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Acremolides A−D, Lipodepsipeptides from an Australian Marine-Derived Fungus, *Acremonium* sp.[⊥]

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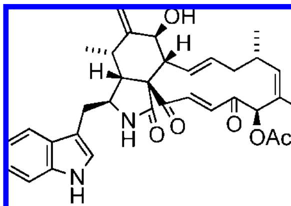
An Australian estuarine isolate of an *Acremonium* sp. (MST-MF588a) yielded the two known compounds 19-*O*-acetylchaetoglobosin D (**1**) and 19-*O*-acetylchaetoglobosin B (**2**), as the sole cytotoxic principles, along with the known aromatic metabolite RKB 3564S (**3**), and a novel family of lipodepsipeptides, acremolides A−D (**4**–**7**). Structures were assigned to **4**–**7** on the basis of detailed spectroscopic analysis and chemical derivatization and by application of a new C_3 Marfey's method for amino acid analysis.

During our investigations into the chemistry of Australian microbes, we routinely undertake the chemical fractionation of microbial extracts that display promising biological properties. Many of these extracts are rich in multiple classes of metabolites, of which some are responsible for the biological activity driving the fractionation process, while others are *seemingly* biologically inactive (at least as pure metabolites and with respect to the bioassay of record). The isolation of suites of biosynthetically related co-metabolites displaying varying potency and selectivity against a given bioassay(s) provides valuable insights into structure–activity relationships (SAR), advancing knowledge of novel natural pharmacophores. Likewise, the isolation of biosynthetically diverse co-metabolites with unknown biological properties represents an opportunity to acquire new chemical knowledge as a *prelude* to future revelations into their biological potential and purpose.

During our studies into the chemistry of Australian marine-derived microbes our attention was drawn to an estuarine isolate of an *Acremonium* sp. (MST-MF588a) obtained from a sediment sample collected in the Huon River, near Franklin, Tasmania. The MeOH extract from a solid phase culture of this fungus displayed significant cytotoxic activity against NS-1 cells (LD_{99} 16 μg/mL), which was concentrated in an EtOAc partition fraction (LD_{99} 1.6 μg/mL). Preliminary HPLC-DAD-ELSD analysis of this extract indicated an interesting array of structurally diverse co-metabolites. Subsequent fractionation studies identified the known mycotoxins 19-*O*-acetylchaetoglobosin D (**1**)[1] and 19-*O*-acetylchaetoglobosin B (**2**)[1] as the sole cytotoxic agents, together with the known small-molecule aromatic compound RKB 3564S (**3**).[2] Whereas chaetoglobosins are a well-known family of mycotoxins, RKB 3564S was first reported in a 2003 patent[2] that noted its antitumor and antiangiogenesis activity under low-oxygen conditions. Despite its structural simplicity, RKB 3564S resurfaced in a 2004 patent[3] for the treatment of diabetes, obesity, and neuroses, as well as Alzheimer's and Parkinson's diseases. In our hands, **3** did not show cytotoxic properties against NS-1 cells and, thus, did not appear to contribute to the cytotoxic properties of the crude *Acremonium* extract. Also detected and isolated during this investigation was a family of novel noncytotoxic lipodepsipeptides, attributed the trivial names acremolides A−D (**4**–**7**). This report presents an account of the characterization and structure elucidation of these acremolides.



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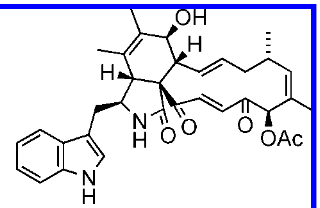

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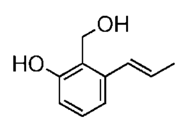

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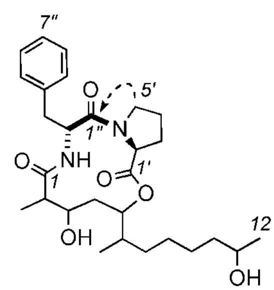

