ₃ , hexane, acetone, AcOEt
Insoluble:	H ₂ O	H ₂ O

^a Measured in a tablet of KBr.

small scale revealed that a mixture of crude metabolites in one of them, a strain of *Trichoderma harzianum* OUPS-N115, showed cytotoxic activity against the P388 lymphocytic leukemia test system in cell culture.¹¹⁾ In assays, the AcOEt extract of the culture filtrate showed ED₅₀ value of 12 µg/ml, whereas ED₅₀ value of a methanolic extract of the mycelia was >100 µg/ml, suggesting that the activity was concentrated in the AcOEt soluble fraction of the culture filtrate. Consequently, full-scale experiments were carried out as follows.

The fungal strain was cultured at 27°C for 3 weeks in a medium containing 1% malt extract, 1% glucose and 0.05% peptone in artificial seawater. The AcOEt extract of the culture filtrate was purified by bioassay-directed fractionation employing a combination of Sephadex LH-20 and silica gel column chromatographies and HPLC to afford trichodenones A~C (**1**~**3**), harzialactone A (**4**) and B (**5**), and *R*-mevalonolactone (**6**). It was thought that the three trichodenones might be not obtained in reasonable yield due to their volatility; especially, trichodenone A which had highest volatility. The last known compound (**6**) was identified by comparison of spectral and other physical data with published values.^{12,13)}

Trichodenone A (**1**) is a colorless oil with the properties listed in Table 1. The molecular formula of **1** was established as C₇H₈O₂ by HR EI-MS (*m/z* 124.0524 [M]⁺, Δ 0.0 mmu). Its UV and IR spectra exhibited

absorption bands, characteristic of a conjugated enone (Table 1). A close inspection of the ¹H and ¹³C NMR spectra of **1** (Table 2) by DEPT and ¹H-¹³C COSY experiments revealed the presence of a 1, 2-disubstituted double bond (C-2 and C-3), a vinyl group (C-6 and C-7), an *sp*³-hybridized methylene (C-5), a 5-membered conjugated ketone (C-1) and a tertiary alcohol (C-4). Splitting patterns of the proton signals of the proton-bearing functional groups implied that no protons are present on carbons adjacent to these groups. Connection of these functional groups was clarified by long-range ¹H-¹³C COSY correlations (C-1/H-5, C-4/H-3, C-4/H-6, C-4/H-7, and C-6/H-5) to lead to the planar structure **1** for trichodenone A.

Trichodenone B (**2**) was isolated as a colorless oil with the molecular formula C₇H₉O₃Cl determined by [M + H]⁺ data (*m/z* 177.0332, Δ 1.5 mmu) in HR EI-MS. The presence of one chlorine atom was supported by an intensity ratio of isotope peaks ([M + H]⁺/[M + H + 2]⁺ = 3/1). The general features of its UV, IR, ¹H and ¹³C NMR spectra (Table 1 and 2) closely resembled those of **1** except that the proton and carbon signals of the disubstituted olefin and the vinyl group in the NMR spectra of **1** were replaced by those of a trisubstituted olefin (C-2 and C-3) and a hydroxyethyl group (C-6 and C-7) [δ_H 7.46 (H-3), δ_C 156.38 (C-3) and 137.27 (C-2); δ_H 1.23 (H-7) and 3.92 (H-6), δ_C 17.81 (C-7) and 76.60 (C-6)] in **2**, respectively. Long-range ¹H-¹³C COSY correlations (C-1/H-3, C-4/H-5 and C-4/H-7) in **2** implied

Table 2. ¹H and ¹³C NMR data of **1**~**3** in CDCl₃^a.

1					2					3				
No.	δ _H	<i>J</i> Hz	δ _C	LR ¹ H- ¹³ C COSY(C) ^b	δ _H	<i>J</i> Hz	δ _C	LR ¹ H- ¹³ C COSY(C)	δ _H	<i>J</i> Hz	δ _C	LR ¹ H- ¹³ C COSY(C)		
1			206.9	3, 5			197.9	3			201.4			
2	6.18 d	5.5(3) ^c	133.5				137.3				129.2			
3	7.38 d	5.5(2)	164.7		7.46 s		156.4				175		7	
4			78.8	2, 6, 7			78.9	5A, 7	2.54 t	4.5(5A)	24.3			
5A	2.60 d	18.3(5B)	49.6		2.50 d	18.9(5B)	43.9		2.71 dt	19.2(5B), 4.5(4)	32.7			
B	2.63 d	18.3(5A)			2.76 d	18.9(5A)			2.76 d	19.2(5A)				
6	6.00 dd	17.2(7B), 10.6(7A)	140.3	5	3.92 q	6.5(7)	76.6	7	5.08 q	6.7(7)	65.4		7	
7A	5.24 d	10.6(6)	114.5		1.23 d	6.5(6)	17.8		1.44 d	6.7(6)	20.8			
B	5.37 d	17.2(6)												
4-OH	2.87 br s				2.73 br s									
6-OH					2.09 br s				2.43 br s					

