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NOTE

Isolation of new fuzanins, carbamate-containing natural products, from *Kitasatospora* sp. IFM10917

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During our studies on the search for bioactive natural products from various sources,[1] we have investigated bioactive metabolites of actinomycete strains and isolated a new tyramine derivative[2] or teleocidin derivative[3] in our screening studies targeting tumor necrosis-factor-related apoptosis-inducing ligand signaling.[4] Conversely, we recently investigated the chemical constituents of *Kitasatospora* sp. IFM10917, based on TLC examination using anisaldehyde as a spray reagent, and isolated new carbamate- and pyridine-containing compounds, named fuzanins A–D.[5] We further investigated the culture of *Kitasatospora* sp. IFM10917 and performed chemical studies on its extract, resulting in the isolation of further carbamate-containing metabolites, named fuzanins E–I (**1**–**5**) (Figure 1). Herein we describe the isolation and structural elucidation of these new compounds.

The cultured supernatant of *Kitasatospora* sp. IFM10917 was partitioned between ethyl acetate (EtOAc) and water. The EtOAc-soluble fraction was subjected to silica gel column chromatography, followed by purification with Sephadex LH-20 column (GE Healthcare Bio-Sciences, Uppsala, Sweden) and reversed-phase HPLC on ODS to give five new compounds, fuzanins E–I (**1**–**5**).

Fuzanin E (**1**) was revealed to have the molecular formula of $C_{14}H_{21}NO_4$ on the basis of high-resolution fast atom bombardment mass spectroscopy (HRFABMS) at *m/z* 268.1560 ($(M+H)^+$, Δ +1.1 mmu). Absorption at 3360 and 1740 cm^{-1} in the IR spectrum suggested the presence of hydroxyl and carbonyl groups, respectively. The ^{13}C NMR spectrum (Table 1) showed 14 signals, which were assigned to two methyls (δ_C 23.7 and 18.0), three aliphatic methylenes (δ_C 28.2, 37.9 and 44.6), four heteroatom-bearing methines (δ_C 60.5, 62.9, 67.4 and 80.6), three sp^2 methines (δ_C 127.7, 126.8 and 137.8), one sp^2 quaternary carbon (δ_C 133.2) and one carbonyl carbon (δ_C 156.4); the ^{13}C chemical shift of this carbonyl group indicated that it was located between oxygen and nitrogen atoms, suggesting that **1** had a carbamate moiety. The ^1H NMR spectrum of **1** (Table 1) revealed signals due to two methyls (δ_H 1.21 (3H, d, J=6.4 Hz) and 1.69 (3H, s)), one heteroatom-bearing methylene (δ_H 2.69 and 4.20), four heteroatom-bearing methines (δ_H 3.82, 3.88, 4.35 and 4.48) and three olefinic protons (δ_H 5.58, 5.64 and 5.94). These ^1H and ^{13}C NMR spectral data were similar to those of fuzanin B (**6**) (Figure 1), except for the number of double bonds. Fuzanin B (**6**) possessed a two disubstituted diene, whereas fuzanin E (**1**) had one disubstituted olefin and two more sp^3 methylene groups. The ^1H-^1H COSY spectrum of **1** revealed ^1H-connectivities from H_2-2 to H-3 and from H-6 to H-13, and the disubstituted olefin was shown to be at C-8 and C-9 and a secondary hydroxyl group at C-12 position from the COSY correlation data. The large coupling constants ($J_{8,9}$=14.9 Hz) suggested the *E*-configurations of $\Delta^{8,9}$-double bonds. The HMBC spectrum of **1** also suggested that fuzanin E (**1**) had the same bicyclic ring system as fuzanin B (**6**) containing a carbamate group (H_2-2/C-3, H_2-2/C-4, H_2-2/C-6, H_2-2/C-15, H-4/C-6, H-7/C-5, H-7/C-8, H_2-10/C-8, H_2-10/C-9, H_2-10/C-11, H_2-10/C-12, H_3-13/C-11, H_3-13/C-12, H_3-14/C-4, H_3-14/C-5 and H_3-14/C-6). The relative configuration of **1** was suggested by ^1H-^1H coupling constants and NOE experiments. A large vicinal coupling constant (J=9.4 Hz) was observed between H-2β and H-3, indicating that H-2β and H-3 were *trans*-diaxial. NOE correlations were observed for H-2α/H-3, H-2β/H-6 and H-6/H-8 revealed that H-2β, H-6 and H-8 were on the same side of the plane, whereas H-2α and H-3 were on the other side. The absolute configurations at C-3 and C-12 of **1** were elucidated by applying the modified Mosher's method.[6] Differences in the chemical shifts ($\Delta\delta$ values: δ_S–δ_R) of the ^1H NMR spectra of (*S*)- and (*R*)-MTPA (α-methoxy-α-(trifluoromethyl)phenylacetic acid) diesters are shown in Table 2. 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 and H_2-11. These findings led to assignment of the 3*R*,12*R*-configuration. On the basis of these results, the structure of fuzanin E was concluded as **1**, including the absolute configurations assigned as 3*R*, 6*S*, 7*S*, 12*R*.