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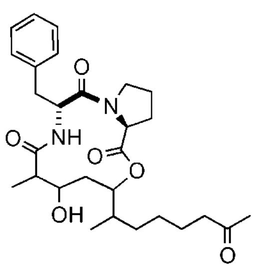

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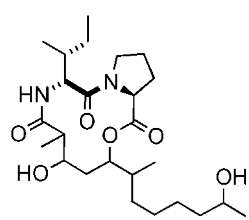

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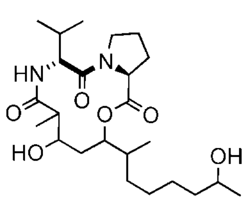

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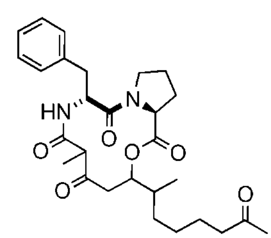

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Results and Discussion

The crude EtOAc fraction obtained from the methanol extract of a mycelial culture of an Australian marine-derived *Acremonium*

[⊥] Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products.

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Table 1. NMR (d_6-DMSO, 500 MHz) Data for Major *cis* (**4**) and Minor *trans* (**4a**) Acremolide A Prolinyl Amide Bond Conformers

	4			4a
#	δ_H (m, J (Hz))	DQF-COSY	gHMBC (^{1}H−^{13}C)	δ_H [m, J (Hz)][a]
1				
2	2.34 (dq, 7.0, 1.3)	H-3, Me-2	4, 3, 1, Me-2	2.57 (dq, 7.0, 3.3)
3	3.72 (m)	H-2,	5, 1	3.93 (m)
4a	1.84 (ddd, 15.0, 6.4, 1.9)	H-5, H-4b, H-3	6, 5, 3, 2	1.51 (m)
4b	1.73 (ddd, 15.0, 7.0, 3.7)	H-5, H-4a, H-3	5, 3, 2	
5	4.55 (m)	H-6, H-4a, H-4b		4.61 (ddd, 9.9, 2.8, 3.3)
6	1.69 (m)	Me-6, H-3	Me-6	1.94 (m)
7a	1.57 (m)	[b]	[b]	1.34 (m)
7b	1.53 (m)	[b]	[b]	1.03 (m)
8	[b]	[b]	[b]	[b]
9a	1.24 (m)	[b]	11, 10, 7	1.24 (m)
9b	1.02 (m)	[b]	11, 10, 7	1.02 (m)
10a	1.33 (m)	H-10b, H-12	12, 11, 9	1.33 (m)
10b	1.26 (m)	H-10a, H-11	12, 11, 9	1.26 (m)
11	3.56 (m)	H-12, H-10a, OH-11	10, 9	3.55 (m)
12	1.02 (d, 6.1)	H-11, H-10b	11, 10	1.02 (d, 6.0)
Me-2	0.73 (d, 7.0)	H-2	3, 2, 1	0.83 (d, 7.0)
Me-6	0.83 (d, 6.6)	H-6	7, 6, 5	0.73 (d, 7.7)
OH-3				4.41 (d, 6.0)
OH-11	4.29 (d, 4.6)	H-11	12, 11, 10	4.27 (d, 4.6)
L-Pro				
1′				
2′	5.00 (brd, 2.8)	H-3a′, H-3b′	5′, 4′, 3′, 1′	4.83 (brd, 6.9)
3a′	2.25 (m)	H-3b′, H-2′	5′, 4′, 2’, 1′	2.18 (ddd, 12.0, 11.2, 5.7)
3b′	1.89 (m)	H-3a′, H-2′	5′, 4′, 2′, 1′	1.59 (m)
4a′	1.78 (m)	H-3a′, H-3b′	5′, 3′, 2’	1.77 (m)
4b′	1.90 (m)	H-3a′, H-5a′, H-5b′	5′, 3′, 2’	1.60 (m)
5a′	3.58 (brdd, 12.0, 7.8)	H-4b′	4′, 3′, 2′, 1″	3.87 (ddd, 9.5, 9.0, 2.2)
5b″	3.47 (ddd, 12.0, 7.8, 7.6)	H-4b′	4′, 3′, 2′, 1″	3.13 (brdd, 9.0, 8.8)
D-Phe				
1′				
2′	4.53 (dt, 11.0, 3.8)	H-3a′, H-3b′, 2’-NH	4′, 3′, 1	4.73 (ddd, 9.1, 8.1, 8.0)
3a′	3.20 (dd, 13.9, 3.8)	H-2′	9′/5′, 2′, 1″	2.87 (dd, 13.5, 8.0)
3b′	2.82 (dd, 13.9, 11.0)	H-2′	9′/5′, 4′, 2′, 1′	2.82 (dd, 13.5, 8.1)
4′				
5′	7.20 (d, 7.5)	H-6″	7″	7.26 (d, 7.0)
6′	7.16–7.28 (m)	[b]	[b]	7.16–7.28 (m)
7′	7.16–7.28 (m)	[b]	[b]	7.16–7.28 (m)
8′	7.16–7.28 (m)	[b]	[b]	7.16–7.28 (m)
9′	7.20 (d, 7.5)	H-8′	7′	7.26 (d, 7.0)
NH-2″	8.22 (d, 8.7)	H-2″	C-3″, C-2″, C-1	8.43 (d, 9.1)

