A Rare Type of Sesquiterpene and β -Santalol Derivatives from *Santalum album* and Their Cytotoxic Activities

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A rare type of sesquiterpene with a spiro bicyclic system (1) and seven new (2–8) and four known (9–12) β -santalol derivatives were isolated from the heartwood of *Santalum album* (Santalaceae). The structures of these new compounds were determined by analysis of extensive spectroscopic data. The isolated compounds and derivatives were evaluated for their cytotoxic activity against HL-60 human promyelocytic leukemia cells, A549 human lung adenocarcinoma cells, HSC-2 and HSC-4 human oral squamous cell carcinoma cell lines, and TIG-3 normal human diploid fibroblasts. *cis*- β -Santalol (9) and β -santalol (10) induced apoptotic cell death in HL-60 cells.

Key words *Santalum album*; Santalaceae; β -santalol; sandalwood; apoptosis; HL-60 cell

Santalum album L. (Santalaceae) is a mid-sized evergreen tree that grows in east India and Indonesia. Sandalwood oil is the volatile substance obtained through steam distillation of the heartwood of *S. album* and is widely used in aromatherapy and traditional medicine.^{1–4} *S. album* contains a variety of aromatic compounds⁵ and sesquiterpenes,^{6–8} of which α -santalol is a major volatile compound that possesses various biological activities.^{9–11} However, β -santalol, another major volatile sesquiterpene from *S. album* with the same bicyclo[2.2.1]heptane skeleton, shows few biological activities, including anti-*Helicobacter pylori*¹² and antiviral effects.¹³ We have reported that a methanolic extract of *S. album* heartwood exhibited cytotoxicity against HL-60 cells with an IC₅₀ value of 2.1 μ g/mL and isolated new α -santalol derivatives¹⁴ and lignans,¹⁵ some of which induced apoptotic cell death in HL-60 and A549 cells. These results prompted us to investigate the heartwood of *S. album* further. In this study, we isolated a rare type of sesquiterpene with a spiro bicyclic system (1), and seven new (2–8) and four known (9–12) β -santalol derivatives. The structures of these new β -santalol derivatives were determined by analysis of extensive spectroscopic data, including two-dimensional NMR data. Here we report the structural determination of 1–12 and their cytotoxic activities against HL-60, A549, HSC-2 and HSC-4 tumor cells, and TIG-3 normal cells. The apoptosis-inducing properties of the β -santalol derivatives with tumor selective cytotoxicity are also described.

Results and Discussion

The heartwood of *S. album* (1.0 kg) was extracted with hot MeOH. The MeOH extract was passed through a porous polystyrene resin (Diaion HP-20) column. The MeOH-eluted fraction was separated by column chromatography (CC) using silica gel and octadecylsilylanized (ODS) silica gel, and by reverse-phase preparative HPLC, giving 1–12 (Fig. 1). Compounds 9–12 were identified as *cis*- β -santalol (9),⁶ β -santalol (10),¹⁶ (9*E*)-11-hydroxy- β -santalol (11),⁷ and (10*Z*)-neosandalol (12),¹² respectively, by comparison of their physical and spectroscopic data with literature values.

Compound 1 was obtained as a colorless oil ($[\alpha]_D$ –23.3 in CHCl₃), and its molecular formula was determined as C₁₅H₂₆O₃ based on the high-resolution electrospray ionization time-of-flight (HR-ESI/TOF)-MS (m/z 277.1772 [M+Na]⁺, Calcd for 277.1780) and ¹³C-NMR (15 carbon signals) data (Table 1). The IR spectrum of 1 showed a broad absorption band from the hydroxy groups at 3335 cm^{–1}. The spectral features of 1 were closely related to those of elviranol,¹⁷ a rare type of sesquiterpene with a spiro bicyclic system. The presence of two hydroxymethylene groups in the molecule was indicated by the ¹H- and ¹³C-NMR signals at δ_H 3.73 (1H, dd, *J*=10.7, 3.8 Hz, H-12a) and 3.61 (1H, dd, *J*=10.7, 4.2 Hz, H-12b), and δ_C 62.9 (C-12); and δ_H 3.73 (1H, dd, *J*=10.7, 3.8 Hz, H-13a) and 3.60 (1H, dd, *J*=10.7, 4.3 Hz, H-13b), and δ_C 63.0 (C-13) in 1. The proton spin-coupling correlations in the ¹H–¹H correlation spectroscopy (COSY) spectrum of 1 revealed that the two hydroxymethylene protons had spin-couplings with the H-11 methine proton at δ_H 1.39 (m). The signals for the C-2 hydroxymethylene group were observed at δ_H 3.54 (1H, dd, *J*=7.8, 3.9 Hz, H-2) and δ_C 79.7 (C-2). Thus, 1 was identified as 13-hydroxy-elviranol. The absolute configuration of the C-2 hydroxy group of 1 was determined by the following procedures. In the nuclear Overhauser effect spectroscopy (NOESY) of 1, NOE correlations between H-2 (δ_H 3.54) and H-3 α (δ_H 1.74), H-6 α (δ_H 0.96), and Me-15 (δ_H 0.91), and between H-4 (δ_H 1.75) and H-8 α (δ_H 2.04) and H-14b (δ_H 1.07), indicated the 2*β*-relative hydroxy configuration. Next, after the C-12 and C-13 hydroxy moieties of 1 were masked with 2,2-dimethoxypropane, the obtained 12,13-isopropylidene derivative (1a) was converted to (*S*)-methylphenylacetic acid (MPA)¹⁸ ester (1b) by treating 1a with (*S*)-MPA in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC-HCl). Following the same procedure, (*R*)-MPA ester (1c) was prepared from 1a using (*R*)-MPA as shown in Fig. 2. When the ¹H-NMR spectrum of 1b was compared with that of 1c, the resonances corresponding to H₂-3, H-4, and H₂-8 of 1b were observed at lower fields than those of 1c, whereas the resonances corresponding to Me-15 and H₂-6 of 1b appeared at higher fields. Thus, the absolute configuration at C-2 of 1 was determined as *S*. The relative configuration at C-10 of 1 was assigned to be *R**, supported by

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Fig. 1. Spiro Bicyclic System (**1**) and β -Santalol Sesquiterpenes (**2**–**12**) Isolated from *Santalum album*

Fig. 2. Determination of the Absolute Configuration at C-2 of **1**

NOE correlations as shown in Fig. 3. Accordingly, the structure of **1** was assigned as shown in Fig. 1.

