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Two Novel Cytotoxic Cyclodepsipeptides from a Mycoparasitic *Cladobotryum* sp.

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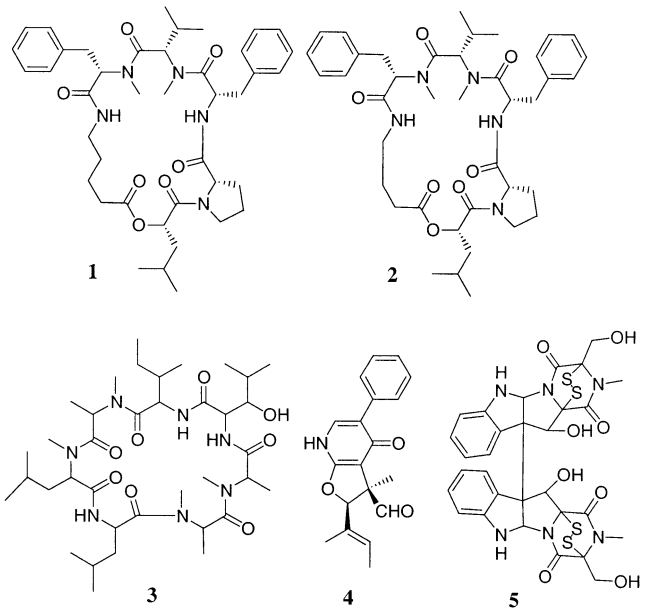
Abstract: Two novel cyclodepsipeptides (**1** and **2**) along with three known compounds (**3**−**5**) were isolated from a New Zealand isolate of a *Cladobotryum* sp. The structures of **1** and **2** were elucidated from NMR and HRESIMS experiments, and the absolute stereochemistry of **2** was established by X-ray crystallography and chemical degradation. Compounds **1** and **2** were both cytotoxic against murine P388 leukemia cells.

In the course of screening for new bioactive natural products from New Zealand-derived fungi, it was established that the fermentation broth of a *Cladobotryum* sp. (CANU-T987) exhibited strong toxicity against murine P388 leukemia cells. Mycoparasitic *Cladobotryum* species have frequently been reported as a source of bioactive metabolites and clearly possess a remarkably diverse biosynthetic capability. This genus produces a wide variety of secondary metabolites including furopyridines (CJ-15,696),[1] azatricyclic phosphate esters (FR 901483),[2] oxidized tricyclic derivatives (rubrobramide),[3] substituted pyridinediones (flavipucine),[4] α-pyrone aldehydes (cladobotrins),[5] and polypeptides.[6] The furopyridine, CJ-15,-696, and its analogues were found to exhibit potent activity against a number of common multi-drug-resistant bacteria,[1] and FR 901483 had potent immunosuppressive activity.[7]

As a result of a bioassay-guided fractionation of a fermentation extract of *Cladobotryum* sp. (CANU-T987) we are able to report the first isolation of the cyclodepsipeptide class of compound, T987 A (**1**) and T987 B (**2**), from this genus along with three known bioactive compounds, ternatin (**3**),[8] cladobotryal (**4**),[9] and melinacidin IV (**5**).[10]



Cladobotryum sp. (CANU-T987) was isolated from a decaying wood sample collected in Christchurch, New Zealand. After 4 weeks fermentation in half-strength Sabouraud dextrose yeast broth (SDY) under static conditions at 26 °C, a combined ethyl acetate extract of both the mycelium and culture filtrate (1 L) was chromatographed on a flash reverse phase (rp) column with use of a sharp, stepped gradient from water through methanol to dichloromethane. Repeated diol chromatography on selected fractions from the rp column yielded T987 A (**1**), T987 B (**2**), ternatin (**3**), cladobotryal (**4**), and melinacidin IV (**5**).

T987A(**1**) was obtained as an amorphous solid. The ES positive ion mass spectrum of **1** showed strong $M + H^+$ and $M + Na^+$ peaks at *m*/*z* 732 and 754. HRESIMS measurement on the $M + H^+$ (*m*/*z* 732.4337), in combination with ^1H and ^{13}C NMR data (Table 1), supported the molecular formula $C_{41}H_{57}N_5O_7$ (16 double bond equivalents).