[a] Assignments are based on 2D NMR correlations. [b] Extensive overlap of ^{1}H NMR signals at ~1.2 to ~2.0 and ~7.16 to ~7.28 prevented unambiguous assignment of COSY and gHMBC correlations.

sp. (MST-MF588a) isolate displayed cytotoxic activity against NS-1 cells (LD_{99} 1.6 μg/mL). Bioassay and HPLC-DAD-MS analysis of solvent-partitioned materials identified two CH_2Cl_2-soluble fractions that displayed similar cytotoxicity (LD_{99} 1.8 and 1.2 μg/mL) and comparable suites of co-metabolites. Bioassay-guided C_{18} solid-phase extraction (SPE) and C_8 HPLC of the CH_2Cl_2-soluble fraction yielded the cytotoxic principles as 19-*O*-acetylchaetoglobosin D (**1**) (LD_{99} 0.8 μg/mL) and 19-*O*-acetylchaetoglobosin B (**2**) (LD_{99} 1.6 μg/mL), together with the noncytotoxic co-metabolite RKB 3654S (**3**). Structures **1**–**3** were confirmed by literature spectroscopic comparisons.[1,2] Estimated yields of **1** and **2** in the crude EtOAc extract (0.16 and 0.17%, respectively) fell well short of that required to explain the observed cytotoxicity and prompted us to look more closely at the co-metabolite profile, in the expectation that we may find other cytotoxic agents and/or co-metabolites capable of synergizing the cytotoxic properties of chaetoglobosins. While exhaustive HPLC fractionation of the *Acremonium* sp. extract failed to reveal additional cytotoxins, it did afford a set of four nontoxic lipodepsipeptides, acremolides A−D (**4**−**7**).

The (+)-HRESIMS of acremolide A (**4**) revealed a pseudomolecular ion [M + Na] corresponding to a molecular formula ($C_{28}H_{42}O_6N_2$, $\Delta = 1.2$ mmu) requiring nine double-bond equivalents (DBE). Preliminary analysis of the ^{1}H NMR (d_6-DMSO) data revealed resonance doubling (ratio 2:1) that coalesced at elevated temperature, suggesting the presence of equilibrating isomers. Careful analysis of the NMR (d_6-DMSO) data allowed tabulation of the resonances attributable to both major (**4**) and minor (**4a**) isomers (Tables 1 and 2) and indicated the presence of phenylalanine and proline residues. The amino acid content in **4** was confirmed by hydrolysis and C_3 Marfey's analysis (see below)[4,5] as L-Pro and D-Phe, while a key gHMBC correlation between these residues (H_2-5′ to C-1″) confirmed the amide linkage as shown. Amides attached through proline nitrogen are known to be capable of existing as equilibrating rotamers, with literature empirical rules establishing that ^{13}C NMR chemical shift differences between proline β and γ carbon resonances are characteristic of *cis* ($\Delta\beta\gamma$ ~8–12 ppm) vs *trans* ($\Delta\beta\gamma$ ~2–6 ppm) rotamers, respectively.[6] On the basis of these considerations the equilibrating acremolide A isomers were attributed in turn to *cis* (**4**) ($\Delta\beta\gamma = 10.9$ ppm) and *trans* (**4a**) ($\Delta\beta\gamma = 3.1$ ppm) prolinyl amide rotamers. The full NMR data (Tables 1 and 2) for **4** were consistent with this L-Pro-D-Phe-NH dipeptide substructure. The remaining structural feature of **4** was attributed to a substituted fatty acid, attached via an amide bond to D-Phe [gHMBC correlations from D-Phe-N<u>H</u> to the C-1 amide carbonyl δ_C 175.9)]. In the absence of further sp^2 carbons, and having accounted for eight of nine DBEs, acremolide A (**4**) was determined to be monomacrocyclic.