^a Measured at 300 and 75.4 MHz for ¹H and ¹³C, respectively. ^b LR ¹H-¹³C COSY correlations from C to H.

^c Figures in parentheses indicate a proton coupling with that question.

that the hydroxyethyl group is located at C-4 and the olefin links to C-1 and C-4. The carbon signal of the conjugated ketone (C-1) was found shifted upfield by *ca.* 10 ppm, relative to a general conjugated cyclopentenone (δ_c 209)¹⁴⁾ and **1**. It has been observed that carbon signals of α -haloketones such as 2-chlorocyclopentanone, 2-chlorocyclohexanone, 2-chloro-*p*-benzoquinone,¹⁵⁾ gymnastatin A¹⁶⁾ *etc.* appear shifted upfield by 8~10 ppm, relative to the corresponding ketone^{15,17)} bearing no halogen atom. These observations suggested that a chlorine atom exists at the α -position of the ketone in **2**, and hence structure **2** was established for trichodenone B.

Trichodenone C (**3**) was assigned a molecular formula of C₇H₉O₂Cl as deduced from [M]⁺ peak in HREI-MS and the intensity ratio of isotope peaks ([M]⁺/[M+2]⁺ = 3/1). The general spectral features of its UV, IR, ¹H and ¹³C NMR spectra showed close correspondence with those of **2**, except that the carbon signal of the tertiary alcohol and the proton signal of the trisubstituted olefin in the NMR spectra of **2** were missing and replaced by those of a methylene group (C-4) and a tetrasubstituted olefin (C-2 and C-3) in **3**, respectively (Table 1 and 2). The methylene protons showed a correlation with the C-5 methylene protons in ¹H-¹H COSY, and one *sp*²-carbon (C-3) of the olefin was correlated with the

methyl protons (H-7) of the hydroxyethyl group in long-range ¹H-¹³C COSY. This evidence led to structure **3** for trichodenone C.

The absolute stereochemistry of the three trichodenones described above could not be established from the spectroscopic data, and hence was determined by their stereospecific total syntheses, which will be reported elsewhere.

Harzialactone A (**4**) had the molecular formula C₁₁H₁₂O₃ established by HREI-MS. Its IR spectrum contained absorption bands, characteristic of a hydroxyl group, a γ -lactone and an aromatic ring (Table 1). A close inspection of the ¹H and ¹³C NMR spectral data (Table 2) of **4** by DEPT and ¹H-¹³C COSY experiments revealed the presence of one γ -lactone (C-1), one phenyl group (C-6~C-11), two *sp*³-hybridized methylenes (C-3 and C-5) including one benzylic methylene, and two *sp*³-methines (C-2 and C-4) linking to the hydroxyl and carbonyloxy groups, respectively. Analysis of coupling relationships of the proton signals, ¹H-¹H COSY and HMBC (C-2/H-3, C-3/H-5, C-4/H-5 and C-7/H-5) correlations led to planar structure of **4**.

The relative stereochemistry for **4** was established by comparison of the observed coupling constants (Table 3) and NOE data with those of two related compounds, antafumicins A (**7**) and B (**8**) (Fig. 2), which have been

Table 3. ¹H and ¹³C NMR data of **4** and **5** in CDCl₃^a.

