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Isolation of new carbamate- or pyridine-containing natural products, fuzanins A, B, C, and D from *Kitasatospora* sp. IFM10917

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ABSTRACT

New carbamate or pyridine-containing natural products, fuzanins A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from the culture supernatant of *Kitasatospora* sp. IFM10917. These compounds were purified by solvent separation, column chromatography, and preparative HPLC, consecutively. The chemical structure of each compound was established on the basis of a spectroscopic analysis and chemical means. Fuzanin D (**4**) showed cytotoxicity against DLD-1 cells (IC_{50}, 41.2 μM) and moderate inhibition of Wnt signal transcription at 25 μM.

1. Introduction

Actinomycetes from soil and marine source are widely recognized to produce secondary metabolites including a number of antimicrobials as penicillin, streptomycin, erythromycin, and tetracycline with original and ingenious structures and potent biological activities.[1] Therefore, it is thought that actinomycete is a potent resource for new lead or seed compounds in the drug development. During our studies on search for bioactive natural products,[2,3] we explored actinomycetes collected from soil and sea water of different areas of Japan. Culture extracts of 323 actinomycete strains were subjected to preliminary screening for antimicrobial activity. The active strain, *Kitasatospora* sp. IFM10917 isolated from a soil sample collected at Toyama city, produced an inhibition zone of 92 mm at 50 mg/mL against *Bacillus subtilis*. This strain was then selected for investigation of antimicrobials using a chemical screening. The genus *Kitasatospora* was proposed taxonomically by Ōmura et al. in 1982,[4] subsequently subsumed within the genus *Streptomyces*,[5,6] re-established by Zhang et al.[7] and now embraces 19 species[8] with published names, including *Kitasatospora putterlickiae*, *Kitasatospora arboriphila*, and *Kitasatospora viridis*.[9–11] This genus has a history of producing biologically active metabolites, including terpentecin with cytotoxic activity against tumor cells from *Kitasatospora* sp. MF730-N6,[12] carbolines with cytotoxic activity against tumor cells from *Kitasatospora setae*,[13,14] tyropeptins A and B, which are proteasome inhibitors from *Kitasatospora* sp. MK9930dF2,[15] and talosins A and B with antifungal activity from *Kitasatospora kifunensis* MJM341.[16]

In this study, we report the TLC-guided isolation and structure determination of four new compounds, fuzanins A (**1**), B (**2**), C (**3**), and D (**4**) from culture extract of *Kitasatospora* sp. IFM10917. In addition, the cytotoxicity of **1**–**4** was evaluated and **4** showed cytotoxicity against DLD-1 cells (IC_{50}, 41.2 μM). Moreover **4** exhibited moderate inhibition of Wnt signal transcription at 25 μM.

2. Results and discussion

The culture supernatant of *Kitasatospora* sp. IFM10917 was partitioned between EtOAc and 10% aqueous MeOH, and the aqueous phase was further extracted with *n*-BuOH to give three fractions. The EtOAc-soluble fraction was subjected to Sephadex LH-20, silicagel column chromatography, followed by final purification with reversed-phase HPLC on ODS to give four compounds, fuzanins A (**1**), B (**2**), C (**3**), and D (**4**).

Fuzanin A (**1**) was obtained as colorless oil. The molecular formula of **1** was determined as $C_{14}H_{19}NO_4$ on the basis of HRFABMS at *m*/*z* 304.0976 [$(M+K)^+$, Δ +2.4 mmu]. Absorption at 1734 and 3369 cm^{-1} in the IR spectrum was characteristic of carbonyl and hydroxyl groups, respectively, and the UV absorption maximum at λ_{max} 240 nm was suggestive of the presence of a diene group. The ^{13}C NMR spectrum showed 14 resolved peaks, which were assigned to two methyls (δ_C 19.1 and 24.1), one aliphatic methylene (δ_C 44.8),

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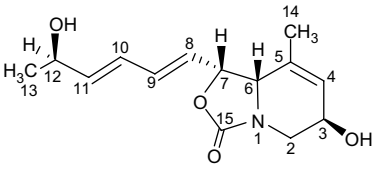