A set of COSY correlations from H-2 (δ_H 2.34) to H-6 (δ_H 1.69) (see Table 1), together with ^{13}C NMR data (see Table 2), identified a significant structure fragment and positioned the Me-2, 3-oxy, 5-oxy, and Me-6 substituents as shown. gHMBC correlations augmented this correlation sequence to include C-1 and C-7 and

Table 2. ^{13}C NMR (d_6-DMSO, 150 MHz) Data for Major (**4–8**) and Minor (**4a–8a**) Acremolide A–D and 3,11-Dioxoacremolide A Prolinyl Amide Bond Conformers

	δ_C[a]									
#	**4**	**4a**	**5**	**5a**	**8**	**8a**	**6**	**6a**	**7**	**7a**
1	175.9	173.5	175.7	173.0	165.8	169.2	173.7	176.8	173.9	177.0
2	41.9	46.5	41.7	46.2	53.9	52.8	46.5	41.5	46.8	41.3
3	69.1	67.5	69.2	67.4	204.8	204.9	67.6	69.9	68.0	69.5
4	35.4	32.8	35.1	36.3	41.6	44.8	32.8	35.7	33.0	35.5
5	75.4	77.2	75.3	77.0	76.3	75.2	77.3	75.6	77.0	75.4
6	34.6	33.6	34.7	33.4	33.8	36.1	33.6	34.9	33.3	35.1
7	31.8	31.2	30.4	31.0	29.7	31.5	31.8	31.3	32.1	31.7
8	[c]	[c]	[c]	[c]	25.6	26.2[c]	[c]	[c]	[c]	23.7
9	25.5	25.5	[c]	[c]	23.1	23.2	25.6	25.6	25.2	25.2
10	38.9	38.9	42.4	42.4	42.5	42.5	38.9	38.9	39.4[b]	39.1[b]
11	65.6	65.7	208.0	208.1	208.6	208.4	65.8	65.8	66.1	66.1
12	23.6	23.6	29.4	29.4	29.7	29.5	23.6	23.6	24.0	24.0
Me-2	15.5[b]	7.2	15.1	6.8	11.9	13.9	7.4	16.9	14.6	16.7
Me-6	15.4	15.3[b]	15.0	15.1	15.5	14.7	15.6	15.4	15.2	15.0
	L-Pro		L-Pro		L-Pro		L-Pro		L-Pro	
1′	171.5	170.5	171.2	170.8	170.4	171.5	170.6	171.5	171.0	171.8
2′	57.5	56.4	57.6	56.2	56.8	58.0	56.5	57.9	56.0	57.5
3′	31.5	26.3	31.3	25.9	26.3	31.6	26.4	31.5	26.7	31.9
4′	20.6	23.2	20.2	22.9	23.5	20.5	23.3	20.6	23.7	20.9
5′	48.6	45.4	48.5	45.1	45.6	48.6	45.7	48.6	45.2	48.5
	D-Phe		D-Phe		D-Phe		D-*allo*-Ile		D-Val	
1′	169.1	169.8	168.8	169.4	169.3	168.9	170.3	169.0	170.7	169.9
2′	55.1	52.1	54.9	52.0	51.7	55.9	55.6	58.3	58.0	58.9
3′	38.7	35.2	38.5	34.8	35.1	38.3	33.1	37.3	27.8	31.0
4′	137.8	137.9	137.5	137.9	137.9	137.6	24.3	29.0	18.4	18.7
5′	129.1	129.1	128.9	128.8	129.0	129.1	10.3	11.4		
6′	127.9	128.2	[c]	[c]	128.2	128.1	15.2	15.6	18.3	17.0
7′	126.3	126.3	[c]	126.0	126.3	126.4				
8′	127.9	128.2	[c]	[c]	128.2	128.1				
9′	129.1	129.1	128.9	128.8	129.0	129.1				