4					5		
No.	δ_H	<i>J</i> Hz	δ_C	LR ¹ H- ¹³ C COSY(C) ^b	δ_H	<i>J</i> Hz	δ_C
1			177.1				169.6
2 α	3.99 td	8.3(3 α , 3 β), 1.9(OH) ^c	67.0	3 β	2.68 d	16.6(2 β)	42.4
β					2.66 d	16.6(2 α)	
3 α	2.38 ddd	13.2(3 β), 8.3(2), 3.5(4)	34.3	5			70.4
β	2.29 dt	13.2(3 α), 8.3(2, 4)					
4 α			78.1	5	2.09 ddd	14.2(4 β), 10.3(5 β), 5.4(5 α)	34.3
β	4.92 dtd	8.3(3 β), 5.5(5A,B), 3.5(3 α)			1.93 dt	14.2(4 α), 4.1(5 α , 5 β)	
5 α (A)	2.98 dd	15(5B), 5.5(4)	41.1		4.36 ddd	11.3(5 β), 5.4(4 α), 4.1(4 β)	65.7
β (B)	3.00 dd	15(5A), 5.5(4)			4.65 ddd	11.3(5 α), 10.3(4 α), 4.1(4 β)	
6			135.2	5	5.98 dd	17.3(7), 10.7(7)	141.9
7	7.22 d	8.1(8), 1.7(9)	129.6	5	5.24 d	10.7(6)	114.4
					5.35 d	17.3(6)	
8	7.34 m		128.9	10			
9	7.31 m		127.3	7, 11			
10	7.34 m		128.9	8			
11	7.22 d	8.1(10), 1.7(9)	129.6	5			
OH	2.46 br d	1.9(2)			1.88 br s		

^a Measured at 300 and 75.4 MHz for ¹H and ¹³C, respectively.
from C to H.

^b Long range ¹H-¹³C COSY correlations

^c Figures in parentheses indicate a proton coupling with that question.

Fig. 2. Structures of antafumicins.

Fig. 3. ^1H chemical shift differences ($\Delta\delta = \delta_S - \delta_R$) between the (*R*)- and (*S*)-MTPA esters (**9a** and **9b**).

$\Delta\delta$ values are expressed in hertz (500 Hz).

isolated from the fermentation broth of *Aspergillus niger* NH-401 by FUJIMOTO *et al.*^{18,19)} The coupling relationship of H-2 and H-4 to H-3 ($J_{2,3\alpha} = J_{2,3\beta} = J_{3\beta,4} = 8.3$ Hz and $J_{3\beta,4} = 3.5$ Hz) in **4** was closely similar to that of antafumicin A (**7**) [$(J_{2,3A} = 10.7$ Hz, $J_{2,3B} = 9.1$ Hz, $J_{3A,4} = 9.4$ Hz and $J_{3B,4} = 5.2$ Hz)] rather than that of antafumicin B (**8**) [$(J_{2,3A} = 8.8$ Hz, $J_{2,3B} = 4.9$ Hz, $J_{3A,4} = 8.6$ Hz and $J_{3B,4} = 10.4$ Hz)]. Furthermore, in NOESY experiments of **4**, NOEs between H-2 and H-3 α , and between H-4 and H-3 β were observed, whereas an NOE was not observed between H-2 and H-4. An NOE between H-2 and H-4 has been observed in antafumicin B (**8**), but not in **7**. Similarity of the coupling and NOE relationship between **4** and **7** as described above suggested that **4** contains *trans*- γ -lactone. The absolute configuration of this compound (**4**) was established by application of the modified MOSHER's method.²⁰⁾ The ^1H chemical shift differences between the (*R*)- and (*S*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) esters (**9a** and **9b**) were observed as shown in Fig. 3. This result suggested the 2*S* and 4*S* configurations and consequently led to the absolute stereostructure **4** for harzialactone A.

Harzialactone B (**5**) had the molecular formula $\text{C}_7\text{H}_{10}\text{O}_3$ established by HREI-MS. Its IR spectrum

contained absorption bands, characteristic of a hydroxyl group, a δ -lactone and a double bond. Analysis of the ^1H and ^{13}C NMR spectral data (Table 3) of **5** by DEPT and ^1H - ^{13}C COSY experiments revealed the presence of one δ -lactone (C-1), vinyl group (C-6~C-7), tertiary alcohol (C-3) each and three sp^3 -hybridized methylenes (C-2, C-4 and C-5) including one oxygen-bearing methylene. Chemical shifts, coupling relationships and ^1H - ^1H COSY correlations for the three sp^3 -methylene protons led to planar structure of **5**. The coupling constants between H-4 and H-5 ($J_{4B,5B} = 10.3$ Hz, $J_{4B,5A} = 5.4$ Hz and $J_{4A,5A} = J_{4A,5B} = 4.1$ Hz) suggested **5** to exist in a chair conformation in CDCl_3 solution. In the circular dichroism (CD) spectrum of **5**, there was a negative band at 213 nm (Table 1) due to an $n \rightarrow \pi^*$ transition in a carbonyl chromophore, comparable in wave length and strength to that of the related compound **6** (see Experimental), showing **5** to have the same configuration (3*R*) as that of **6**.