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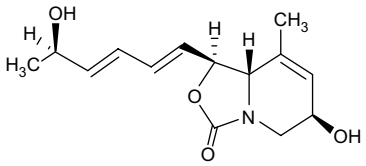

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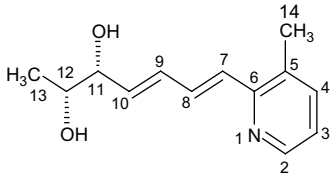

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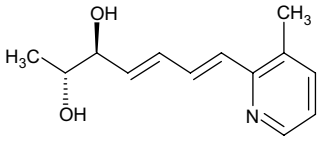

4

four heteroatom-bearing methines (δ_C 58.0, 62.8, 66.5, and 78.3), five olefinic methines (δ_C 125.4, 126.8, 131.1, 135.5, and 142.4), one olefinic quaternary carbon (δ_C 129.5), and one carbonyl carbon (δ_C 155.9) by analysis of the DEPT spectrum. The connectivity of carbon and hydrogen atoms was established from the HMQC spectrum. The ^{1}H NMR spectrum obtained in DMSO-d_6 solution also supported this molecular formula. It displayed 19 proton signals, including two methyls (δ_H 1.10 (3H) and 2.34 (3H)), one heteroatom-bearing methylene (δ_H 2.49 and 3.80), four heteroatom-bearing methines (δ_H 4.03, 4.15, 4.38, and 5.17), and five olefinic protons (δ_H 5.46, 5.56, 5.81, 6.11, and 6.35). Also two signals observed at δ_H 5.27 (d, J=4.9 Hz) and 4.79 (d, J=4.3 Hz), which had no HMQC correlations with any carbon signals, were assignable to secondary hydroxyl protons (3-OH and 12-OH). The formula of **1** indicated 6 degrees of unsaturation, four of which were accounted for by one carbonyl group and three double bonds (δ_C 125.4, 126.8, 129.5, 131.1, 135.5, and 142.4). Hence the remaining two degrees of unsaturation indicated the presence of two rings. The ^{13}C chemical shift of the carbonyl group (C-15, δ_C 155.9) indicated that this carbonyl group was located between oxygen and nitrogen atoms, suggesting that **1** has a carbamate moiety. The planar structure of **1** was established by HMBC, HMQC, and COSY analyses. The COSY spectrum revealed two partial structures, a C-2 to C-3 residue and a C-6 to C-13 chain containing one diene moiety (C-8 to C-11) and one methyl group (C-13). The large coupling constants ($J_{8,9}$=15.2 Hz and $J_{10,11}$=15.0 Hz) established both *E*-configurations of $\Delta^{8,9}$ and $\Delta^{10,11}$-double bonds (literature values:[17] *Z*-configuration: 5–12 Hz, *E*-configuration: 14–16 Hz). The HMBC spectrum showed ^{1}H–^{13}C long-range correlations for H_2-2/C-15, H-7/C-15, and H_2-2/C-6, indicating the presence of a five-membered ring containing a carbamate group. Also, the presence of a 3-methyltetrahydropyridine ring was deduced from HMBC correlations for H_3-14/C-4, H_3-14/C-5, H_3-14/C-6, and H-4/C-2 and a COSY correlation for H_2-2/H-3. The relative configuration of **1** was confirmed by NOE difference experiments. The NOE correlations observed between H-2α (δ_H 3.80) and H-3 (δ_H 4.03), H-2β (δ_H 2.49) and H-6 (δ_H 4.38), and H-6 and H-7 (δ_H 5.17) revealed that H-2β, H-6, and H-7 were on the same side of the plane, while H-2α and H-3 were on the other side. This was also supported by a large vicinal coupling constant (J=11.4 Hz) between H-2β and H-3, indicating that H-2β and H-3 were *trans*-diaxial. The absolute configurations at C-3 and C-12 of **1** were elucidated by applying the modified Mosher's method.[18] Compound **1** was treated separately with (*R*)- and (*S*)-MTPA-Cl in the presence of triethylamine and 4-(dimethylamino)pyridine to yield the (*S*)- and (*R*)- MTPA diesters (**1a** and **1b**), respectively, and the differences in the chemical shifts ($\Delta\delta$ values: $\delta_S-\delta_R$) in the ^{1}H NMR spectra of **1a** and **1b** are shown in Figure 1. Diagnostic positive $\Delta\delta$ values were observed for H-4, H_3-13, and H_3-14, and negative $\Delta\delta$ values for H-2α, H-2β, H-10, and H-11. These findings led to assignment of the 3*R*,12*R*-configuration. Based on these results, the structure of fuzanin A was concluded as **1**, including the absolute configurations assigned as 3*R*, 6*S*, 7*R*, and 12*R*.