The high-resolution electron spray ionization mass spectra (HRESIMS) of fuzanin F (**2**) exhibited a quasi-molecular ion peak at *m/z* 290.1358 ($(M+Na)^+$, Δ –0.5 mmu), indicating the molecular formula $C_{14}H_{21}NO_4$. The ^1H and ^{13}C NMR spectra of **2** (Table 1)

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were almost identical to those of **1**, except for the signals of positions C-6, C-7 and C-8. The ^{1}H NMR chemical shifts due to H-6 (δ_H 4.31), H-7 (δ_H 4.98) and H-8 (δ_H 5.33) for **2** were significantly different from those for **1** (δ_H 3.88 (H-6), δ_H 4.48 (H-7) and δ_H 5.64 (H-8)) (Table 2), whereas the three carbon signals at δ_C 58.0 (C-6), δ_C 78.6 (C-7) and δ_C 122.9 (C-8) in **2** resonated in higher fields than those of **1** (δ_C 60.5 (C-6), δ_C 80.6 (C-7) and δ_C 126.8 (C-8)). From these findings, **2** was suggested as a stereoisomer of **1** at position C-7, which was also supported by the significant NOE correlations between H-6 and H-7 for **2**, instead of the correlation between H-6 and H-8 observed for **1**. The absolute configuration of **2** was determined as 3*R*,12*R* configurations using the modified Mosher's method (Table 3).

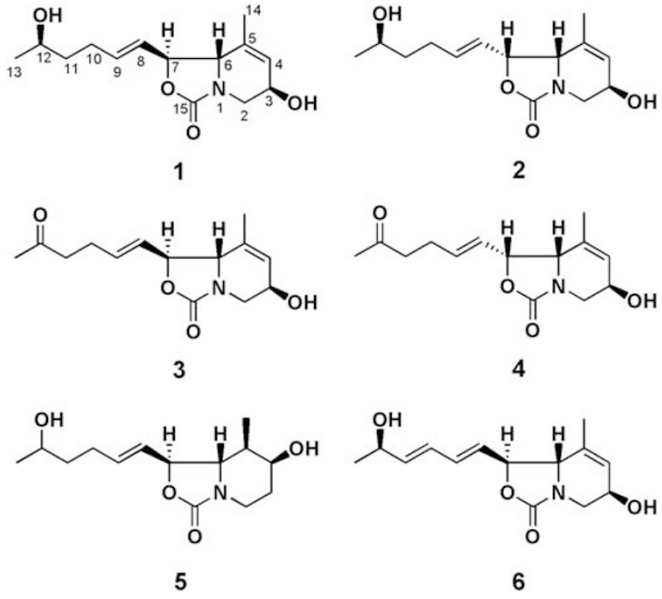

Figure 1 Structures of **1–6**.

From these results, the structure of fuzanin F was revealed as **2** with 3*R*,6*S*,7*S*,12*R* configurations.