Cytotoxic activities of compounds (**1**~**6**) described above were examined in the P388 lymphocytic leukemia test system in cell culture.¹¹⁾ The results are listed in Table 4, and imply that **1**~**3** exhibited significant cytotoxicity.

Experimental

General

UV spectra were recorded on a Shimadzu spectrophotometer and IR spectra on a Perkin Elmer FT-IR spectrometer 1720X. Optical rotations were obtained on a JASCO ORD/UV-5 spectropolarimeter. NMR spectra were recorded at 27°C on a Varian XL-300 spectrometer, operating at 300 and 75.4 MHz for ^1H and ^{13}C , respectively, in CDCl_3 with TMS as an internal reference. EI-MS was determined using a Hitachi M-4000H mass spectrometer. Liquid chromatography was performed over Sephadex LH-20 and silica gel 60 (mesh 230~400, Nacalai Tesque) in an ordinary and a medium pressure, respectively. HPLC was run on a Waters ALC-200 instrument equipped with a differential refractometer (R

Table 4. Cytotoxicity of compounds **1**~**6** against P388 cell line^a.

Compound	ED ₅₀ (μg/ml)
Trichodenone A (1)	0.21
Trichodenone B (2)	1.21
Trichodenone C (3)	1.45
Harzialactone A (4)	> 100
Harzialactone B (5)	60
R-Mevalonolactone (6)	> 100
Mitomycin C ^b	0.05
5-FU ^b	0.07

^a DMSO was used as vehicle in the tests of all compounds. For significant activity, an ED₅₀ value ≤ 4 μg/ml is required.²¹⁾ ^b Used as standard.

401) and Shim-pack PREP-ODS (25 cm × 20 mm i.d.). Analytical TLC was performed on precoated Merck aluminium sheets (DC-Alufolien Kieselgel 60 F₂₅₄, 0.2 mm) and precoated plates RP-18 F_{254S}, and compounds were viewed under UV lamp and sprayed with 10% H₂SO₄ followed by heating.

Fermentation

The sponge *Halichondria okadai*, collected in the Tanabe Bay of Japan, was homogenized with sterile artificial seawater and applied to the surface of nutrient agar layered in a Petri dish. Serial transfers of one of the resulting colonies provided a pure strain of *Trichoderma harzianum* Rifai OUPS-N115. The fungal strain was grown in a liquid medium (300 liters) containing 1% glucose, 0.05% peptone and 1% malt extract in artificial seawater adjusted to pH 7.5 for three weeks at 27°C.

Isolation of Metabolites

The culture filtrate was extracted thrice with AcOEt. The combined extracts were evaporated *in vacuo* to give a mixture of crude metabolites (17.7 g). The mixture was passed through Sephadex LH-20, using CH₂Cl₂-MeOH (1:1) as the eluent. The second fraction (A, 10.2 g), in which cytotoxic activity was concentrated, was chromatographed on a silica gel column with a CH₂Cl₂-MeOH gradient (100:0→0:100) as the eluent. Fractions B (237 mg) and C (216.1 mg), eluted with CH₂Cl₂ and MeOH-CH₂Cl₂ (1:99), respectively, and two fractions D (534.5 mg) and E (137.9 mg), eluted with MeOH-CH₂Cl₂ (1:49), were used to next purification. The fraction B was further chromatographed on silica gel with a CH₂Cl₂-MeOH gradient (100:0→0:100) as the eluent. The MeOH-CH₂Cl₂ (2:98) eluate (F, 131.1 mg)

was separated by HPLC using MeOH-H₂O (1:4) as the eluent to afford two fractions G (47.1 mg) and H (17.4 mg). The fraction H afforded **4** (7.3 mg) after purification by repeated HPLC. The fraction G was purified by HPLC using MeOH-H₂O (2:3) as the eluent to afford **1** (18.6 mg) and **3** (7.8 mg). The MeOH-CH₂Cl₂ (1:49) eluate (I, 145.8 mg) from silica gel column chromatography of the fraction C was repeatedly purified by HPLC using MeOH-H₂O (1:4, 2:3 and 3:2) as the eluent to afford **5** (8.4 mg). The MeOH-CH₂Cl₂ (1:99) eluate (J, 413.7 mg) from silica gel column chromatography of the fraction D afforded **6** (23.2 mg) after purification by HPLC using MeOH-H₂O (1:4) as the eluent. The MeOH-CH₂Cl₂ (1:99) eluate (K, 52.3 mg) from silica gel column chromatography of the fraction E was repeatedly purified by HPLC using MeOH-H₂O (3:7, 2:3 and 1:1) as the eluent to afford **2** (6.0 mg).