Fuzanin B (**2**) was isolated as colorless oil. The HRFABMS of **2** exhibited a quasi-molecular ion peak at m/z 266.1403, [$(M+H)^+$, Δ +1.0 mmu], indicating the molecular formula $C_{14}H_{19}NO_4$. The IR spectrum showed absorption bands at 1742 and 3375 cm^{-1}, suggesting the presence of carbonyl and hydroxyl groups, respectively. The ^{1}H and ^{13}C NMR spectra of **2** were almost similar to those of **1**, except for the signals of positions C-6, C-7, and C-8. Three signals of protons at δ_H 4.38 (H-6), δ_H 5.17 (H-7), and δ_H 5.46 (H-8) of **1** appeared instead of those at δ_H 3.98 (H-6), δ_H 4.66 (H-7), and δ_H 5.81 (H-8) of **2**, while the three carbon signals at δ_C 60.3 (C-6), δ_C 79.9 (C-7), and δ_C 128.8 (C-8) in **2** resonated in a lower field than those of **1** (δ_C 58.0 (C-6), δ_C 78.3 (C-7), and δ_C 125.4 (C-8)). Analysis of the COSY and HMBC spectral data revealed that the planar structure of **2** is identical to that of **1**. From these findings, it was suggested that **2** is a stereoisomer of **1** at position C-7. This assumption was also supported by the significant NOE correlations between H-6 and H-8 for **2**, instead of the correlation between H-6 and H-7 observed for **1**. Compound **2** showed NOE correlations for H-2α/H-3 and H-2β/H-6, which were also observed for **1**, and the coupling constant between H-2α and H-3 was also large for **2** ($J_{2\alpha,\ 3}$=11.6 Hz). Thus, the configurations at C-3 and C-6 of **2** were suggested to be the same as those of **1**.

The absolute configuration of **2** was elucidated using the modified Mosher's method in the same manner as that of **1**. Compound **2** was converted into (*S*)- and (*R*)-MTPA esters (**2a** and **2b**). The differences between the ^{1}H NMR chemical shifts for **2a** and **2b** ($\Delta\delta$ values: $\delta_S-\delta_R$) led to the assignment of the 3*R*,12*R*-configuration as shown in Figure 2. From these results, fuzanin B was revealed to have the structure of **2** with 3*R*,6*S*,7*S*,12*R*-configurations.

Fuzanin C (**3**) was obtained as colorless oil. Its molecular formula was determined as $C_{13}H_{17}NO_2$ on the basis of HREIMS at m/z

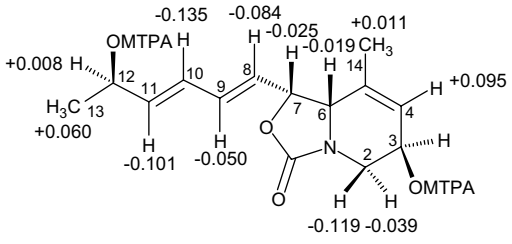

Figure 1. $\Delta\delta$ values [$\Delta\delta$ (in ppm)=$\delta_S-\delta_R$] obtained for (*S*)-, and (*R*)-MTPA esters (**1a** and **1b**).

