Novel Cytotoxic Thiodiketopiperazine Derivatives from a *Tilachlidium* sp.

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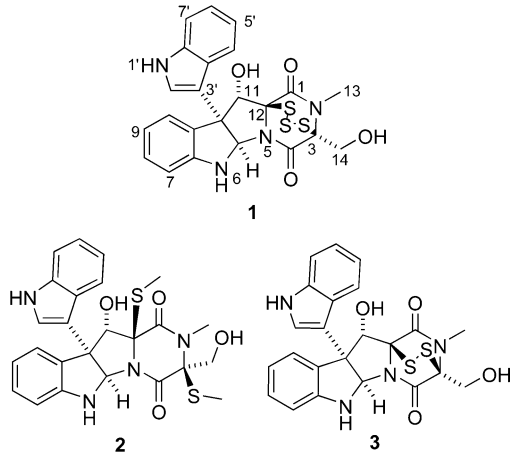
Three novel thiodiketopiperazine derivatives, T988 A, B, and C (**1**−**3**), were isolated from a *Tilachlidium* sp. Their structures were elucidated by spectroscopic analyses using various 1D and 2D NMR techniques. All the compounds showed potent cytotoxicity against cultured P388 leukemia cells.

The hyphomyceteous fungal genus *Tilachlidium* is morphologically closely related to the genus *Verticillium*. Previous studies suggest that *Verticillium* spp. produce a variety of biologically active secondary metabolites including polythiodiketopiperazine derivatives, such as the cytotoxic verticillins[1,2] and the antimalarial bioxanthracenes.[3−5] In contrast, *Tilachlidium* spp. have rarely been studied and the only secondary metabolites reported have been polyketides[6] and synnematin.[7] In our continuing studies on bioactive natural products from New Zealand fungi, a *Tilachlidium* sp. (CANU-T988) attracted our attention, not only for its unusual isolation in our sampling protocol but also for the potent cytotoxicity of the extract against cultured P388 cells. Bioassay-guided fractionation of a fermentation extract led to the discovery of three novel cytotoxic thiodiketopiperazine derivatives, T988 A (**1**), B (**2**), and C (**3**). The isolation, structure elucidation, and activity evaluation of **1**−**3** are reported here.



Tilachlidium sp. (CANU-T988) was isolated from a decaying wood sample collected in Christchurch, New Zealand. After 4 weeks fermentation in half-strength malt-peptone-yeast (MPY) broth under static conditions at 26 °C, the culture broth was filtered through Celite and the mycelium and filtrate (2 L) were extracted with EtOAc, respectively. Both extracts showed potent cytotoxicity against P388 cultured cells and gave similar HPLC traces. The mycelial extract was washed with petroleum ether, the residue was combined with the filtrate extract, and the combined extract (250 mg) was chromatographed on a flash reversed-phase (rp) column using a sharp, stepped gradient from water through methanol to dichloromethane. Repeated LH-20 Sephadex chromatography on a selected bioactive fraction from the rp column yielded T988 A (**1**), T988 B (**2**), and T988 C (**3**).

T988 A (**1**) was obtained as an amorphous solid. The molecular formula was determined to be $C_{23}H_{20}N_4O_4S_3$ by HRESIMS measurement and was consistent with the ^{1}H and ^{13}C NMR data (Table 1).

The ^{1}H NMR spectrum of **1** showed signals due to one NH, nine aromatic protons, and one methyl along with several methine and methylene groups. The chemical shifts and coupling constants in the aromatic region, in combination with the UV data (210, 230, 258, 292 nm), indicated a 1,2-disubstituted benzene and 3-substituted indole systems. All 23 carbons were observed in the ^{13}C NMR experiments (HSQC and CIGAR).[8] In addition to 14 carbon signals for the benzene and indole systems, two amide (or ester) carbonyl carbons, three sp^3 quaternary carbons, two heteroatom-substituted methines, one heteroatom-substituted methylene, and one *N*-methyl carbon were also observed.