The ^{13}C-APT sequence experiment on **1** displayed a total of 37 carbon signals with six methyl, nine methylene, fourteen methine, and eight quaternary carbons. Four carbon signals at 128.6, 128.7, 128.8, and 129.1 ppm each represented two symmetric aromatic methine carbons. The presence of six amide (or ester) carbonyl resonances in the range of 168−175 ppm and the characteristic signals of two NCH_3 singlets (3.01 and 3.06

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TABLE 1. ^{1}H and ^{13}C NMR data for 1 and 2[a]

		δ_C[b]		δ_H[b] (mult., *J* in Hz)	
residue	position	**1**	**2**	**1**	**2**
N-MePhe	CO	168.2	168.2		
	α	61.1	61.6	5.06 (1H, dd, 4.5, 10.5)	5.13 (1H, dd, 4.0, 11.0)
	β	36.7	37.0	2.66 (1H, dd, 4.0, 13.5)	2.65 (1H, dd, 4.0, 13.0)
				3.55 (1H, dd, 11.0, 13.5)	3.52 (1H, dd, 11.0, 13.0)
	γ	137.0	137.0		
	δ	129.1	129.2	7.20−7.30*	7.20−7.30*
	ϵ	128.6	128.7	7.20−7.30*	7.20−7.30*
	ϕ	126.9	127.0	7.20−7.30*	7.20−7.30*
	NCH_3	29.0	29.0	3.06 (3H, s)	3.07 (3H, s)
N-MeVal	CO	169.7	169.3		
	α	57.3	57.5	5.09 (1H, d, 11.0)	5.15 (1H, d, 10.5)
	β	27.7	27.2	2.28 (1H, m)	2.33 (1H, m)
	γ	18.9	18.6	0.85 (3H, d, 6.5)	0.84 (3H, d, 6.0)
	γ′	19.4	19.3	0.76 (3H, d, 6.5)	0.77 (3H, d, 6.5)
	NCH_3	30.1	29.7	3.01 (3H, s)	3.04 (3H, s)
Phe	CO	174.0	173.9		
	α	53.0	52.9	4.70 (1H, dd, 7.5, 16.0)	4.75 (1H, m)
	β	36.0	35.7	2.96 (2H, m)	2.81 (1H, dd, 10.5, 14.0), 2.94 (1H, m)
	γ	136.2	136.0		
	δ	128.8	128.8	7.15 (1H, d, 7.0)	7.14 (1H, d, 6.0)
	ϵ	128.7	128.7	7.20−7.30*	7.20−7.30*
	ϕ	127.3	127.3	7.20−7.30*	7.20−7.30*
	NH	-	-	8.14 (1H, d, 7.0)	7.85 (1H, d, 7.5)
Pro	CO	171.3	171.7		
	α	60.4	60.6	4.08 (1H, d, 7.0)	4.06 (1H, d, 9.0)
	β	32.2	32.3	2.11 (1H, m), 2.32 (1H, m)	2.10 (1H, m), 2.24 (1H, m)
	γ	21.8	21.9	1.37 (1H, m), 1.78 (1H, m)	1.29 (1H, m), 1.74 (1H, m)
	δ	47.2	47.2	3.46 (2H, m)	3.49 (2H, m)
α-HIHA	CO	170.0	170.3	-	-
	α	72.1	72.0	4.94 (1H, d, 11.5)	4.96 (1H, d, 10.5)
	β	38.6	38.7	1.18 (1H, dd, 11.5, 14.0), 1.87 (1H, m)	1.15 (1H, dd, 12.0, 14.0), 1.84 (1H, m)
	γ	24.5	24.4	1.88 (1H, m)	1.85 (1H, m)
	δ	23.4	23.4	0.98 (3H, d, 7.0)	1.00 (3H, d, 7.0)
	δ′	20.5	20.2	0.87 (3H, d, 6.5)	0.88 (3H, d, 6.5)
δ-APA/*δ*-ABA	CO	174.9	174.1	-	
	α	32.9	27.8	2.22 (1H, m), 2.48 (1H, m)	1.70 (1H, m), 2.38 (1H, m)
	β	21.7	21.0	1.33 (1H, m), 1.64 (1H, m)	1.58 (1H, m), 1.76 (1H, m)
	γ	27.5	35.7	1.53 (1H, m), 1.68 (1H, m)	3.16 (1H, m), 3.76 (1H, m)
	δ	38.8	-	2.94 (1H, m), 3.48 (1H, m)	
	NH	-	-	7.03 (1H, br d, 3.0)	6.95 (1H, dd, 8.0, 4.0)

[a] An asterisk indicates signals overlapped. [b] Spectra were recorded at 500 MHz for ^{1}H and at 125 MHz for ^{13}C with $CDCl_3$ as solvent and TMS as internal standard.