Compound **2** ($C_{15}H_{26}O_4$) was obtained as a colorless oil ($[\alpha]_D -7.6$ in $CHCl_3$). When the 1H - and ^{13}C -NMR spectra of **2** were compared with those of **12**,¹²⁾ the signals for the C-9 methylene group at δ_H 2.14 (2H, m, H_2 -9) and δ_C 24.7 (C-9), and the C-13 methyl group δ_H 1.80 (3H, s, Me-13) and δ_C 21.8 (C-13) in **12** were replaced by the signals for the hydroxymethylene group at δ_H 4.47 (1H, ddd, $J=11.6, 7.5$ and 5.2 Hz, H-9) and δ_C 69.7 (C-9) and the hydroxymethylene group at δ_H 4.34 and 4.20 (each 1H, d, $J=12.6$ Hz, H_2 -13) and δ_C 59.9 (C-13), respectively, in **2**. In addition, the molecular formula of **2** had two more oxygen atoms than that of **12**. These data suggested that **2** was the 9,13-dihydroxy derivative of **12**, which was supported by the 1H -detected heteronuclear multiple-bond connectivity (HMBC) correlations between δ_H 4.47 (H-9) and δ_C 141.8 (C-11), 128.5 (C-10), 49.9 (C-3), and 49.9 (C-8), and between δ_H 4.34 and 4.20 (H_2 -13) and δ_C 141.8 (C-11), 128.5 (C-10), and 66.6 (C-12) (Fig. 4). The relative configurations at C-2 and C-3 were identified as 2α and 3α , respectively, by the NOE correlations between H-6 β (δ_H 1.41) and H-7 β (δ_H

Fig. 3. Important NOE Correlations of **1**

1.01), between H-7a (δ_H 1.88) and H-8b (δ_H 1.64), and between Me-15 (δ_H 1.14) and H-6a (δ_H 1.22) and Me-14 (δ_H 0.99) in the NOESY spectrum of **2**. Accordingly, the structure of **2** was assigned as shown in Fig. 1.

The 1H - and ^{13}C -NMR spectral data for **3** ($C_{15}H_{28}O_4$) suggest a **12**-derivative with two additional hydroxy groups at the

Fig. 4. Bold Lines Indicate the ^1H - ^1H -Couplings and Arrows Indicate the ^1H - ^{13}C Long-Range Correlations

saturated side chain moiety. When the ^{13}C -NMR spectrum of **3** was compared with that of **12**,¹²⁾ the olefinic carbon signals at δ_{C} 129.5 (C-10) and δ_{C} 134.0 (C-11) in **12** were replaced by the hydroxymethine carbon signal at δ_{C} 84.5 (C-10) and the quaternary carbon signal bearing a hydroxy group at δ_{C} 71.4 (C-11), respectively. Through the ^1H -detected heteronuclear multiple-quantum coherence (HMQC) spectrum of **3**, the C-10 carbon signal was associated with the one-bond coupled hydroxymethine proton signal at δ_{H} 3.77 (1H, dd, $J=12.3$, 4.2 Hz, H-10), which exhibited HMBC correlations with the carbon signals at δ_{C} 71.8 (C-12), 71.4 (C-11), 34.5 (C-8), 20.0 (C-13), and 19.1 (C-9) (Fig. 4). In addition, HMBC correlations between δ_{H} 3.80 and 3.38 (each 1H, d, $J=11.2$ Hz, H₂-12) and δ_{H} 0.96 (3H, s, Me-13) and δ_{C} 84.5 (C-10), 71.8 (C-12), 71.4 (C-11), and 20.0 (C-13) allowed two hydroxy groups to be located at C-10 and C-11 of the side chain moiety, respectively. The 2α and 3α relative configurations of **3** were verified by NOE correlations between H-6 β (δ_{H} 1.43) and H-7b (δ_{H} 1.11), between H-7a (δ_{H} 2.00) and H-8b (δ_{H} 1.35), and between Me-15 (δ_{H} 0.89) and H-6 α (δ_{H} 1.32) and Me-14 (δ_{H} 1.25). Thus, **3** was assigned as shown in Fig. 1.

Compound **4** ($\text{C}_{15}\text{H}_{26}\text{O}_3$) was isolated as a colorless oil. The IR spectrum of **4** suggested the presence of hydroxy groups (3433 cm^{-1}) and an olefinic group (1643 cm^{-1}). The ^1H - and ^{13}C -NMR data for **4** were analogous to those of **3**, except for the signals belonging to the terminal portion of the side chain moiety. Instead of the Me-13 singlet proton signal and C-11 deshielded quaternary carbon signal, the signals for an exomethylene group were observed at δ_{H} 5.12 and 5.06 (each 1H, d, $J=1.1$ Hz, H₂-13) and δ_{C} 150.6 (C) and 111.5 (CH₂). In the HMBC spectrum of **4**, the exomethylene proton signals at δ_{H}

5.12 and 5.06 showed long-range correlations with the carbon signals at δ_{C} 150.6 (C-11), 69.3 (C-10), and 64.3 (C-12). The relative configurations at C-2 and C-3 of **4** were also identified as 2α and 3α , respectively, by similar NOE correlations to those observed for **3**. Thus, **4** was identified as a rare type of a β -neosandalnol derivative with a 11(13)-exomethylene moiety (Fig. 1). The relative stereochemistry at C-2 and C-3 of **2–4** were assigned as $(2\alpha, 3\alpha)$. However, all the absolute configurations of **2–4** remain to be determined.