Detailed analysis of the ^{1}H and ^{13}C NMR spectra with the aid of COSY, HSQC, and CIGAR experiments (Table 2) established the partial structures **a**, **b**, and **c** (Figure 1, solid lines). The presence of a dihydroindole system **a** was established by the CIGAR correlations from the methine singlet at 6.17 ppm to the aromatic carbons at 128.5 and 149.6 ppm and the quaternary carbon at 59.1 ppm. It was also confirmed by the CIGAR correlation from H-10 to the carbon at 59.1 ppm. The substructure **b** was deduced mainly by the CIGAR correlations from the *N*-methyl and CH_2OH methylene protons to the two carbonyl carbons and the quaternary carbon (74.8 ppm). The 3-substituted indole substructure **c** was also confirmed by a detailed analysis of CIGAR correlations (Table 2).

The connectivities between **a**, **b**, and **c** (Figure 1, dashed lines) were afforded by further analysis of the CIGAR correlations. The correlation from the methine singlet at 6.17 ppm in **a** to the carbonyl carbon at 166.1 ppm in **b** and the carbon signal at 113.4 ppm in **c** established the connectivity between **a** and **b**, and **a** and **c**. The further connectivity between **a** and **b** through a CHOH−C residue was defined by the correlations from the methine singlet at 5.40 ppm in the CHOH−C residue to the carbons at 59.1, 81.5, and 128.5 ppm in **a** and 169.2 ppm in **b**. This connectivity was also confirmed by the correlation from the proton singlet at 6.17 ppm in **a** to the carbon at 84.8 ppm in the CHOH−C residue. No correlations were observed that could be directly related to the connectivities of the three sulfur atoms in the molecule. The formation of the

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1**−**3**

	1[a]		**2**[b]		**3**[b]	
position	δ_H (mult., *J* Hz)	δ_C	δ_H (mult., *J* Hz)	δ_C	δ_H (mult., *J* Hz)	δ_C
1		169.2		166.4		166.3
2						
3		74.8		72.8		77.1
4		166.1		164.5		162.5
5						
5a	6.17 (1H, s)	81.5	6.46 (1H, s)	82.3	6.29 (1H, s)	82.4
6						
6a		149.6		149.3		148.0
7	6.75 (1H, d, 7.0)	111.2	6.57 (1H, d, 8.0)	108.9	6.65 (1H, d, 7.5)	109.8
8	7.15 (1H, m)[c]	130.3	6.99 (1H, m)[c]	128.0	7.04 (1H, m)[c]	128.7
9	6.77 (1H, t, 6.5)	119.9	6.61 (1H, t, 7.0)	117.8	6.71 (1H, t, 7.0)	118.8
10	7.32 (1H, d, 7.5)	125.4	7.37 (1H, d, 7.0)	122.8	7.44 (1H, d, 8.0)	123.9
10a		128.5		133.1		132.3
10b		59.1		59.9		61.5
11	5.40 (1H, s)	83.4	5.23 (1H, s)	80.2	5.46 (1H, s)	80.3
12		84.8		73.3		77.3
13	3.25 (3H, s)	27.8	3.09 (3H, s)	27.6	3.14 (3H, s)	26.4
14a	4.00 (1H, d, 13.0)	62.4	3.87 (1H, d, 12.0)	63.5	4.28 (1H, d, 12.5)	58.9
14b	4.25 (1H, d, 13.0)		4.31 (1H, d, 11.5)		4.41 (1H, d, 12.5)	
1′	7.97 (1H, br s)					
2′	7.01 (1H, br s)	123.3	7.13 (1H, br s)	122.5	7.14 (1H, br s)	122.9
3′		113.4		115.1		113.5
3′a		126.0		126.3		126.1
4′	7.87 (1H, d, 8.0)	121.0	7.88 (1H, d, 7.5)	121.0	7.93 (1H, d, 8.0)	121.0
5′	7.14 (1H, m)[c]	120.1	7.02 (1H, m)[c]	118.9	7.04 (1H, m)[c]	118.7
6′	7.16 (1H, m)[c]	122.5	7.05 (1H, m)[c]	121.0	7.08 (1H, m)[c]	121.1
7′	7.28 (1H, d, 8.0)	111.8	7.29 (1H, d, 7.5)	111.1	7.31 (1H, d, 8.0)	111.1
7′a		137.2		137.6		137.7
3-SCH_3			2.28 (3H, s)	12.3		
12-SCH_3			2.08 (3H, s)	14.9		