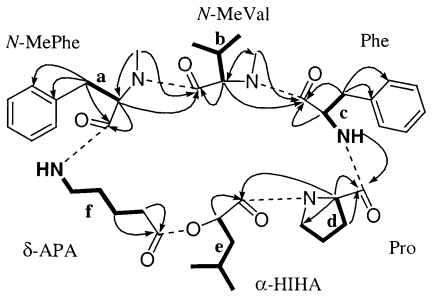

FIGURE 1. Subunits **a** to **f** and CIGAR correlations for **1**.

ppm) and two NH doublets (7.03 and 8.14 ppm) in the ^{1}H NMR spectrum suggested that **1** contained peptide linkages. This was also supported by IR absorption bands in the region of 3320−3340 and 1620−1730 cm^{-1}.

A series of COSY, TOCSY, and HSQC experiments established a number of partial connectivities and defined fragments **a** to **f** (Figure 1 in bold). A detailed analysis of the CIGAR[11] experimental data (Figure 1) established *N*-MePhe (*N*-methylphenylalanine), *N*-MeVal (*N*-methylvaline), Phe (phenylalanine), Pro (proline), α-HIHA (α-hydroxylisohexanoic acid), and *δ*-APA (*δ*-aminopentanoic acid) residues. The connectivities between the *N*-MePhe, *N*-MeVal, Phe, Pro, and α-HIHA residues were established by CIGAR correlations from the NH, *N*-Me, or α-protons in each residue to the carbonyl carbon in another (Figure 1). However, no correlations were observed to or from the *δ*-APA residue. The final definition of the structure **1** was therefore based on consideration of the molecular formula, double bond equivalents, chemical shifts, and valency requirements.

T987 B (**2**) was also isolated as an amorphous solid. HRESIMS indicated **2** had a molecular formula of $C_{40}H_{55}N_5O_7$ (16 double bond equivalents). The comparison of the ^{1}H and ^{13}C NMR spectral data between **1** and **2** (Table 1) suggested **2** had the same *N*-MePhe, *N*-MeVal, Phe, Pro, and α-HIHA residues in the molecule, while the *δ*-APA residue in **1** had been replaced by a *γ*-ABA (*γ*-aminobutanoic acid) residue. Further analysis of the 2D NMR data (COSY, HSQC, and CIGAR) confirmed that T987 B had the structure **2**.

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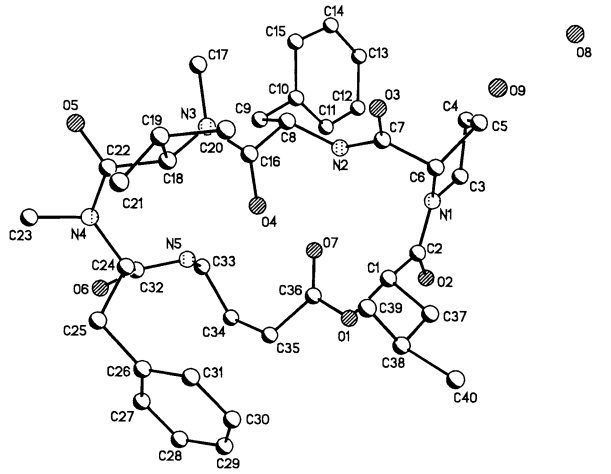

FIGURE 2. Crystal structure of **2**.

The absolute stereochemistry of **2** was determined by a combination of X-ray crystallography and chemical degradation. Acid hydrolysis followed by derivatization with Marfey's reagent and HPLC analysis[12] indicated that the Phe and Pro residues in **1** and **2** both had *S*-configurations. Several solvent systems were used in attempts to crystallize **1** and **2**. Suitable crystals for **2** only were obtained by solution in methanol and standing in the refrigerator for two months. The relative stereochemistries of **2** were thus established by X-ray crystallography. On the basis of the absolute stereochemistry of the Phe and Pro and the X-ray structure, all the stereogenic centers in **2** were assigned as *S*.