Compound **5** ($\text{C}_{15}\text{H}_{24}\text{O}_2$) was isolated as a colorless oil. The ^1H - and ^{13}C -NMR spectral features of **5**, particularly signals from the side chain moiety, were different from those of **2–4**. The COSY and HMQC spectra of **5** revealed that the partial structural unit $-\text{C}_{(8)}\text{H}_2-\text{C}_{(9)}\text{H}_2-\text{C}_{(10)}\text{H}-\text{C}_{(14)}\text{H}_2-$ showed long-range correlations from the proton signals at δ_{H} 1.91 (dd, $J=11.9$, 7.5 Hz, H-8a) and 1.42 (br d, $J=11.9$ Hz, H-8b), and δ_{H} 1.65 (ddd, $J=12.3$, 6.0, 2.1 Hz, H-14a) and 1.40 (dd, $J=12.3$, 12.1 Hz, H-14b) to the carbon signals at δ_{C} 79.9 (C-2), 57.2 (C-3), and 45.1 (C-4) in the HMBC spectrum. These data indicated that a five-membered cyclic side chain moiety was attached to C-3 to form a spiro-cyclic skeleton. Furthermore, the ^1H - and ^{13}C -NMR signals at δ_{H} 5.01 and 4.92 (each 1H, dd, $J=1.0$, 1.0 Hz) and δ_{C} 106.9 and 152.8 were assigned to the C-11(13) exomethylene group, and those at δ_{H} 4.11 (2H, brs) and δ_{C} 65.6 were attributed to the C-12 hydroxymethylene group. NOE correlations between H-7a (δ_{H} 1.91) and H₂-8 (δ_{H} 1.91 and 1.42), and between Me-15 (δ_{H} 1.17) and H-5 (δ_{H} 1.32), H-6 α (δ_{H} 1.36), and H₂-14 (δ_{H} 1.65 and 1.40) provided evidence that **5** had the relative stereochemistry $(2\alpha, 3S^*)$. An NOE correlation between H-4 (δ_{H} 1.93) and H-10 (δ_{H} 2.48) indicated that H-10 was on the same side of the H-4 bridgehead

Table 1. ^1H - and ^{13}C -NMR (500 MHz) Data for **1–8** in CDCl_3 at 300 K

	1^{a)}			2			3			4		
	^1H	J (Hz)	^{13}C	^1H	J (Hz)	^{13}C	^1H	J (Hz)	^{13}C	^1H	J (Hz)	^{13}C
1	—		49.7	2.07 d	3.8	47.5	1.86 dd	5.1, 1.1	52.4	1.98 dd	4.3, 1.1	50.6
2	3.54 dd	7.8, 3.9	79.7	—		90.0	—		84.3	—		82.9
3	1.53 ddd	13.2, 7.7, 3.9	40.9	—		49.9	—		43.1	—		41.7
	1.74 dd	13.2, 7.8	—			—			—			
4	1.75 brs		46.0	1.91 d	2.9	47.6	1.73 d	2.1	49.4	1.72 brd	1.9	49.2
5	1.66 m		28.4	1.28–1.33 m		22.7	1.58 m		24.0	1.58 m		23.9
	1.03 m		—			—	1.29 m		—	1.28 m		
6 β	1.35 ddd	12.2, 10.8, 4.6	35.2	1.41 m		22.5	1.43 m		23.3	1.42 m		23.0
6 α	0.96 ddd	12.2, 10.8, 3.0	—	1.22m		—	1.32 m		—	1.31 m		
7a	—		58.5	1.88 ddd	10.0, 1.8, 1.8	33.7	2.00 ddd	9.8, 2.1, 2.1	34.8	2.24 ddd	10.9, 2.2, 2.2	34.4
7b	—		—	1.01 ddd	10.0, 1.5, 1.5	—	1.11 ddd	9.8, 1.5, 1.5	—	1.12 brd	10.9	
8a	2.04 ddd	13.2, 8.9, 2.6	29.6	1.78 dd	12.3, 5.2	49.9	1.42 m		34.5	1.60 dddd	12.8, 12.8, 3.1, 3.1	30.4
8b	1.39 m		—	1.64 dd	12.3, 11.6	—	1.35 t	12.8	—	1.06 ddd	12.8, 3.1, 3.1	
9	1.83 m		32.5	4.47 ddd	11.6, 7.5, 5.2	69.7	1.64 dddd	13.1, 13.0, 12.3, 2.3	19.1	1.89 dddd	13.9, 9.7, 9.5, 3.1	24.1
	1.11 t	9.8	—	—		—	1.49 m		—	1.49 ddd	13.9, 4.5, 3.1, 2.1	
10	1.79 m		38.3	5.67 d	7.5	128.5	3.77 dd	12.3, 4.2	84.5	4.39 dd	9.7, 2.1	69.3
11	1.39 m		49.9	—		141.8	—		71.4	—		150.6
12	3.73 dd	10.7, 3.8	62.9	4.21 s		66.6	3.80 d	11.2	71.8	4.29 d	12.8	64.3
	3.61 dd	10.7, 4.2	—	—		—	3.38 d	11.2	—	4.08 d	12.8	
13	3.73 dd	10.7, 3.8	63.0	4.34 d	12.6	59.9	0.96 s		20.0	5.12 d	1.1	111.5
	3.60 dd	10.7, 4.3	—	4.20 d	12.6	—	—		—	5.06 d	1.1	
14	1.65 dd	11.5, 6.0	36.0	0.99 s		20.3	1.25 s		23.0	1.16 s		15.5
	1.07 dd	11.5, 11.5	—	—		—	—		—	—		
15	0.91 s		12.3	1.14 s		16.9	0.89 s		22.0	0.86 s		19.3

^{a)} CD_3OD .