[a] Assignments made with the aid of HSQC and gHMBC correlations. [b] Assignments may be interchanged. [c] Due to extensive overlap of the corresponding ^{1}H NMR signals, some uncertainty exists in gHSQC and gHMBC correlations.

extended the structure fragment to include the L-Pro-D-Phe-NH residue. The deshielded ^{1}H NMR chemical shift for H-5 (δ_H 4.55) required a lactone linkage to the L-Pro residue, thereby establishing a macrocyclic structure and accounting for the remaining DBE. A sequence of COSY correlations from H_3-12 (δ_H 1.02) to H_2-10 (δ_H 1.33 and 1.26) was extended by a gHMBC correlation to include C-9 (δ_C 25.5). The deshielded nature of the ^{13}C NMR chemical shift for C-11 (δ_C 65.6) confirmed substitution by oxygen, while a COSY correlation from an exchangeable O$\underline{H}$ resonance (δ_H 4.29) to H-11 (δ_H 3.56) confirmed placement of a hydroxy at C-11. The remaining C-8 methylene was positioned as indicated, to complete the structure for acremolide A (**4**) as shown.

(+)-ESIMS analysis of a mild base hydrolysis (aqueous NH_4OH at room temperature) of **4** indicated an increase in MW of 18 amu, consistent with ring opening of the depsipeptide lactone bond. Similarly, HPLC-DAD-MS analysis of a 4 h pyridinium dichromate (PDC) oxidation of **4** indicated two major products, assigned to the two anticipated monoketone analogues. After 24 h the oxidation reaction was worked up to recover a 3,11-diketone (**8**) as the sole product. Spectroscopic analysis of **8** (see Tables 2 and 3) was in full accord with the assigned structure including the presence of major (**8**) and minor (**8a**) rotamers in a ratio of 2:1. Notably, the ^{1}H NMR resonances for H-2 and H_2-4 were significantly deshielded in **8** by comparison to acremolide A (**4**) (δ_H 1.4 and 1.58/0.78, respectively) due to the influence of a C-3 ketone moiety, as were H_2-10 and H_3-12 (δ_H 1.13 and 1.06, respectively) due to the influence of a C-11 ketone. gHMBC correlations in **8** from Me-2 to C-1 (δ_C 165.8) and C-3 (δ_C 204.8), and from H_3-12 to C-11 (δ_C 208.6), further confirmed the placement of C-3 and C-11 ketones and reasserted the structure assigned to acremolide A (**4**) as shown. Curiously, while the diketone existed, as expected, as an equilibrating inseparable 2:1 mixture of rotamers about the prolinyl amide bond, the isomeric preference was inverted compared to acremolide A (**4**), favoring a major *trans* (**8**) ($\Delta\beta\gamma = 2.8$ ppm) over a minor *cis* (**8a**) ($\Delta\beta\gamma = 11.1$ ppm) rotamer. It would appear that this *configurational* preference is finely balanced such that the inclusion of an additional sp^2 atom in the macrocycle (C-3 ketone) reversed the preference.

In an attempt to assign absolute stereochemistry about the two chiral secondary alcohols, acremolide A (**4**) was subjected to a Mosher's analysis. Despite repeated attempts, neither of these OH moieties proved capable of reacting with (*S*)-Mosher's reagent, returning unreacted starting material, such that the relative and absolute stereochemistry about the fatty acid substructure of **4** remains unassigned.