The crystal structure of **2** (Figure 2) included two water molecules. The oxygen atoms in the water molecules were designated O-8 and O-9. Oxygen O-8 is 2.82 Å from oxygen O-2 in one molecule and 2.77 Å from oxygen O-6 in another molecule. Oxygen O-9 is 2.70 Å from oxygen O-8 and 3.15 Å from oxygen O-3 in a third molecule. These close oxygen−oxygen distances suggested that hydrogen bonding was involved between molecules in the crystal lattice and this factor probably contributed to the crystallization of this molecule.

Within the molecule (see Figure 2 for the numbering scheme), oxygen O-4 was 2.93 Å from N-5. This suggested hydrogen bonding between O-4 and the hydrogen (not shown in Figure 2) on N-5. In addition to this, N-2 is 2.77 Å from N-1 and 3.03 Å from O-7. This suggested a strong hydrogen bonding interaction between N-1 and the hydrogen on N-2. There may also be a weaker interaction between O-7 and the hydrogen on N-2. It is also interesting to note that the macrocyclic part of **2**, rather than being more-or-less planar, was folded like a taco shell. The hydrogen bonding described above was probably responsible for this folding of the ring.

The absolute stereochemistry of **1** was proposed to be the same as that of **2** based on their apparent common biosynthetic origin.

It is rare to find a single species producing so many interesting bioactive compounds. This *Cladobotryum* sp. is indeed one such organism and produces three different classes of compounds. **1** and **2** both contain rare amino acid residues (δ- and γ-amino acids respectively), and they are the first cyclodepsipeptides reported from the genus *Cladobotryum*. The only other structurally related compound, M-6124, was found from a *Beauveria* sp.[13] and has a β-amino acid residue in the molecule. In terms of biological activity, compounds **1**−**5** all possessed notable cytotoxicity against murine P388 leukemia cells[14−16] with ID_{50} values of 0.14, 3.91, 1.63, 8.74, and 0.05 μM, respectively, with compound **3**, ternatin, also exhibiting strong antimicrobial activity[15,16] against *Candida albicans* and *Trichophyton mentagrophytes*.

Experimental Section

General Experimental Procedures. 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 using Merck Diol TLC plates. All solvents for extraction and chromatography were distilled prior to use. Culture media included Sigma cycloheximide and chloramphenicol, Gibco yeast extract and meat peptone, Oxoid soya peptone and tryptose, and BDH glucose.

Mycology. *Cladobotryum* sp. was isolated from a decaying tree stump from Christchurch, New Zealand. Identification was based on the characteristic microscopic features of short, fragile chains of oval septate, Hyaline conidia which have a marked basal scar and borne on phialides on verticillately branched conidiophores distinctive of *Cladobotryum*. *Cladobotryum* sp. are frequently found as saprophytes or parasites on the fructifications of higher fungi and have a *Hypomyces* teleomorph. A voucher of the fungus has been deposited in the University of Canterbury fungal herbarium and assigned as CANU-T987. 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 malt-extract 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 SDY broth (500 mL) (2.5 g of yeast extract, 1 g of soya peptone, 2 g of meat peptone, 2 g of tryptose, and 10 g of glucose per liter of distilled water) by aseptically transferring agar plugs (10; 6 mm in diameter) from the growing margin of a colony on a malt extract agar plate. The culture was incubated under static conditions at 26 °C for 4 weeks.

Isolation and Purification. The whole culture broth (1 L) was homogenized and filtered through Celite. The mycelium was extracted by stirring with ethyl acetate overnight (3 × 200 mL), as was the broth (3 × 1 L). The combined ethyl acetate extracts were concentrated under vacuum, yielding a dark brown residue (139 mg). The residue was chromatographed on C18 chromatographic phase (20 g) with a a steep, stepped solvent gradient from 10% MeOH/H_2O to MeOH to CH_2Cl_2. The fraction that eluted with 90% MeOH/H_2O (53.9 mg) was repeatedly chromatographed on Diol (8 g) with a petroleum ether/EtOAc gradient (4:1 to 3:2 to 1:1) to yield T987 A (**1**) (5.0 mg), T987 B (**2**) (5.0 mg), ternatin (**3**) (8.0 mg), and cladobotryal (**4**) (2.6 mg).