Table 1. (Continued)

	5			6			7			8		
	^1H	J (Hz)	^{13}C	^1H	J (Hz)	^{13}C	^1H	J (Hz)	^{13}C	^1H	J (Hz)	^{13}C
1	1.96 dd	4.3, 1.1	51.0	1.96 dd	4.5, 1.4	51.1	2.67 brd	2.7	46.1	2.01 brs		47.9
2	—		79.9	—		79.9	—		166.6	—		50.6
3	—		57.2	—		57.5	—		54.6	—		49.8
	—		—	—		—	—		—	—		
4	1.93 brd	2.0	45.1	1.99 d	1.8	45.0	1.95 brs		46.8	1.72 brs		47.0
5 β	1.32 m	(2H)	23.1	1.36 m	(2H)	23.8	1.43 m		24.0	1.25 m		22.7
5 α	—		—	—		—	1.51 m		—	1.41 ddd	10.9, 2.5, 2.5	
6 β	1.42 m		23.8	1.43 m		23.2	1.21 m		29.9	1.48 ddd	10.8, 2.1, 2.1	23.1
6 α	1.36 m		—	1.29 m		—	1.64 m		—	1.23 m		
7	1.91 brd	10.6, 1.7	34.4	1.93 d	10.4	34.5	1.56 brd	9.4	37.5	1.82 d	10.4	34.1
	1.16 dd	10.6, 1.7	—	1.17 d	10.4	—	1.24 brd	9.4	—	0.99 d	10.4	
8	1.91 dd	11.9, 7.5	32.3	1.94 dd	10.9, 10.9	32.3	1.56 m		41.0	1.36 dd	8.2, 3.0	40.1
	1.42 brd	11.9	—	1.43 brd	10.9	—	—		—	—		
9	1.88 brd	13.6	28.2	1.98 m		28.3	1.81 m		30.1	1.53 ddd	8.2, 5.9, 2.2	25.0
	1.49 brd	13.6	—	—		—	1.40 m		—	1.31 m		
10	2.48 ddd	12.1, 9.1, 6.0	40.7	2.84 m		36.0	1.79 m		36.9	1.84 dd	12.7, 5.8	55.1
11	—		152.8	—		154.4	1.62 m		47.9	—		75.4
12	4.11 brs	(2H)	65.6	9.52 s		195.0	3.91 brd	10.5	66.0	3.60 d	10.7	70.0
	—		—	—		—	3.71 brdd	10.5, 9.0	—	3.40 d	10.7	
13	5.01 dd	1.0, 1.0	106.9	6.28 brs		131.9	3.90 brd	9.4	66.0	1.24 s		23.6
	4.92 dd	1.0, 1.0	—	5.95 brs		—	3.74 brdd	9.4, 8.5	—	—		
14	1.65 ddd	12.3, 6.0, 2.1	37.0	1.72 ddd	12.2, 6.5, 1.6	37.1	1.93 dd	11.6, 7.3	40.0	0.94 s		16.2
	1.40 dd	12.3, 12.1	—	1.33 dd	12.2, 7.2	—	1.29 dd	11.6, 11.6	—	—		
15	1.17 s		22.8	1.15 s		22.8	4.73 brs		99.3	0.84 s		20.9
							4.53 brs					

proton. Thus, the relative stereochemistry of **5** was assigned as ($2\alpha,3S^*,10R^*$) and the structure of **5** was assigned as shown in Fig. 1.

The spectroscopic features of **6** ($C_{15}H_{22}O_2$) and **7** ($C_{15}H_{24}O_2$) were closely related to those of **5**, suggesting that **6** and **7** were the β -santalol derivatives with the spirocyclic skeleton. The 1H - and ^{13}C -NMR spectra of **6** differed from those of **5** because of aldehyde signals at δ_H 9.52 (1H, s) and δ_C 195.0 instead of the C-12 primary alcohol group. The HMBC correlations between δ_H 9.52 and δ_C 154.4 (C-11), 131.9 (C-13), and 36.0 (C-10) confirmed that an aldehyde group was located at C-12 in **6**, and NOE correlations between H-7a (δ_H 1.93) and H₂-8 (δ_H 1.94 and 1.43), between Me-15 (δ_H 1.15) and H-5 (δ_H 1.36), H-6a (δ_H 1.29), and H₂-14 (δ_H 1.72 and 1.33), and between H-4 (δ_H 1.99) and H-10 (δ_H 2.84) indicated that the relative configurations of **6** were the same as those of **5**. Treatment of **5** with Dess–Martin periodinane (DMP) in dry CH_2Cl_2 yielded **6**. Thus, the structure of **6** was assigned as shown in Fig. 1.

When the 1H -NMR spectrum of **7** was compared with that of **5**, the signal at δ_H 1.17 (3H, s), which was assigned to the Me-15 group bearing a geminal hydroxy group in **5**, was replaced by exomethylene group signals at δ_H 4.73 and 4.53 (each 1H, s, H₂-15) in **7**. The exomethylene protons showed long-range correlations with the carbons of δ_C 166.6 (C-2), 54.6 (C-3), and 46.1 (C-1) in the HMBC spectrum. Furthermore, the exomethylene signals belonging to C-11(13) were not observed in the 1H - and ^{13}C -NMR spectra of **7**. Instead, the signals of two hydroxymethylene groups appeared at δ_H 3.91 (1H, brd, $J=10.5$ Hz, H-12a) and 3.71 (1H, brdd, $J=10.5$, 9.0 Hz, H-12b) and δ_H 3.90 (1H, brd, $J=9.4$ Hz, H-13a) and 3.74

(1H, brdd, $J=9.4$, 8.5 Hz, H-13b), which had a spin-coupling correlation with the H-11 methine proton signal at δ_H 1.62 (1H, m). These data indicated that **7** was the C-2/C-15 dehydrated and C-13 hydroxylated derivative of **5**. In the NOESY spectrum of **7**, NOE correlations were observed between H-14a (δ_H 1.93) and H-5a (δ_H 1.51), between H-14b (δ_H 1.29) and H-15b (δ_H 4.53), between H-1 (δ_H 2.67) and H-15a (δ_H 4.73), and between H-10 (δ_H 1.79) and H-4 (δ_H 1.95). Therefore, the structure of **7** was assigned as shown in Fig. 1.