(+)-HRESIMS analysis of acremolide B (**5**) revealed a pseudomolecular ion [M + Na] corresponding to a molecular formula ($C_{28}H_{40}O_6N_2$, $\Delta = 0.7$ mmu) requiring 10 double-bond equivalents and suggestive of a didehydro analogue of **4**. Analytical scale acid hydrolysis followed by C_3 Marfey's analysis (see below)[4,5] confirmed the presence of D-Phe and L-Pro residues. The NMR data for **5** (Tables 2 and 3) were almost identical to those of **4** (Tables 1 and 2) and revealed a 2:1 ratio of major *cis* (**5**) ($\Delta\beta\gamma = 11.1$ ppm) versus minor *trans* (**5a**) ($\Delta\beta\gamma = 3.0$ ppm) rotamers. As might be expected, the major rotamer in acremolide B matched that found in acremolide A. Notable differences in the NMR data for **5** compared to **4** were significantly deshielded resonances for H_2-10 (δ_H 2.40), H_3-12 (δ_H 2.06), and C-11 (δ_C 208.6), characteristic of a C-11 ketone (as observed above for **8**). More detailed analysis of these NMR data (Tables 2 and 3) was in full accord with the structure as shown for acremolide B (**5**).

Acremolides C (**6**) ($C_{25}H_{44}O_6N_2$, $\Delta = 0.4$ mmu) and D (**7**) ($C_{24}H_{42}O_6N_2$, $\Delta = 1.4$ mmu) were obtained as minor co-metabolites, both of which existed as a 2:1 mixture of equilibrating rotamers incorporating both the L-Pro and substituted fatty acid substructures common to acremolide A (**4**) (see Tables 1, 2, 3, 4). Acremolides

Table 3. ^{1}H NMR (d_6-DMSO, 600 MHz) Data for Major (**8** and **5**) and Minor (**8a** and **5a**) 3,11-Dioxoacremolide A and Acremolide B Prolinyl Amide Bond Conformers, Respectively