Fuzanin G (**3**) was shown to have a molecular formula of $C_{14}H_{19}NO_4$ from HRFABMS data at *m/z* 288.1199 ($(M+Na)^+$, Δ −2.2 mmu). The ^{1}H and ^{13}C NMR spectra of **3** (Table 1) were similar to those of **1** except for the presence of a ketone (δ_C 207.3) for **3** in place of an oxymethine at C-12 for **1**. The C-13 methyl signal of **3** was observed as a singlet (δ_H 2.15, (3H, s)). Thus, the structure of fuzanin G (**3**) was inferred as a 12-dehydro derivative of fuzanin E (**1**), which was further supported by the ^{1}H-^{1}H COSY and HMBC spectra (data not shown). The relative configuration between H-6 and H-7 of **3** was deduced as *anti* from the comparison of the ^{1}H chemical shifts of H-6 (δ_H 3.87), H-7 (δ_H 4.46) and H-8 (δ_H 5.63) as discussed above in the case of fuzanin E (**1**) and F (**2**), as shown in Table 2. This configuration was also consistent with the observation of significant NOE from H-6 to H-8 for **3**. The absolute configuration was determined as 3*R* based on the modified Mosher's method (Table 3). Thus, the structure **3** was assigned to fuzanin G.

The molecular formula of fuzanin H (**4**), $C_{14}H_{19}NO_4$, determined by the HRESIMS data at *m/z* 288.1201 ($(M+Na)^+$, Δ –0.5 mmu) was the same as that of fuzanin G (**3**). The ^{1}H and ^{13}C NMR spectral data of **4** (Table 1) were almost identical to those of **3**, except for the chemical shifts of H-6, H-7 and H-8 positions, which were diagnostic for the configuration for H-6/H-7 (vide supra). On the basis of the comparison data in Table 2, the relative configuration of H-6/H-7 was suggested as *syn*. Thus, fuzanin H (**4**) was deduced as a C-7 epimer of

Table 2 Comparison of the ^{1}H NMR chemical shifts of H-6, H-7 and H-8, and assignment of relative configurations between H-6 and H-7

Positions	***1***	***2***	***3***	***4***	***5***
H-6	3.88	4.31	3.87	4.30	3.39
H-7	4.48	4.98	4.46	4.96	4.49
H-8	5.64	5.33	5.63	5.32	5.57
Configuration of H-6/H-7	*Anti*	*Syn*	*Anti*	*Syn*	*Anti*

Table 1 ^{1}H and ^{13}C NMR spectral data for fuzanins E (1), F (2), G (3), H (4) and I (5) in $CDCl_3$