R-Mevalonolactone (**6**)

Colorless oil, [α]_D²⁵ -25.6° (25°, *c* 0.2, EtOH); EI-MS *m/z*: 130 [M]⁺. UV λ_{max}^{EtOH} nm (log ε): 226 (3.27), 279 (3.93). IR ν_{max}^{film} cm⁻¹: 3419, 1738. CD λ_{max}^{EtOH} nm (Δε) (*c* 4.05 × 10⁻⁴ M in EtOH): 219 (-0.56). ¹H NMR δ ppm (CDCl₃): 1.40 (3H, s, H-6), 1.91 (1H, m, H-4β), 1.96 (1H, m, H-4α), 2.41 (1H, d, *J* = 17.3 Hz, H-2α), 2.67 (1H, dd, *J* = 17.3, 2.2 Hz, H-2β), 2.78 (1H, br s, OH), 4.36 (1H, ddd, *J* = 11.3, 5.0, 4.0 Hz, H-5α), 4.61 (1H, ddd, *J* = 11.3, 8.7, 6.4 Hz, H-5β); ¹³C NMR δ ppm (CDCl₃): 29.8 (C-6), 35.9 (C-4), 44.7 (C-2), 66.0 (C-5), 68.3 (C-3), 170.5 (C-1). These ¹H and ¹³C NMR data and specific rotation were identical with published values,^{12,13)} though detailed coupling relationships of proton signals were not shown in the previous paper.

Formation of the (*R*)- and (*S*)-MTPA esters **9a** and **9b** from harzialactone A (**4**)

(*R*)-MTPA (5 mg), DCC (5 mg) and DMAP (3 mg) was added to a CH₂Cl₂ solution (1 ml) of harzialactone A (**4**) (0.7 mg), and the reaction mixture was left at room temperature for 2 hours. The solvent was evaporated off under reduced pressure, and the residue was purified by HPLC (ODS) using 80% MeOH in H₂O to afford **9a** (0.8 mg). The same reaction with **4** (0.7 mg) using (*S*)-MTPA (5 mg) gave **9b** (0.9 mg).

9a: Obtained as colorless oil; *m/z* (EI) 390 (M⁺). ¹H NMR δ_H (500 MHz; CDCl₃): 2.43 (1H, dt, *J*=13.7 and 8.0, 3β-H), 2.49 (1H, ddd, *J*=13.7, 9.0 and 3.9, 3α-H), 3.01 (1H, dd, *J*=14.2 and 5.5, 5-H_A), 3.05 (1H, dd, *J*=14.2 and 5.5, 5-H_B), 3.49 (3H, s, OMe), 5.00 (1H, dtd, *J*=8.0, 5.5 and 3.9, 4β-H), 5.02 (1H, dd, *J*=9.0 and 8.0, 2α-H), 7.23 (2H, m, Ar-H), 7.35 (6H, m, Ar-H), 7.52 (2H, m, Ar-H).

9b: Obtained as colorless oil; *m/z* (EI) 390 (M⁺). ¹H NMR δ_H (500 MHz; CDCl₃): 2.26 (1H, dt, *J*=13.8 and 7.9, 3β-H), 2.46 (1H, ddd, *J*=13.8, 9.0 and 3.7, 3α-H), 3.00 (1H, dd, *J*=14.2 and 5.6, 5-H_A), 3.03 (1H, dd, *J*=14.2 and 5.6, 5-H_B), 3.57 (3H, s, OMe), 4.91 (1H, dtd, *J*=7.9, 5.6 and 3.7, 4β-H), 5.15 (1H, dd, *J*=9.0 and 7.9, 2α-H), 7.22 (2H, m, Ar-H), 7.36 (6H, m, Ar-H), 7.50 (2H, m, Ar-H).

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