	δ_H (m, J (Hz))^a			
#	**5**	**5a**	**8**	**8a**
2	2.32 (dq, 7.2, 1.7)	2.57 (dq, 6.7, 3.1)	3.74 (q, 6.5)	3.50 (q, 6.9)
3	3.73 (m)	3.92 (m)		
4a	1.82 (m)	1.50 (m)	3.42 (dd, 16.9, 10.9)	2.77 (dd, 14.0, 7.8) 2.69 (dd, 14.0, 2.8)
4b	1.72 (m)	2.69 (dd, 14.0, 2.8)	2.51 (m)	
5	4.52 (m)	4.60 (ddd, 9.8, 3.0, 2.7)	4.79 (ddd, 10.0, 3.9, 1.0)	4.73 (m)
6	1.67 (m)	1.93 (m)	1.96 (m)	1.61 (m)
7a	1.18 (m)	1.01 (m)	1.53 (m) 0.95 (m)	1.30 (m) 1.03 (m)
7b	0.95 (m)	1.03 (m)		
8a	^{b}	^{b}	1.24 (m) 1.16 (m)	1.32 (m) 1.16 (m)
8b	1.16 (m)	1.16 (m)		
9a	1.43 (m)	1.43 (m)	1.49 (m)	1.49 (m)
9b	1.38 (m)	1.38 (m)	1.36 (m)	1.36 (m)
10	2.40 (brt, 7.5)	2.40 (brt, 7.5)	2.47 (brt, 7.5)	2.39 (brt, 7.2)
12	2.06 (d, 5.7)	2.06 (d, 5.7)	2.08 (s)	2.06 (s)
Me-2	0.73 (d, 7.3)	0.83 (d, 6.7)	0.98 (d, 6.5)	0.74 (d, 6.9)
Me-6	0.83 (d, 6.7)	0.73 (d, 7.3)	0.81 (d, 6.9)	0.85 (d, 66.8)
3-OH	4.41 (d, 6.2)	Not observed		
	L-Pro		L-Pro	
2′	5.00 (dd, 8.2, 2.8)	4.83 (brd, 7.0)	4.71 (brd, 7.6)	5.05 (dd, 8.3, 2.6)
3a′	2.26 (m)	2.18 (m)	2.13 (m)	2.31 (m)
3b′	1.91 (m)	1.58 (m)	1.70 (m)	1.95 (m)
4a′	1.90 (m)	1.77 (m)	1.79 (m)	1.82 (m)
4b′	1.78 (m)	1.61 (m)	1.45 (m)	1.75 (m)
5a′	3.58 (dd, 12.0, 7.8, 4.7)	3.87 (dd, 11.8, 9.3, 3.0)	3.69 (ddd, 12.6, 9.6, 3.0)	3.56 (ddd, 12.1, 8.5, 3.7)
5b′	3.47 (ddd, 12.0, 7.7, 7.6)	3.13 (m)	3.24 (m)	3.47 (ddd, 12.1, 8.2, 3.7)
	D-Phe		D-Phe	
2″	4.54 (m)	4.73 (dd, 8.0, 7.8)	4.93 (m)	4.48 (ddd, 10.5, 8.4, 4.4)
3a″	3.20 (dd, 13.8, 3.7)	3.02 (dd, 13.0, 8.0)	3.11 (dd, 14.0, 7.4)	3.17 (dd, 13.9, 4.4)
3b″	2.81 (dd, 13.8, 11.1)	2.87 (dd, 13.0, 7.8)	2.93 (dd, 14.0, 7.9)	2.87 (dd, 13.9, 10.5)
5″	7.21 (d, 7.2)	7.25 (d, 7.5)	7.23–7.29 (m)	7.16–7.21 (m)
6″	7.16–7.28 (m)	7.16–7.28 (m)	7.23–7.29 (m)	7.16–7.21 (m)
7″	7.16–7.28 (m)	7.16–7.28 (m)	7.19 (m)	7.27 (m)
8″	7.16–7.28 (m)	7.16–7.28 (m)	7.23–7.29 (m)	7.16–7.21 (m)
9″	7.21 (d, 7.2)	7.25 (d, 7.5)	7.23–7.29 (m)	7.16–7.21 (m)
NH-2″	8.22 (brd, 8.4)	8.44 (brd, 9.1)	8.64 (d, 9.5)	8.37 (d, 8.4)

^{a} Assignments are based on 2D NMR correlations. ^{b} Extensive overlap of ^{1}H NMR signals at ∼1.2 to ∼2.0 prevented unambiguous assignment.

C (**6**) and D (**7**) differed from acremolides A (**4**) and B (**5**) in replacement of the D-Phe residue with an Ile and a Val residue, respectively. Confirmation of this assignment and a determination of the absolute stereochemistry of these amino acid residues were achieved by C_3 Marfey's analysis (see below).[4,5]

Marfey's analysis[4] relies on the hydrolysis of a peptide- or amino acid-containing substance (i.e., acremolides) followed by *in situ* conversion of the resulting amino acids using a chiral derivatizing agent (CDA) (i.e., (1-fluoro-2,4-dinitrophenyl)-5-L-alanine amide, also known as L-FDDA) to form 2,4-dinitrophenyl-5-L-alanine amide (DNP) derivatives. The C_{18} HPLC retention times of the resulting DNP derivatives monitored by UV at 340 nm can be diagnostic for both a given amino acid and a given stereochemistry. Marfey's analysis is a powerful method of choice for many natural products researchers faced with the challenge of identifying amino acids in scarce and very valuable analytes (bioactive metabolites). While comparisons to DNP derivatives obtained from authentic amino acid standards makes this approach highly sensitive and accurate, success ultimately relies on the resolving power of the HPLC method. In a 2003 study[7] directed at characterizing novel depsipeptides from an Australian isolate of *Aspergillus carneus* we observed that the standard C_{18} Marfey's HPLC method was incapable of resolving and hence differentiating D-Ile from D-*allo*-Ile, or L-Ile from L-*allo*-Ile. This observation was reinforced in a 2004 study by Hess *et al.*,[8] who examined the merits of HPLC-ESIMS, together with a range of CDAs (including FDDA), as a means to identify and assign absolute stereochemistry to amino acids. Hess *et al.* documented the C_{18} HPLC resolution of many amino acids against four CDAs and concluded "diasteromers such as D-*allo*-Ile and D-Ile. . .were not completely resolved with any of the CDAs". In our earlier *A. carneus* study[7] we addressed this deficiency of the Marfey's method by developing a chiral Marfey's HPLC method (Phenomenex Chirex urea type 3010) that successfully resolved all Ile stereoisomers and confirmed the presence of D-*allo*-Ile in the aspergillicins. We later built on these findings to develop and describe a C_3 Marfey's method capable of resolving the stereoisomers of all proteogenic amino acids (including Ile).[5]