Compound **8** ($C_{15}H_{26}O_2$) showed spectral features similar to those of β -cyclosantalol,¹⁹⁾ a β -santalol analogue containing an C-12 aldehyde group, where the C-10 carbon of the side-chain moiety is linked to the C-2 carbon to form a five-membered ring. Instead of the signals for the C-12 aldehyde group, those from the hydroxymethylene group were observed at δ_H 3.60 and 3.40 (each 1H, d, $J=10.7$ Hz, H₂-12) and δ_C 70.0 (C-12), indicating that **8** was the C-12 hydroxy-reduced derivative of β -cyclosantalol.¹⁹⁾ This was supported by the HMBC correlations from the hydroxymethylene proton signals at δ_H 3.60 and 3.40 to the carbon signals at δ_C 75.4 (C-11), 55.1 (C-10), and 23.6 (C-13). The NOE correlations between H-10 (δ_H 1.84) and H-9 β (δ_H 1.53), between H-10 and H-1 (δ_H 2.01), between Me-15 (δ_H 0.84) and H-5a (δ_H 1.41), H-9a (δ_H 1.31), and Me-14 (δ_H 0.94), and between Me-14 and H-9a, H-6a (δ_H 1.23), and Me-15, supported the 2α , 3α , and $10S^*$ relative configurations of **8**. The absolute configurations of **5**–**8** have not been determined. Accordingly, the structure of **8** was assigned as shown in Fig. 1.

Isolated compounds **1**–**12** were evaluated for their cytotoxic activities against HL-60, A549, HSC-2, and HSC-4 tumor cells, and TIG-3 normal cells (Table 2). Etoposide and cis-

Fig. 5. (A) Morphological Observations of Representative Fields of HL-60 Cell Stained with DAPI to Evaluate Fragmented and Condensed Nuclear Chromatins after Treatment with **9**, **10** ($40\mu M$) or Etoposide ($15\mu M$) for 24h; (B) Caspase-3 Activity in **9**, **10**, or Etoposide-Treated HL-60 Cell Lysates; HL-60 Cells Were Incubated at $37^\circ C$ for 16h, without (Control) or with **9**, **10** ($40\mu M$) or Etoposide ($15\mu M$); Data Are Presented as the Mean $\pm$ S.E.M. from Measurements in Triplicate

Statistical significance is indicated as $*p < 0.05$ as determined by Dunnett's multiple comparison test.

platin were used as positive controls, and had IC_{50} values of 0.5 ± 0.03 and $1.8 \pm 0.05 \mu M$ against HL-60 cells; 4.4 ± 0.4 and $2.4 \pm 0.1 \mu M$ against A549 cells; and 14.5 ± 1.0 and $2.6 \pm 0.2 \mu M$ against TIG-3 cells, respectively. Etoposide was not cytotoxic to HSC-2 cells and HSC-4 cells even at a concentration of $20 \mu M$. As in the case of the α -santalol derivatives,¹⁴⁾ the β -santalol derivative with an aldehyde group at the terminal side chain moiety (**6**) showed the most potent cytotoxic activities. In the neosandalnol skeleton (**2–6** and **12**), the introduction of an exomethylene group in the side chain (**4–6**) seem to contribute partially to the cytotoxicity, whereas hydrogenation of the double-bond in the side chain (**3** and **7**) decreased in the activity. *cis*- β -Santalol **9**, differs from **12** in structure with the presence of an exomethylene group at C-2, was more cytotoxic compared to **12**. These data imply that the presence of an exomethylene group and double bond (**9** and **10**) might be important and contribute to the cytotoxic activity. In particular, **9** and **10** were cytotoxic to the adherent tumor cells (A549, HSC-2, or HSC-4) with IC_{50} values ranging from 11.0 ± 0.5 to $39.3 \pm 1.2 \mu M$ for **9** and 29.0 ± 0.1 to $>40 \mu M$ for **10**, and they did not affect the cell growth of the TIG-3 normal cells at $40 \mu M$. Moreover, **9** showed moderate cytotoxicity against both HSC-2 and HSC-4 cells, which are resistant to most anti-cancer agents.^{20,21)} Morphological observation of HL-60 cells stained with 4',6-diamidino-2-phenylindole (DAPI) suggested that **9** and **10** induced apoptosis in HL-60 cells; the cell displayed nuclear chromatin condensation and apoptotic bodies (Fig. 5A). Furthermore, an increase of caspase-3 activity in HL-60 cells was evident after 16h of treatment with **9** and **10** (Fig. 5B). Thus, **9** and **10** induced apoptotic cell death in HL-60 cells through caspase-3 activation.

In conclusion, *cis*- β -santalol (**9**) and β -santaldiol (**10**) induced apoptotic cell death in HL-60 cells. It has been reported that α -santalol have induced apoptosis in tumor cells by various mechanism.^{22–25)} On the other hand, the cytotoxic activities of the β -santalol derivatives against cultured tumor cell lines were examined in detail for the first time.