[a] Spectra were recorded at 500 MHz for ^{1}H and at 125 MHz for ^{13}C using $CDCl_3$ as solvent. [b]Spectra were recorded at 500 MHz for ^{1}H and at 125 MHz for ^{13}C using CD_3OD as solvent. [c]Signals overlapped.

Table 2. CIGAR and NOE Correlation Data for **1**−**3**

	CIGAR correlations			NOE correlations		
proton at	**1**	**2**	**3**	**1**	**2**	**3**
5a	C-4, C-6a, C-10a, C-10b, C-12, C-3′	C-4, C-6a, C-10a, C-10b, C-11, C-12, C-3′	C-6a, C-10a, C-10b, C-12, C-3′	H-2′, H-4′	H-2′, H-4′	H-2′, H-4′
7	C-6a, C-9, C-10a	C-9, C-10a	C-9, C-10a	H-8		
8	C-6a, C-10	C-6a, C-10	C-6a, C-10			
9	C-7, C-10	C-7, C-10a	C-7, C-10a	H-8, H-10		H-8, H-10
10	C-6a, C-8, C-10b	C-6a, C-8, C-10b	C-6a, C-8, C-10b		H-11, 12-SCH_3, H-2′, H-4′	H-9, H-11
11	C-1, C-5a, C-10a, C-10b, C-12	C-5a, C-10a	C-5a, C-10a, C-12	H-10, H-4′	H-10, 12-SCH_3, H-4′	H-10, H-4′
13	C-1, C-3, C-14	C-1, C-3	C-1, C-3	H-14a	3-SCH_3, H-14a	H-14a
14a	C-3, C-4	C-3, C-4	C-3, C-4	H-13, H-14b	H-13, H-14b	H-13, H-14b
14b	C-3, C-4	C-3, C-4	C-3, C-4	H-14a	3-SCH_3, H-14a	H-14a
2′	C-10b, C-3′, C-3′a, C-7′a	C-10b, C-3′, C-3′a, C-7′a	C-10b, C-3′, C-3′a, C-7′a	H-5a	H-5a, H-10	H-5a
4′	C-3′, C-6′, C-7′a	C-6′, C-7′a	C-3′, C-6′, C-7′a	H-5a, H-11	H-5a, H-10, H-11	H-5a, H-11
5′	C-7′	C-6′, C-7′a	C-6′, C-7′a			
6′	C-4′, C-7′a	C-4′, C-7′a	C-4′, C-7′a			
7′	C-3′a, C-5′	C-3′a, C-5′	C-3′a, C-5′			H-6′
3-SCH_3		C-3			H-13, H-14b	
12-SCH_3		C-12			H-10, H-11	

trisulfide bridge between C-3 and C-12 and the final definition of **1** were therefore based on the consideration of the molecular formula, double-bond equivalents, valency requirements, and chemical shifts, and by analogy with the previously reported polythioketopiperazines.[1,2]

T988 B (**2**) was also obtained as an amorphous solid. The comparison of the ^{1}H and ^{13}C NMR data (Table 1) suggested that **2** had structural features similar to **1**. The only difference was that two extra methyl singlets at 2.28 and 2.08 ppm were observed in the ^{1}H NMR spectrum of **2.** The corresponding carbon signals were observed at 12.3 and 14.9 ppm. Further analysis of the CIGAR experimental data suggested that the trisulfide bridge between C-3 and C-12 in **1** had been replaced by two SCH_3 groups in **2**. The molecular formula $C_{25}H_{26}N_4O_4S_2$ from HRESIMS corroborated the structure.