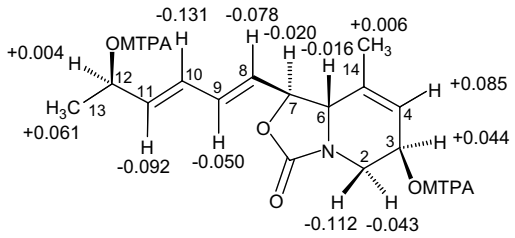

Figure 2. $\Delta\delta$ values [$\Delta\delta$ (in ppm)=$\delta_S-\delta_R$] obtained for (*S*)-, and (*R*)-MTPA esters (**2a** and **2b**).

219.1293 [M^+] (calcd 219.1259). The IR spectrum showed a strong band at 3351 cm^{-1}, suggesting the presence of hydroxyl groups. The presence of a conjugated diene was also indicated by the IR spectrum (ν_{max} 1616 cm^{-1}), and the UV absorption maxima at λ_{max} 269 and 314 nm were indicative of the presence of an aromatic or a long-conjugation system. The ^{13}C NMR and DEPT spectra confirmed the presence of 13 carbons, including two oxymethines (δ_C 71.0 and 77.3), seven olefinic methines (δ_C 122.3, 128.8, 130.9, 132.7, 132.9, 135.2, and 138.8), two quaternary carbons (δ_C 130.9 and 153.3), and two methyl groups (δ_C 18.6 and 19.0). The ^1H NMR and HMQC spectra displayed 17 proton signals, including two methyl groups (δ_H 1.18 (3H) and 2.34 (3H)), two oxymethine protons (δ_H 3.68 and 3.96), and seven olefinic or aromatic protons (δ_H 5.93–8.40). The planar structure of **3** was established by HMBC, HMQC, and COSY analyses. The COSY spectrum revealed the presence of the partial structures of C-4 to C-6 residue and C-7 to C-13 residue, the latter of which is a long chain with a conjugated diene moiety (C-7 to C-10), a vicinal diol moiety (C-11 to C-12), and one methyl group (C-13). The conjugated diene was shown to have both *E*-configurations based on *J*-values ($J_{7,8}$=$J_{9,10}$=15.2 Hz). The HMBC correlations observed for H-2/C-4, H-2/C-6, H-3/C-5, and H-4/C-2 indicated the presence of a disubstituted pyridine ring. Also, the HMBC correlations were observed for H-4 (δ_H 7.42)/C-14 (δ_C 19.0) and H_3-14 (δ_H 7.42 (3H))/C-6 (δ_C 153.3), indicating that a methyl group is attached to the C-5 position. Moreover, the HMBC correlations from H-7 (δ_H 6.80) to C-5 (δ_C 130.9) and from H-8 (δ_H 7.40) to C-6 (δ_C 153.3) indicated that the side chain of C-7 to C-13 was connected at C-6. The relatively large vicinal coupling constant ($J_{11,12}$=6.5 Hz) of the 1,2-diol moiety in **3** revealed a *syn* relationship between H-11 and H-12, since *anti* and *syn* 1,2-diols, having the corresponding partial structure [CH_3–CH(OH)–CH(OH)–CH=CH–] were described to have the coupling constants, 3.0 Hz for *anti*[19] and 6.2 Hz for *syn*.[20] The absolute configuration of **3** was deduced from the CD spectrum of **3** obtained in the presence of $Ni(acac)_2$, which showed a positive Cotton effect at 313 nm and negative Cotton effect at 286 nm, indicating the absolute configuration of **3** as 11*R* and 12 *R*, according to the literature for *syn* 1,2-diols.[21]