Experimental

Optical rotations were measured using an automatic digital polarimeter (P-1030, JASCO, Tokyo, Japan). IR spectra were recorded on a spectrophotometer (FT-IR 620, JASCO). ¹H-NMR spectra were recorded at 500 MHz for using standard Bruker pulse programs at 300 K (DRX-500, Bruker, Karlsruhe, Germany). Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard. HR-ESI/TOF-MS data were recorded on an LCT mass spectrometer (Waters-Micromass, Manchester, U.K.). Diaion HP-20 (50 mesh, Mitsubishi-Chemical, Tokyo, Japan), BW-300 silica gel (200–300 mesh, Fuji-Silysia Chemical, Kasugai, Japan), and ODS silica gel COSMOSIL 75C₁₈-OPN (75 μm , Nacalai Tesque, Kyoto, Japan) were used for CC. TLC was carried out on precoated silica gel 60F₂₅₄ (0.25 mm thick, Merck, Darmstadt, Germany) and RP₁₈ F₂₅₄S plates (0.25 mm thick, Merck), and the spots were visualized by spraying the plates with 10% H₂SO₄ (aq) and then heating. HPLC was performed with a system consisting of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh), and a Rheodyne injection port (Rohnert Park, CA, U.S.A.). A TSK gel ODS-100Z column (10 mm i.d. $\times$ 250 mm, 5 μm , Tosoh) was used for preparative HPLC.

The purities of all the isolated compounds were confirmed by their NMR spectra, optical rotations, mass spectrometry, and TLC analysis. The following materials and reagents were used for the cell cultures and the cytotoxicity assays: a microplate reader (Spectra Classic, Tecan, Salzburg, Austria); a 96-well flat-bottom plate (Iwaki Glass, Funabashi, Japan); JCRB 0085 HL-60 cells, JCRB 0076 A549 cells, and JCRB 0506 TIG-3 cells (Human Science Research Resources Bank, Osaka, Japan); HSC-2 cells and HSC-4 cells (Riken Cell Bank, Tsukuba, Japan); fetal bovine serum (FBS; Bio-Whittaker, Walkersville, MD, U.S.A.); 0.25% trypsin–EDTA solution, RPMI-1640 medium, minimum essential medium (MEM), etoposide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma, St. Louis, MO, U.S.A.); Dulbecco's modified Eagle's medium (DMEM), penicillin G sodium salt and streptomycin sulfate (Gibco, Grand Island, NY, U.S.A.). All other chemicals used were of biochemical reagent grade.

Plant Material The heartwood of *S. album* was obtained from Uchida Wakannyaku¹⁵⁾ (Tokyo, Japan).

Extraction and Isolation The heartwood of *S. album* (1.0 kg dry weight) was extracted with hot MeOH (12 L) for 8 h. After removing the solvent, the MeOH extract (9 L; cytotoxicity IC_{50} 2.1 $\mu g/mL$) was passed through a Diaion HP-20 column (2300 g, 8.5 cm i.d. $\times$ 60 cm) and was successively eluted with 30% MeOH, 50% MeOH, MeOH, EtOH, and EtOAc (each 9 L). CC of the MeOH-eluted fraction (40.0 g; cytotoxicity IC_{50} 2.2 $\mu g/mL$) on silica gel (2000 g, 8.0 cm i.d. $\times$ 40 cm) eluted with a stepwise gradient mixture of hexane–EtOAc (7:1, 5:1, 2:1, 1:1) and finally with pure MeOH, provided 13 fractions (A–M). Fraction B was chromatographed on silica gel column (1000 g, 6.0 cm i.d. $\times$ 20 cm) eluted with hexane–EtOAc (50:1, 15:1, 5:1) to afford **9** (40.1 mg). Fraction D was chromatographed on ODS silica gel (600 g, 4.5 cm i.d. $\times$ 30 cm) eluted with MeCN–H₂O (1:1, 3:1, 5:1) to give **4** (19.4 mg) and **6** (5.4 mg). Fraction E was separated by an ODS silica gel column (200 g, 4.0 cm i.d. $\times$ 15 cm) eluted with MeCN–H₂O (1:1, 2:1) to give **3** (9.4 mg) and **8** (3.4 mg). Fraction F was chromatographed on silica gel (1000 g, 5.0 cm i.d. $\times$ 30 cm) eluted with hexane–EtOAc (3:1, 2:1) and on ODS silica gel (1200 g, 6.0 cm i.d. $\times$ 30 cm) eluted with MeOH–H₂O (3:1) and MeCN–H₂O (2:3, 1:2) to afford **5** (18.6 mg), **11** (6.5 mg), and **12** (7.0 mg). Fraction G was separated by HPLC using hexane–EtOAc (1:2) and MeCN–H₂O (2:1) to give **7** (13.0 mg) and **10** (27.0 mg). Fraction K was chromatographed on ODS silica gel (500 g, 4.0 cm i.d. $\times$ 30 cm) eluted with MeCN–H₂O (1:2, 1:1) and MeOH–H₂O (2:1) to yield **2** (4.2 mg) and **1** (4.9 mg).

Compound 1: Colorless oil, $[\alpha]_D^{23} -23.3$ ($c=0.25$, CHCl₃). IR (film) ν_{max} cm⁻¹: 3335 (OH), 2921 and 2851 (CH). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS m/z : 277.1772 [$M+Na$]⁺ (Calcd for C₁₅H₂₆NaO₃; 277.1780).