T988 C (**3**) also possessed spectral properties very similar to **1**. The molecular formula $C_{23}H_{20}N_4O_4S_2$ from HRESIMS indicated the only difference of their structures was that **3** had a disulfide bridge between C-3 and C-12 instead of a trisulfide bridge as in **1**. Analysis of the 2D spectral data (COSY, HSQC, and CIGAR) confirmed that T988 C has the structure **3**.

The relative configuration of **2** was deduced from a series of 1D NOE experiments (Table 2). NOE correlations were observed between H-5a and H-2′ suggesting a *cis* configuration of the H-5a and C-10b−C-3′ bond. The *cis* relationship between 12-SCH_3 and H-11 was established by

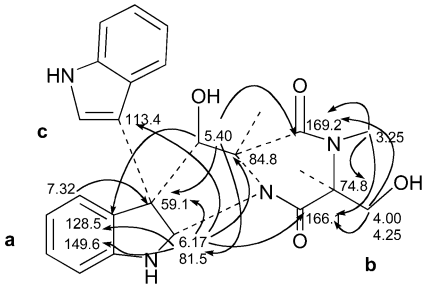

Figure 1. Substructures **a**, **b**, and **c** and key CIGAR correlations for **1**.

observation of an NOE correlation between them. Enhancement of signals for both H-11 and 12-SCH_3 on irradiation of H-10 could be consistent only with H-5a being *trans* to H-11. No information could be obtained on the relative orientation of the 3-SCH_3 group. It was concluded that because of the presence of sulfide bridges between C-3 and C-12 in **1** and **3**, the two SCH_3 groups in **2** should have a *cis* orientation. Thus the relative stereochemistry of **2** was determined as indicated.

Similar NOE correlations were also observed for **1** and **3** (Table 2). Their relative configurations were therefore assigned the same as **2** on the basis of the NOE experimental data and a presumed common biosynthetic origin.

Polythiodiketopiperazine derivatives have frequently been isolated as fungal metabolites with antimicrobial and/or cytotoxic properties, with a high percentage of this class of compounds being dimeric. The three new compounds isolated in the present study had a monomeric diketopiperazine structural feature with an indole substituent on C-10b, so number among the very few known in nature.[9] Compounds **1**−**3** all showed potent cytotoxicity against P388 leukemia cultured cells[10] with ID_{50} values of 0.25, 2.18, and 0.56 μM, respectively. The comparison of their activity data suggested that the sulfide bridge in the molecule may be an important factor for their cytotoxicity, while the number of sulfur atoms in the bridge may not significantly influence the activity.

Experimental Section

General Experimental Procedures. The optical rotation was measured with a Perkin-Elmer 341 polarimeter. UV and IR spectra were measured with a GBC UV/vis 920 spectrometer and a Shimadzu FTIR-8201 PC spectrometer, respectively. ^{1}H and 2D NMR (^{1}H−^{1}H COSY, ^{1}H−^{1}H NOESY, ^{1}H−^{13}C HSQC, ^{1}H−^{13}C CIGAR) spectra were recorded on a Varian INOVA 500 MHz spectrometer. Mass spectra were acquired using a Micromass TOF LCT mass spectrometer. Column chromatography used J.T. Baker 40 μM Prep LC Bakerbond octadecyl (C_{18}) and 40 μM Prep LC Bakerbond diol (COHCOH), and TLC was performed with Merck diol TLC plates. Solvents for extraction and chromatography were distilled prior to use. Culture media included Oxoid malt extract, BBL polypeptone, and DIFCO yeast extract.