Fuzanin D (**4**) was obtained as colorless oil. The molecular formula was determined as $C_{13}H_{17}NO_2$ on the basis of HREIMS at *m*/*z* 219.1242 [M^+] (calcd 219.1259). The IR spectrum showed absorption bands at 1669 and 3381 cm^{-1}, suggesting the presence of a diene and a hydroxyl group, respectively. The ^1H and ^{13}C NMR chemical shifts of **4** were similar to those of **3** except for the signals of positions 11 and 12. The signals of H-11 (δ_H 4.22) and H-12 (δ_H 3.92) of **4** were shifted downfield from those of **3** (H-11, δ_H 3.96; H-12, δ_H 3.68) in the ^1H NMR spectrum, whereas C-11 (δ_C 76.2) and C-12 (δ_C70.4) of **4** were shifted upfield when compared to **3** (C-11, δ_C 77.3; C-12, δ_C 71.0) in the ^{13}C NMR spectrum. The COSY and HMBC spectral data implied that the planar structure of **4** is the same as that of **3**, thus suggesting that **4** was a stereoisomer of **3**. Compound **4** was shown to have a relatively small vicinal coupling constant ($J_{11,12}$=3.8 Hz) for the 1,2-diol moiety. This small vicinal coupling constant suggested the anti relationship between H-11 and H-12 on the basis of comparison with the literature value.[19] Thus, the structure of fuzanin D was concluded as **4**.[22]

Fuzanins A (**1**)–D (**4**) were unique natural products containing a carbamate or pyridine moiety isolated from an actinomycete. No compounds having a related structure are known in *Kitasatospora* sp., although some pyridine-containing metabolites such as piericidins have been obtained from *Streptomyces* sp. and *Nocardioides* sp.[23–25] Moreover the biogenesis of fuzanins is yet to be revealed, and may be clarified by further detailed analysis of secondary metabolites including precursor of these compounds from this strain.

The cytotoxicity of fuzanins A–D (**1**–**4**) was examined by a fluorometric microculture cytotoxicity assay (FMCA).[26] Fuzanin D (**4**) exhibited cytotoxicity against human colon carcinoma DLD-1 cells, with an IC_{50} value of 41.2 μM, while **1**, **2**, and **3** proved to be inactive (IC_{50}, >50 μM).[27] Moreover fuzanin D (**4**) exhibited moderate inhibition of Wnt signal transcription (inhibition: 61.7%) along with mild cytotoxicity (27.5%) at 25 μM when it was tested for its Wnt signal inhibitory activity using a luciferase reporter gene assay in SuperTOP-Flash transfected cells.[28]

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a JASCO P-1020 polarimeter. IR spectra were measured on ATR in a JASCO FTIR 230 spectrophotometer. UV spectra were measured in a Shimadzu UV mini-1240 spectrometer. CD spectra were obtained in a JASCO J-720WI spectropolarimeter. NMR spectra were recorded on JEOL JNM-A400, JEOL JNM-A500, and JEOL JNM-ECP600 spectrometers with a deuterated solvent, the chemical shift of which was used as an internal standard. Electron ionization mass spectra (EIMS) and high-resolution EIMS were measured on a JEOL GC-Mate spectrophotometer, and fast atom bombardment mass spectra (FABMS) and high-resolution FABMS were measured on a JEOL HX-110A spectrometer.

3.2. Microbial strain

Kitasatospora sp. IFM 10917 was separated on humic acid-vitamin (HV) agar,[29] a medium for the selective isolation of soil actinomycetes, from a soil sample collected at Toyama City, Japan in March 2007. The identification was carried out by Professor Yuzuru Mikami of Medical Mycology Research Center, Chiba University, where a voucher specimen is deposited with the code IFM 10917.

3.3. Fermentation

The spores of the strain growing on Waksman medium containing agar were transferred to a flask (500 mL) and inoculated into 100 mL of a Waksman medium consisting of glucose (2 g/100 mL), meat extract (0.5 g/100 mL), peptone (0.5 g/100 mL), dried yeast (0.3 g/100 mL), NaCl (0.5 g/100 mL), and $CaCO_3$ (0.3 g/100 mL), and the mixture cultured at 28 °C for 3 days on a reciprocating shaker. Then the seed culture (10 mL) was transferred into a flask (3 L) containing 500 mL of the Waksman medium and cultured at 28 °C for 5 days on the reciprocating shaker. The cultivation of this strain was repeated two times.