Preparation of (S)-MPA Ester (1b) and (R)-MPA Ester (1c) of 1 A solution of **1** (2.8 mg) and *p*-toluenesulfonic acid (TsOH) (*ca.* 9.0 mg) in dry dimethylformamide (DMF) (1.0 mL) was treated with 2,2-dimethoxypropane (0.1 mL), and the mixture was stirred at room temperature (rt) for 2 h. The reaction mixture was neutralized with saturated NaHCO₃ (aq) (3.0 mL) and extracted with EtOAc (2.0 mL $\times$ 3) to yield the crude isopropylidene derivative (**1a**) of **1**. A mixture of **1a** (2.2 mg), (S)-MPA (10.0 mg), EDC-HCl (40.0 mg), and *N,N*-dimethyl-4-aminopyridine (DMAP) (10.0 mg) in CH₂Cl₂

Table 2. Cytotoxic Activities of Compounds **1–12**, Etoposide, and Cisplatin against HL-60, A549, HSC-2, HSC-4, and TIG-3 Cells

Compound	IC ₅₀ (μM)				
	HL-60	A549	HSC-2	HSC-4	TIG-3
1	>40	>40	>40	>40	>40
2	19.7±0.2	>40	>40	>40	>40
3	>40	>40	>40	>40	>40
4	13.0±0.7	>40	>40	>40	11.5±2.1
5	19.9±0.7	27.5±0.6	>40	>40	13.5±0.5
6	2.1±0.03	19.4±0.5	13.5±0.6	24.4±1.0	12.1±2.2
7	>40	>40	>40	>40	>40
8	36.0±2.2	>40	>40	>40	>40
9	9.9±0.5	39.3±1.2	11.0±0.5	17.7±0.7	>40
10	11.9±0.4	29.0±0.1	>40	>40	>40
11	10.4±0.4	>40	>40	>40	38.5±0.6
12	20.3±0.5	>40	>40	>40	>40
Etoposide	0.5±0.03	4.4±0.4	>40	>40	14.5±1.0
Cisplatin	1.8±0.05	2.4±0.1	1.6±0.03	2.3±0.4	2.6±0.2

(0.7 mL) was stirred at rt for 24 h. The reaction mixture was poured into 1.0 M HCl (2.0 mL) and extracted with CHCl₃ (2.0 mL×3). Evaporation of the solution followed by preparative TLC using hexane–EtOAc (4:1) gave the (*S*)-MPA ester (**1b**, 3.6 mg) of **1a**. Using the same procedure, **1a** (2.2 mg) was converted to the (*R*)-MPA ester (**1c**, 2.2 mg).

(*S*)-MPA ester (**1b**) of **1**: Amorphous solid, ¹H-NMR (CDCl₃, 500 MHz) δ: 7.41–7.26 (5H, m, aromatic protons), 4.70 (1H, s, MPA-H), 4.60 (1H, dd, *J*=7.8, 3.4 Hz, H-2), 3.84 (1H, dd, *J*=10.6, 4.6 Hz, H-13a), 3.82 (1H, dd, *J*=10.6, 4.6 Hz, H-12a), 3.62 (1H, dd, *J*=10.6, 3.0 Hz, H-13b), 3.60 (1H, dd, *J*=10.6, 3.0 Hz, H-12b), 3.41 (3H, s, MPA-OCH₃), 1.78 (1H, dd, *J*=13.6, 7.8 Hz, H-3a), 1.68 (1H, dd, *J*=4.3, 4.3 Hz, H-4), 1.63 (1H, m, H-9a), 1.59 (1H, m, H-11), 1.58 (1H, m, H-5a), 1.55 (1H, m, H-8a), 1.47 (1H, dd, *J*=9.2, 6.8 Hz, H-14a), 1.42 (3H, s, Me-2'), 1.39 (3H, s, Me-3'), 1.33 (1H, ddd, *J*=12.5, 12.5, 4.8 Hz, H-6a), 1.25 (1H, m, H-3b), 1.12 (1H, ddd, *J*=10.9, 9.2, 3.6 Hz, H-14b), 1.06 (1H, m, H-5b), 1.00 (1H, m, H-8b), 0.99 (1H, m, H-9b), 0.91 (1H, ddd, *J*=12.5, 11.9, 0.8 Hz, H-6b), 0.80 (3H, s, Me-15). ¹³C-NMR (CDCl₃, 125 MHz) δ: 170.1 (OC=O), 136.4, 128.5, and 127.2 (aromatic carbons), 97.6 (C-1'), 82.7 (MPA-C-), 81.5 (C-2), 64.3 (C-12), 64.1 (C-13), 57.9 (C-7), 57.3 (OMe), 48.2 (C-1), 44.2 (C-4), 40.3 (C-11), 38.4 (C-3), 37.9 (C-10), 33.6 (C-6), 33.5 (C-14), 30.3 (C-9), 27.7 (C-8), 27.6 (Me-2'), 27.1 (C-5), 20.3 (Me-3'), 11.7 (Me-15). HR-ESI/TOF-MS *m/z*: 465.2630 [M+Na]⁺ (Calcd for C₂₇H₃₈NaO₅; 465.2617).

(*R*)-MPA ester (**1c**) of **1**: Amorphous solid, ¹H-NMR (CDCl₃, 500 MHz) δ: 7.43–7.21 (5H, m, aromatic protons), 4.69 (1H, s, MPA-H), 4.68 (1H, dd, *J*=8.7, 3.5 Hz, H-2), 3.84 (1H, dd, *J*=11.4, 2.6 Hz, H-13a), 3.81 (1H, dd, *J*=11.4, 2.6 Hz, H-12a), 3.63 (1H, dd, *J*=11.4, 8.8 Hz, H-13b), 3.61 (1H, dd, *J*=11.4, 8.8 Hz, H-12b), 3.41 (3H, s, MPA-OCH₃), 1.83 (1H, dd, *J*=13.4, 8.7 Hz, H-3a), 1.75 (1H, dd, *J*=4.3, 4.3 Hz, H-4), 1.68 (1H, m, H-9a), 1.62 (1H, m, H-8), 1.56 (1H, m, H-11), 1.56 (1H, m, H-10), 1.55 (1H, m, H-5a), 1.53 (1H, dd, *J*=13.4, 3.4 Hz, H-3b), 1.45 (1H, dd, *J*=9.1, 5.7 Hz, H-14a), 1.42 (3H, s, Me-2'), 1.39 (3H, s, Me-3'), 1.25 (1H, m, H-6a), 1.25 (1H, m, H-8b), 1.09 (1H, ddd, *J*=9.1, 9.1, 3.0 Hz, H-14b), 1.07 (1H, m, H-5b), 0.92 (1H, m, H-9b), 0.84 (1H, m, H-6b), 0.44 (3H, s, Me-15). ¹³C-NMR (CDCl₃, 125 MHz) δ: 170.1 (OC=O), 136.5, 128.8, and 127.3 (aromatic rings), 97.5 (C-1'), 82.7 (MPA-C-),