Mycology. *Tilachlidium* sp. was isolated from a decaying wood sample from Christchurch, New Zealand. Colonies are slow-growing, reaching 10 mm diameter in 7 days, becoming tufted in the center due to synnema formation. The colonies produce orange pigment. Synnemata are covered over their whole length by conidiophores with verticillate tapering phialides bearing ovate conidia. A voucher of the fungus has been deposited in the University of Canterbury fungal herbarium and assigned as CANU-T988. The strain was initiated from serially diluted homogenized wood fragments on Difco mycological agar containing cycloheximide (100 mg/L) and chloramphenicol (200 mg/L). Pure cultures were subsequently grown on Sabouraud-dextrose-yeast (SDY) agar. Frozen vegetative mycelia (−80 °C) are maintained in the University of Canterbury Microbiology Culture Collection.

Fermentation. The isolate was inoculated into Fernbach flasks containing half-strength MPY broth (2 L) (17 g of malt extract, 3 g of polypeptone, and 1 g of yeast extract per liter of distilled water) by aseptically transferring agar plugs (10 × 6 mm diameter) from the growing margin of a colony on a SDY agar plate. The culture was incubated under static conditions at 26 °C for 4 weeks.

Isolation and Purification. The whole culture broth (2 L) was homogenized and filtered through Celite. The mycelium was extracted by stirring with ethyl acetate overnight (3 × 200 mL), as was the culture filtrate (3 × 2 L). The mycelium extract was washed with petroleum ether (3 × 20 mL), and the residue was combined with the filtrate extract. The combined extracts (250 mg) were chromatographed on C18 chromatographic phase using a steep, stepped solvent gradient from 10% MeOH/H_2O to MeOH to CH_2Cl_2. The fraction that eluted with 70% MeOH/H_2O (18 mg) was repeatedly chromatographed on LH-20 eluting with MeOH to yield T988 A (**1**) (3.0 mg). The fraction that eluted with 60% MeOH/H_2O (9.4 mg) was chromatographed on LH-20 eluting with MeOH to give T988 B (**2**) (1.5 mg) and T988 C (**3**) (0.8 mg).

T988 A (1): amorphous solid; $[\alpha]^{20}_D$ +366° (*c* 0.002, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.07), 230 (4.16), 258 (4.19), and 292 (4.16); IR (chloroform) ν_{max} 3479, 1670, 1608, 1531, 1420, and 1068 cm^{-1}; full ^{1}H and ^{13}C assignments were made from ^{1}H, COSY, HSQC, and CIGAR NMR experiments, and the data are summarized in Table 1; HRESIMS *m/z* 513.0724 (M + H$^+$) (calcd for $C_{23}H_{21}N_4O_4S_3$, 513.0725).

T988 B (2): amorphous solid; $[\alpha]^{20}_D$ +330° (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.14), 232 (4.20), 258 (4.22), and 292 (4.22); IR (chloroform) ν_{max} 3475, 1662, 1612, 1527, 1420, and 1069 cm^{-1}; full ^{1}H and ^{13}C assignments were made from ^{1}H, COSY, HSQC, and CIGAR NMR experiments, and the data are summarized in Table 1; HRESIMS 511.1477 (M + H$^+$) (calcd for $C_{25}H_{27}N_4O_4S_2$, 511.1474).

T988 C (3): amorphous solid; $[\alpha]^{20}_D$ +277° (*c* 0.0006, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.21), 234 (4.29), 282 (4.12), and 354 (3.63); IR (chloroform) ν_{max} 3483, 1678, 1612, 1400, and 1061 cm^{-1}; full ^{1}H and ^{13}C assignments were made from ^{1}H, COSY, HSQC, and CIGAR NMR experiments, and the data are summarized in Table 1; HRESIMS 481.0998 (M + H$^+$) (calcd for $C_{23}H_{21}N_4O_4S_2$, 481.1004).

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Supporting Information Available: 1D and 2D NMR spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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