3.4. Extraction and isolation

The culture broth (3 L in total) was harvested and centrifuged to separate the mycelium and supernatant. The supernatant was concentrated under reduced pressure to 500 mL and partitioned between EtOAc (500 mL×3) and then BuOH (500 mL×3). The EtOAc-soluble fraction (679 mg) was subjected to Sephadex LH-20

column chromatography (20×290 mm) eluted with MeOH to give six fractions: 1A (4.2 mg), 1B (54.0 mg), 1C (463.9 mg), 1D (78.3 mg), 1E (20.3 mg), and 1F (4.9 mg). Fraction 1C was further separated on a Silicagel column (30×300 mm) eluted with a gradient of mixtures (hexane/EtOAc=1:1, 1:2, 1:4, 0:1 and MeOH) to give nine fractions: 2A (15.7 mg), 2B (14.6 mg), 2C (15.5 mg), 2D (74.2 mg), 2E (17.3 mg), 2F (21.6 mg), 2G (78.6 mg), 2H (37.2 mg), and 2I (36.2 mg). Fraction 2D eluted with hexane/EtOAc (2:8) was purified by preparative HPLC (YMC-Pack ODS-AM, 10×250 mm; eluent, 50% MeOH; flow rate, 1.7 mL/min; detection, 254 nm) to afford fuzanin A (**1**, 14.5 mg, t_R 12 min), fuzanin B (**2**, 23.2 mg, t_R 14 min), fuzanin C (**3**, 1.5 mg, t_R 26 min), and fuzanin D (**4**, 1.8 mg, t_R 29 min).

3.4.1. Fuzanin A (*1*)

Colorless oil, $[\alpha]_D^{15}$+30.0 (*c* 1.0, MeOH); IR ν_{max} (ATR) 3369 and 1734 cm^{-1}; UV (MeOH) λ_{max} nm (log ε) 240 (3.9); ^1H and ^{13}C NMR data in Table 1; EIMS *m*/*z* 265 (M^+); HRFABMS *m*/*z* 304.0976, calcd for $C_{14}H_{19}NO_4K$, 304.0952.

3.4.2. Fuzanin B (*2*)

Colorless oil, $[\alpha]_D^{15}$−19.0 (*c* 1.0, MeOH); IR ν_{max} (ATR) 3375 and 1742 cm^{-1}; UV (MeOH) λ_{max} nm (log ε) 233 (3.5); ^1H and ^{13}C NMR data in Table 1; EIMS *m*/*z* 265 (M^+); HRFABMS *m*/*z* 266.1403, calcd for $C_{14}H_{20}NO_4$, 266.1393.

3.4.3. Fuzanin C (*3*)

Colorless oil, $[\alpha]_D^{15}$+34.5 (*c* 0.1, $CHCl_3$); IR ν_{max} (ATR) 3351 and 1616 cm^{-1}; UV ($CHCl_3$) λ_{max} nm (log ε) 269 (4.0), 314 (4.0); ^1H and ^{13}C NMR data in Table 1; FABMS *m*/*z* 220 $(M+H)^+$; HREIMS *m*/*z* 219.1293, calcd for $C_{13}H_{17}NO_2$, 219.1259. CD data of a mixture of $Ni(acac)_2$ and fuzanin C: nm ($\Delta\varepsilon$) 313 (+0.84), 286 (−0.52).

3.4.4. Fuzanin D (*4*)

Colorless oil, $[\alpha]_D^{15}$−32.9 (*c* 0.1, $CHCl_3$); IR ν_{max} (ATR) 3381 and 1669 cm^{-1}; UV ($CHCl_3$) λ_{max} nm (log ε) 269 (4.1), 315 (4.2); ^1H and ^{13}C NMR data in Table 1; FABMS *m*/*z* 220 $(M+H)^+$; HREIMS *m*/*z* 219.1242, calcd for $C_{13}H_{17}NO_2$, 219.1259.