The C18 fractions that had been eluted with 100% MeOH and MeOH/CH_2Cl_2 (1:1) (29.6 mg) were combined and repeatedly

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partitioned between petroleum ether and MeOH. The MeOH soluble fraction afforded melinacidin IV (**5**) (6.0 mg).

T987 A (1): amorphous solid; $[\alpha]_D^{20}$ −109° (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.23); IR (chloroform) ν_{max} 3639, 3344, 3320, 2968, 2937, 2879, 2372, 2341, 1726, 1654, 1637, 1624, 1525, 1516, 1475, 1454, 1348, 1275, 1170, 1085, and 1057 cm^{-1}; full ^1H and ^{13}C assignments were made from ^1H, ^{13}C-APT, COSY, HSQC, and CIGAR NMR experiments, and the data are summarized in Table 1; HRESIMS *m*/*z* 732.4337 ($M + H^+$) (calcd for $C_{41}H_{58}N_5O_7$, 732.4336).

T987 B (2): white crystalline needles (MeOH); $[\alpha]_D^{20}$ −88° (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.77); IR (chloroform) ν_{max} 3634, 3327, 2962, 2360, 2343, 1728, 1654, 1637, 1624, 1543, 1516, 1477, 1458, 1425, 1348, 1280, 1088, and 1014 cm^{-1}; full ^1H- and ^{13}C assignments were made from ^1H, ^{13}C-APT, COSY, HSQC, and CIGAR NMR experiments, and the data are summarized in Table 1; HRESIMS *m*/*z* 718.4184 ($M + H^+$) (calcd for $C_{40}H_{56}N_5O_7$, 718.4180).

Configurations of Phe and Pro in 1 and 2. Compounds **1** and **2** (1.0 mg) were each individually heated in HCl (6M, 10 mL) in sealed glass tubes at 110 °C for 40 h. The resulting hydrolysates were concentrated in vacuo, dissolved in distilled water (200 μL), and derivatized with 1-fluoro-2,4-dinitro-phenyl-5-L-alanine amide (FDAA) (1.8 mg) in acetone (500 μL) and sodium bicarbonate (1 M, 100 μL) at 50 °C for 2 h. Upon completion of the reaction the solutions were acidified with HCl (2 M, 500 μL) and stored in the dark until analyzed. HPLC analysis (250 × 4.6 mm, 5 μm C_{18} column; linear gradient elution, 0.05% TFA solution/acetonitrile, 90:10−50:50 in 40 min, hold at 50:50 for 20 min; 1.0 mL/min; diode array detection) of FDAA-derivatized hydrolysates established the stereochemistry of the constituent amino acids. Each peak in the chromatographic traces was identified by comparing its retention time with that of the FDAA derivative of the pure amino acid standard and by co-injection. The acid hydrolysate of **1** showed peaks at 26.11 and 36.25 min. The hydrolysate of **2** also showed peaks with the same retention times. The amino acid standards gave the following retention times in minutes: 26.07 for *S*-Pro and 27.26 for *R*-Pro; 36.10 for *S*-Phe and 39.12 for *R*-Phe. Co-injection was necessary because of the similarity of the retention times for *S*- and *R*-Pro, together with slight variations in elution times for successive injections.

X-ray Structure Determination of 2. Crystals of **2** suitable for X-ray crystallography were obtained by dissolving **2** in methanol and allowing the solution to stand in the refrigerator for two months. A colorless crystal needle with dimensions (0.55 × 0.10 × 0.06) mm^3 was used for data collection. The final *R*-factor was 10.1%.

A Siemens SMART area detector system equipped with a nitrogen low-temperature gas-flow device was used to collect a full sphere of data with Mo Kα radiation and $\lambda = 0.71073$ Å. This yielded 10 736 data points, of which 5 572 were unique, and 2 742 of the unique reflections had intensities greater than twice their standard deviation. These data were processed with the program SAINT. The structure determination, refinement and diagram were carried out with the SHELXTL suite of programs.[17,18]

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Supporting Information Available: ^1H and ^{13}C-APT, CIGAR NMR spectra for **1** and **2**, tables of crystal data and structure refinement, and the full list of bond lengths and angles from the X-ray crystallographic study of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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