	1		***2***		***3***		***4***		***5***	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2										
(α)	4.20 dd (12.8, 6.7)	44.6	4.19 dd (12.3, 6.3)	44.5	4.19 dd (12.8, 6.8)	44.6	4.19 dd (12.4, 6.3)	44.5	3.71 ddd (12.9, 5.6, 1.7)	36.5
(β)	2.69 dd (12.8, 9.4)		2.64 dd (12.3, 9.3)		2.69 dd (12.8, 9.6)		2.64 dd (12.4, 9.2)		3.22 td (12.9, 3.9)	
3	4.35 m	62.9	4.27 m	63.5	4.35 m	62.8	4.27 m	63.5	1.72-1.81 m (2H)	33.6
4	5.58 s	127.7	5.67 s	129.7	5.58 s	127.8	5.67 s	129.7	3.97 br s	68.2
5		133.2		130.7		133.2		130.7	1.70 m	42.1
6	3.88 d (8.0)	60.5	4.31 d (8.0)	58.0	3.87 d (7.8)	60.5	4.30 d (8.0)	58.0	3.39 dd (10.5, 7.1)	60.4
7	4.48 t (8.0)	80.6	4.98 t (8.0)	78.6	4.46 t (7.8)	80.3	4.96 t (8.0)	78.3	4.49 t (7.1)	80.0
8	5.64 dd (14.9, 8.0)	126.8	5.33 dd (15.4, 8.0)	122.9	5.63 dd (15.5, 7.8)	127.4	5.32 dd (15.4, 8.0)	123.5	5.57 dd (14.2, 7.1)	124.2
9	5.94 dt (14.9, 7.3)	137.8	5.89 dt (15.4, 7.1)	137.9	5.92 dt (15.5, 6.8)	136.2	5.86 dt (15.4, 8.0)	136.5	5.94 dt (14.2, 6.9)	133.3
10	2.22 m (2H)	28.2	2.15 m (2H)	28.5	2.38 m (2H)	26.1	2.32 m (2H)	26.0	2.19 m (2H)	30.6
11	1.54 m (2H)	37.9	1.52 m (2H)	37.9	2.56 t (6.5) (2H)	42.0	2.51 t (7.0) (2H)	42.3	1.60 m (2H)	39.4
12	3.82 sext (6.4)	67.4	3.79 sext (6.1)	67.4		207.3		[a]	3.81 sext (6.3)	67.4
13	1.21 d (6.4) (3H)	23.7	1.19 d (6.1) (3H)	23.6	2.15 s (3H)	29.8	2.14 s (3H)	29.9	1.20 d (6.3) (3H)	23.7
14	1.69 s (3H)	18.0	1.68 s (3H)	19.0	1.68 s (3H)	18.0	1.66 s (3H)	19.0	0.98 d (6.8) (3H)	15.8
15		156.4		[a]		156.2		[a]		[a]

[a]Not observed.

Table 3 $\Delta\delta$ values ($\delta_S-\delta_R$) of (*S*)- and (*R*)-MTPA esters of 1–4

Positions	*1*	*2*	*3*	*4*
2α	−0.037	−0.011	−0.036	−0.018
2β	−0.167	−0.124	−0.119	−0.120
3δ	—	—	—	—
3β	—	—	—	—
4	+0.094	+0.004	+0.036	+0.111
5	—	—	—	—
11 (2H)	−0.043	−0.105	—	—
13 (3H)	+0.005	+0.001	—	—
14 (3H)	+0.023	+0.024	+0.028	[a]

[a]Not assigned due to overlapping.

fuzanin G (**3**), and the absolute configuration was determined as 3*R* from the modified Mosher's method (Table 3).

Fuzanin I (**5**) had a molecular formula of $C_{14}H_{23}NO_4$, revealed from the HRESIMS data at *m/z* 292.1513 ($(M+Na)^+$, Δ −1.2 mmu), having two more hydrogen atoms than fuzanin E (**1**) or F (**2**). The ^1H and ^{13}C NMR data of fuzanin I (**5**) (Table 1) showed no signals due to an olefinic methyl group corresponding to C-14 of **1–4**, whereas two doublet methyl signals were observed and were assigned to C-13 and C-14 positions. The ^1H-^1H COSY spectrum showed that the methylene protons on C-2 (H_2-2, δ_H 3.71 and 3.22) were connected to another methylene group (H_2-3, δ_H 1.81 and 1.72), which in turn was coupled to an sp^3 oxymethine proton at C-4 (H-4, δ_H 3.97). The HMBC spectrum revealed correlations from the methyl protons on C-14 (H_3-14, δ_H 0.98) to the oxymethine carbon (C-4, δ_C 68.2) and two other sp^3 methines (C-5, δ_C 42.1; C-6, δ_C 60.4). From these observations, it was suggested that the C-4/C-5 position of **5** was saturated and a secondary hydroxyl group was located at the C-4 position instead of C-3 position. The ^1H-^1H COSY and HMBC spectra indicated that the structure of the rest of the molecule of **5** (C-6 to C-13 part) was identical to that of fuzanin E (**1**). The coupling constant between H-5 and H-6 was large (J=10.5 Hz), suggesting that these two hydrogens were both axial. The oxymethine proton (H-4) was observed as a broad singlet, thus implying that this hydrogen had small coupling constants with neighboring hydrogens and was therefore equatorial. These observations revealed 4β-OH, 5β-CH_3 and 6β-H configurations for **5**. The H-6/H-7 configuration was suggested as *anti* from the diagnostic ^1H NMR chemical shifts of H-7 and H-8, as shown in Table 2, although the chemical shift of H-6 of **5** was slightly different from those of **1** or **3** due to the C-5 position being sp^3 or sp^2. This configuration was also supported by the observation of significant NOE from H-6 to H-8 for **5**. From these results, the structure **5** was assigned to fuzanin I. The absolute configuration of **5** remained undefined because the modified Mosher's method could not be applied on **5** due to small quantity.