Table 1
NMR spectral data for fuzanins A (**1**), B (**2**), C (**3**), and D (**4**)[a]

Position	**1**		**2**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
2a	3.80 dd (11.4 6.0)	44.8	3.81 dd (12.5, 6.6)	45.0
2b	2.49 t (11.4)		2.58 dd (12.5, 9.9)	
3	4.03 brs	62.8	4.05 brs	62.1
4	5.56 s	131.1	5.50 s	129.1
5		129.5		132.3
6	4.38 d (8.4)	58.0	3.98 d (7.6)	60.3
7	5.17 t (8.4)	78.3	4.66 t (7.8)	79.9
8	5.46 dd (15.2, 8.4)	125.4	5.81 dd (15.2, 8.0)	128.8
9	6.35 dd (15.2, 10.8)	135.5	6.45 dd (15.2, 10.8)	135.4
10	6.11 dd (15.0, 10.8)	126.8	6.20 dd (15.2, 10.8)	126.7
11	5.81 dd (15.0, 5.4)	142.4	5.86 dd (15.2, 5.3)	142.8
12	4.15 m	66.5	4.18 q (5.4)	66.5
13-Me	1.10 d (6.6)	24.1	1.12 d (5.8)	24.0
14-Me	1.56 s	19.1	1.61 s	18.1
15		155.9		156.2
3-OH	5.27 d (4.9)		5.25 d (5.4)	
12-OH	4.79 d (4.3)		4.80 d (4.2)	
Position	**3**		**4**	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
2	8.40 d (4.7)	147.2	8.42 d (4.6)	147.2
3	7.04 dd (5.7, 4.7)	122.3	7.05 dd (7.6, 4.6)	122.4
4	7.42 d (5.7)	138.3	7.43 overlapped	138.6
5		130.9		131.1
6		153.3		153.4
7	6.80 d (15.0)	128.8	6.81 d (15.2)	128.6
8	7.40 dd (15.0, 11.0)	132.9	7.43 overlap	132.8
9	6.56 dd (15.4, 11.0)	132.7	6.57 dd (15.2, 10.9)	132.0
10	5.93 dd (15.4, 6.8)	135.2	6.01 dd (15.2, 6.6)	134.4
11	3.96 t (6.7)	77.3	4.22 dd (6.6, 3.6)	76.2
12	3.68 q (6.5)	71.0	3.92 dd (6.6, 3.6)	70.4
13-Me	1.18 d (6.5)	18.6	1.17 d (6.6)	17.6
14-Me	2.34 s	19.0	2.36 s	18.8

[a] In DMSO-d_6 for **1** and **2**; in $CDCl_3$ for **3** and **4**.