81.0 (C-2), 64.2 (C-12), 64.1 (C-13), 57.9 (C-7), 57.3 (OMe), 48.7 (C-1), 44.4 (C-4), 40.3 (C-11), 38.3 (C-3), 37.9 (C-10), 33.5 (C-6), 33.5 (C-14), 30.4 (C-9), 28.1 (C-8), 27.5 (Me-2'), 27.1 (C-5), 20.4 (Me-3'), 11.2 (Me-15). HR-ESI/TOF-MS *m/z*: 465.2625 [M+Na]⁺ (Calcd for C₂₇H₃₈NaO₅; 465.2617).

Compound **2**: Colorless oil, [α]_D²⁵ –7.6 (*c*=0.21, CHCl₃). IR (film) $\nu_{\max}$ cm^{–1}: 3450 (OH), 2926 (CH). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 275.1634 [MNa–H₂O]⁺ (Calcd for C₁₅H₂₄NaO₃; 275.1623).

Compound **3**: Colorless oil, [α]_D²⁵ –3.5 (*c*=0.48, CHCl₃). IR (film) $\nu_{\max}$ cm^{–1}: 3438 (OH), 2959 and 2874 (CH). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 277.1774 [MNa–H₂O]⁺ (Calcd for C₁₅H₂₆NaO₃; 277.1780).

Compound **4**: Colorless oil, [α]_D²⁵ –5.2 (*c*=0.13, CHCl₃). IR (film) $\nu_{\max}$ cm^{–1}: 3433 (OH), 2929 and 2876 (CH), 1643 (C=C). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 259.1661 [MNa–H₂O]⁺ (Calcd for C₁₅H₂₄NaO₂; 259.1674).

Compound **5**: Colorless oil, [α]_D²⁵ +3.5 (*c*=0.43, CHCl₃). IR (film) $\nu_{\max}$ cm^{–1}: 3421 (OH), 2957 and 2875 (CH). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 219.1748 [M–OH]⁺ (Calcd for C₁₅H₂₃O; 219.1749).

Compound **6**: Colorless oil, [α]_D²⁵ –6.6 (*c*=0.17, CHCl₃). IR (film) $\nu_{\max}$ cm^{–1}: 3444 (OH), 2961 (CH), 1681 and 1650 (C=C). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 257.1517 [M+Na]⁺ (Calcd for C₁₅H₂₂NaO₂; 257.1502).

Preparation of 6 from 5 DMP (10 mg) was added to a solution of **5** (5.0 mg) in dry CH₂Cl₂ (0.5 mL) at 0°C. After stirring at rt for 2 h, the reaction mixture was diluted with EtOAc (2.0 mL) and poured into saturated NaHCO₃ (aq) Na₂S₂O₄ (aq) (1:1, 2.0 mL). After stirring at rt for 1 h, the mixture was extracted with EtOAc (2.0 mL×3). The EtOAc residue was purified by preparative TLC using CHCl₃–MeOH (10:1) to yield **6** (1.3 mg).

Compound **7**: Colorless oil, [α]_D²³ +22.0 (*c*=0.10, CHCl₃). IR $\nu_{\max}$ (film) cm^{–1}: 3431 (OH), 2354, 2323, and 2153 (CH), and 1642 (C=C). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 259.1663 [M+Na]⁺ (Calcd for C₁₅H₂₄NaO₂; 259.1674).

Compound **8**: Colorless oil, [α]_D²⁶ –7.4 (*c*=0.21, CHCl₃). IR (film) $\nu_{\max}$ cm^{–1}: 3432 (OH). ¹H- and ¹³C-NMR, see Table 1. HR-ESI/TOF-MS *m/z*: 261.1813 [M+Na]⁺ (Calcd for

C₁₅H₂₆NaO₂; 261.1831).

Cell Culture Assays Cell growth was measured with an MTT reduction assay as previously described.¹⁵⁾ Briefly, HL-60 cells were maintained in an RPMI-1640 medium; A549 and TIG-3 cells were maintained in MEM; and HSC-2 and HSC-4 cells were maintained in DMEM. The media contained heat-inactivated 10% (v/v) FBS supplemented with L-glutamine, penicillin G sodium salt (100 units/mL), and streptomycin sulfate (100 µg/mL). The HL-60 (4×10⁴ cells/mL), A549 (1×10⁴ cells/mL), TIG-3 (2×10⁴ cells/mL), HSC-2 (1×10⁴ cells/mL), and HSC-4 (2×10⁴ cells/mL) cells were continuously treated with each compound for 72 h, and cell growth was measured with an MTT reduction assay. The concentration (up to 40 µM) resulting in a 50% inhibition (IC₅₀ value) was calculated from the dose-response curve. Data are represented as means±standard error of the mean (S.E.M.) of three experiments that were each performed in triplicate.

Assay for Caspase-3 Activation The activity of caspase-3 was measured using a commercially available kit (Apocyto Caspase-3 Colorimetric Assay Kit, MBL, Nagoya, Japan) as previously described.¹⁵⁾ Briefly, HL-60 cells (2×10⁶ cells/mL) were treated with a compound for 16 h and the cells were centrifuged and collected. The cell lysate (50 µL, equivalent to 200 µg of protein) was mixed with reaction buffer (2×50 µL) containing the substrates for caspase-3 (DEVD-pNA). After incubation for 2 h at 37°C, the absorbance at 405 nm of the liberated p-nitroanilide chromophore was measured using a microplate reader. The activity of caspase-3 was measured in triplicate.

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