We examined the bioactivity of fuzanins E–H (**1–4**) using cell-based assay systems constructed in our laboratories targeting Wnt signaling pathways, but these compounds did not exhibit inhibition of Wnt signal transcription activity even at 100 μM using a luciferase reporter gene assay in SuperTOP-Flash transfected cells.[7] Fuzanins E–I (**1–5**) also did not show antimicrobial activity at 100 μg per disc against *Bacillus subtilis*.

MATERIALS AND METHODS

General

Optical rotations were measured with a JASCOP-1020 polarimeter (JASCO, Tokyo, Japan). IR spectra were measured on ATR (attenuated total reflection) on a JASCO FT-IR 230 spectrophotometer. UV spectra were measured on a Shimadzu UV mini-1240 spectrometer (Shimadzu, Kyoto, Japan). NMR spectra were recorded on JEOL JNM-A400 and JEOL JNM-ECP600 spectrometers (JEOL, Tokyo, Japan) with a deuterated solvent, the chemical shift of which was used as an internal standard. FABMS were obtained on a JEOL AX-500 spectrophotometer, and HRFABMS were obtained on a JEOL HX-110 mass spectrometer. HRESIMS were obtained on an Exactive (Thermo Scientific, Kanagawa, Japan).

Microbial strain

Kitasatospora sp. IFM 10917 was separated from a soil sample collected in Toyama City, Japan in March 2007, as described previously.[5] The identification was carried out by Professor Yuzuru Mikami of the Medical Mycology Research Center, Chiba University, where a voucher specimen is deposited with code IFM 10917.

Extraction and isolation

The culture broth (6 l total), obtained as described previously,[5] was harvested and centrifuged to separate the mycelia and supernatant. The supernatant was concentrated under reduced pressure to 500 ml and partitioned between EtOAc (500 ml×3) to give the EtOAc-soluble fraction (937 mg). This material was subjected to silica gel column chromatography (25×450 mm) eluted with a gradient of mixtures (hexane/EtOAc=1:1, 4:6, 3:7, 1:4, and MeOH) to give 14 fractions 1A to 1N, which were subjected to TLC examination, and three fractions (1I, 1J and 1K) were selected as anisaldehyde-positive fractions, which were suggested to contain previously isolated fuzanins A–D[5] and other related compounds. Fraction 1I (26.8 mg) eluted with hexane/EtOAc (2:8) was purified by Sephadex LH-20 column chromatography (10×450 mm) eluted with MeOH, followed by preparative HPLC (YMC-Pack ODS-AM, 10×250 mm; eluent, 50% MeOH; flow rate, 1.0 ml min^{-1}; UV detection at 254 nm; YMC Co Ltd, Kyoto, Japan) to afford fuzanin G (**3**, 2.2 mg, Rt (retention time) 24.0 min) and fuzanin H (**4**, 2.4 mg, Rt 20.5 min). Fraction 1J (27.1 mg) eluted with hexane/EtOAc (2:8) was fractionated by Sephadex LH-20 column chromatography (10×450 mm) eluted with MeOH, followed by preparative HPLC (YMC-Pack ODS-AM, 10×250 mm; eluent, 50% MeOH; flow rate, 1.0 ml min^{-1}; UV detection at 254 nm) to give fuzanin E (**1**, 3.5 mg, Rt 27.0 min), fuzanin F (**2**, 2.2 mg, Rt 22.0 min) and fuzanin H (**4**, 0.9 mg, Rt 20.0 min). Fraction 1K (25.6 mg) eluted with hexane/EtOAc (0:1) was separated by Sephadex LH-20 column chromatography (13×400 mm) eluted with MeOH, followed by preparative HPLC (Develosil ODS UG-5, 10×250 mm; eluent, 50% MeOH; flow rate, 1.0 ml min^{-1}; UV detection at 254 nm) to afford fuzanin F (**2**, 2.4 mg, Rt 23.0 min) and fuzanin I (**5**, 1.2 mg, Rt 31.5 min).