3.5. Preparation of Mosher's esters derivatives

A mixture of fuzanin A (**1**) (0.5 mg), (*R*)-MTPA-Cl (1 μL (=1.3 mg)), dichloromethane (200 μL), triethylamine (0.6 μL (=0.4 mg)), and 4-(dimethylamino)pyridine (2.5 mg) was stirred at rt for 24 h and then poured into water and extracted with EtOAc. The organic layer was evaporated dry in vacuo. The residue was purified by silicagel chromatography (hexane/EtOAc=3:2) to give the 3,12-*O*-(*S*)-MTPA diester (**1a**, 0.1 mg). Also the corresponding 3,12-*O*-(*R*)-MTPA diester (**1b**, 0.1 mg) was prepared by the same procedures using (*S*)-MTPA-Cl from **1**. Through a similar procedure, the 3,12-*O*-(*S*)-MTPA diester (**2a**, 0.3 mg) and 3,12-*O*-(*R*)-MTPA diester (**2b**, 0.4 mg) were also prepared from fuzanin B (**2**, 0.5 mg). Compound **1a**: ^1H NMR ($CDCl_3$) δ_H 6.33 (1H, dd, *J*=15.2 10.5 Hz, H-9), 6.22 (1H, dd, *J*=15.2 10.5 Hz, H-10), 5.79 (1H, dd, *J*=15.2 and 6.6 Hz, H-11), 5.61 (1H, t, *J*=6.3 Hz, H-12), 5.53 (1H, br s, H-4), 5.48 (1H, dd, *J*=15.2 and 8.4 Hz, H-8), 5.07 (1H, t, *J*=8.2 Hz, H-7), 4.35 (1H, dd, *J*=12.2 and 7.2 Hz, H-2α), 4.34 (1H, d, *J*=8.2 Hz, H-6), 2.84 (1H, dd, *J*=12.2 and 9.3 Hz, H-2β), 1.52 (3H, s, H-14), 1.36 (3H, d, *J*=6.0 Hz, H-13). Compound **1b**: ^1H NMR ($CDCl_3$) δ_H 6.33 (1H, dd, *J*=15.2 and 10.5 Hz, H-9), 6.22 (1H, dd, *J*=15.2 and 10.5 Hz, H-10), 5.79 (1H, dd, *J*=15.2 and 6.6 Hz, H-11), 5.61 (1H, t, *J*=6.3 Hz, H-12), 5.53 (1H, br s, H-4), 5.48 (1H, dd, *J*=15.2 and 8.4 Hz, H-8), 5.07 (1H, t, *J*=8.2 Hz, H-7), 4.35 (1H, dd, *J*=12.2 and 7.2 Hz, H-2α), 4.34 (1H, d, *J*=8.2 Hz, H-6), 2.84 (1H, dd, *J*=12.2 and 9.3 Hz, H-2β), 1.52 (3H, s, H-14), 1.36 (3H, d, *J*=6.0 Hz, H-13). Compound **2a**: ^1H NMR ($CDCl_3$) δ_H 6.33 (1H, dd, *J*=15.3 and 10.5 Hz, H-9), 6.16 (1H, dd, *J*=15.5 and 10.5 Hz, H-10), 5.73 (1H, dd, *J*=15.5 and 6.3 Hz, H-11), 5.69 (1H, dd, *J*=15.2 and 7.2 Hz, H-8), 5.57 (1H, br s, H-4), 5.54 (1H, m, H-3), 4.55 (1H, t, *J*=7.3 Hz, H-7), 4.30 (1H, dd, *J*=12.6 and 6.9 Hz, H-2α), 3.89 (1H, d, *J*=7.4 Hz, H-6), 2.79 (1H, dd, *J*=12.6 and 9.3 Hz, H-2β), 1.50 (3H, s, H-14), 1.43 (3H, d, *J*=6.6 Hz, H-13). Compound **2b**: ^1H NMR ($CDCl_3$) δ_H 6.38 (1H, dd, *J*=14.9 and 11.0 Hz, H-9), 6.29 (1H, dd, *J*=15.2 and 11.0 Hz, H-10), 5.82 (1H, dd, *J*=15.2 and 6.6 Hz, H-11), 5.77 (1H, d, *J*=14.9 and 7.5 Hz, H-8), 5.63 (1H, dd, *J*=6.6 and 6.2 Hz, H-12), 5.55 (1H, m, H-3), 5.49 (1H, br s, H-4), 4.57 (1H, t, *J*=7.7 Hz, H-7), 4.35 (1H, dd, *J*=12.8 and 6.9 Hz, H-2α), 3.90 (1H, d, *J*=7.4 Hz, H-6), 2.90 (1H, dd, *J*=12.8 and 9.6 Hz, H-2β), 1.50 (3H, s, H-14), 1.36 (3H, d, *J*=6.2 Hz, H-13).

3.6. Cell culture

DLD-1 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were cultured in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum (FBS) at 37 °C under an atmosphere of 5% CO_2.

3.7. Cytotoxicity

The assay procedure was the same as previously described.[2,3] Briefly, DLD-1 cells (6×10^3 per well) were treated with different concentrations of each isolated compound for 24 h at 37 °C. After the medium containing the samples was removed, cell growth inhibitory activity was determined by the Fluorometric Micro Culture Assay (FMCA) method[24] using a fluorescence plate reader. The ratio of living cells was determined as the fluorescence in sample wells

expressed as a percentage of that in the control wells, and inhibitory activity was indicated as IC_{50} values. Each experiment was conducted in triplicate.

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