Fuzanin E (1)

Colorless oil, $[\alpha]_D^{24}$ –2.9 (*c* 1.0, MeOH); IR ν_{max} (ATR) 3360 and 1736 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; FABMS *m/z* 268 $(M+H)^+$ and 306 $(M+K)^+$; HRFABMS *m/z* 268.1560 $(M+H)^+$, calcd for $C_{14}H_{22}NO_4$, 268.1549.

Fuzanin F (2)

Colorless oil, $[\alpha]_D^{16}$ +10.6 (*c* 1.0, MeOH); IR ν_{max} (ATR) 3368 and 1734 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; HRESIMS *m/z* 290.1358 $(M+Na)^+$, calcd for $C_{14}H_{21}NO_4Na$, 290.1363.

Fuzanin G (3)

Colorless oil, $[\alpha]_D^{24}$ –2.5 (*c* 1.0, MeOH); IR ν_{max} (ATR) ca. 3300, 1739 and 1713 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; FABMS *m/z* 265 $[M^+]$ and 304 $(M+K)^+$; HRFABMS *m/z* 288.1199 $(M+Na)^+$, calcd for $C_{14}H_{19}NO_4Na$, 288.1212.

Fuzanin H (4)

Colorless oil, $[\alpha]_D^{16}$ +20.2 (*c* 1.0, MeOH); IR ν_{max} (ATR) ca. 3300, 1741 and 1715 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; HRESIMS *m/z* 288.1201 $(M+Na)^+$, calcd for $C_{14}H_{19}NO_4Na$, 288.1212.

Fuzanin I (5)

Colorless oil, $[\alpha]_D^{24}$ –4.8 (*c* 1.0, MeOH); IR ν_{max} (ATR) 3383 and 1732 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; HRESIMS *m/z* 292.1513 $(M+Na)^+$, calcd for $C_{14}H_{23}NO_4Na$, 292.1525.

Preparation of Mosher's esters derivatives

Fuzanin E (**1**) (1.1 mg) was treated with (*R*)-MTPA-Cl (8 µl, 10.4 mg) in pyridine (8 µl) at room temperature for 24 h, and the mixture was evaporated under reduced pressure. The residue was purified by silica gel chromatography (7×65 mm, hexane/EtOAc=1:1) to give the (*S*)-MTPA diester of **1** (2.8 mg): ESIMS, *m/z* 722 $(M+Na)^+$. Fuzanin E (**1**) was also treated with (*S*)-MTPA-Cl by the same procedures as above to afford (*R*)-MTPA diester of **1**: ESIMS, *m/z* 722 $(M+Na)^+$. Corresponding (*S*)- and (*R*)-MTPA esters of **2**–**5** were prepared by the same procedures as above. (*S*)-MTPA ester of **2**: ESIMS, *m/z* 722 $(M+H)^+$. (*R*)-MTPA ester of **2**: ESIMS, *m/z* 722 $(M+Na)^+$. (*S*)-MTPA ester of **3**: ESIMS, *m/z* 504 $(M+Na)^+$. (*R*)-MTPA ester of **3**: ESIMS, *m/z* 504 $(M+Na)^+$. (*S*)-MTPA ester of **4**: ESIMS, *m/z* 504 $(M+Na)^+$. (*R*)-MTPA ester of **4**: ESIMS, *m/z* 504 $(M+Na)^+$.

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