Analysis of the Marfey's DNP derivatives obtained from acremolide C (**6**) and acremolide D (**7**) using the C_3 Marfey's method unambiguously identified L-Pro and D-Ile, and L-Pro and D-Val, respectively. In addition to unambiguously differentiating all Ile DNP derivative stereoisomers (D, L, *allo*), the C_3 Marfey's method is superior in its resolution of Pro DNP derivatives. Under the traditional C_{18} Marfey's method, the L-Pro DNP derivative can elute with the same retention time (subject to eluant pH and HPLC column performance) to a peak corresponding to a Marfey's reagent contaminant (MW 270 amu, speculated to be the hydrolyzed product, 5-defluoro-5-hydroxy Marfey's reagent), making comparisons to authentic standards problematic. This situation is further complicated by the coelution of D-Pro with residual Marfey's reagent (MW 272 amu). By contrast, the C_3 Marfey's method provides clear baseline resolution between L- and D-Pro DNP derivatives and residual reagent peaks.

Close analysis of the ^{13}C NMR data for **6** and **7** confirmed a preference for major *trans* ($\Delta\beta\gamma = 3.1$ and 3.0 ppm) versus minor *cis* ($\Delta\beta\gamma = 10.9$ and 11.0 ppm) prolinyl amide bond conformers, in contrast to acremolides A (**4**) and B (**5**). This observation suggests that replacement of the bulky D-Phe (**4** and **5**) residue with either D-Ile (**6**) or D-Val (**7**) inverts the preferred *cis/trans* bias in acremolide macrocycles.

Table 4. ^{1}H NMR (d_6-DMSO, 600 MHz) Data for Major *trans* (**6** and **7**) and Minor *cis* (**6a** and **7a**) of Acremolide C and D Prolinyl Amide Bond Conformers, Respectively

	δ_H (m, *J* (Hz))[a]			
#	**6**	**6a**	**7**	**7a**
2	2.59 (dq, 6.8, 3.3)	2.66 (dq, 7.2, 2.1)	2.59 (dq, 6.8, 3.3)	2.68 (dq, 7.1, 1.6)
3	3.89 (m)	3.81 (m)	3.87 (m)	3.81 (m)
4a	1.51(m)	1.89 (m)	1.51 (m)	1.77 (m)
4b		1.77 (m)		1.87 (m)
5	4.61 (ddd, 9.8, 3.0, 2.9)	4.56 (m)	4.60 (ddd, 9.8, 3.1, 2.7)	4.58 (m)
6	1.92 (m)	1.70 (m)	1.91 (m)	1.70 (m)
7a	1.54 (m)	1.37 (m)	1.55 (m)	1.03 (m)
7b	1.05 (m)	1.23 (m)		
8	[b]	[b]	[b]	[b]
9	[b]	[b]	[b]	[b]
10a	1.33 (m)	1.34 (m)	1.33 (m)	1.33 (m)
10b	1.25 (m)	1.25 (m)	1.26 (m)	1.26 (m)
11	3.54 (m)	3.54 (m)	3.54 (m)	3.54 (m)
12	1.02 (d, 6.1)	1.02 (d, 6.1)	1.02 (d, 6.1)	1.02 (d, 6.1)
Me-2	0.85 (d, 6.8)	1.18 (d, 7.2)	0.85 (d, 6.7)	1.20 (d, 7.2)
Me-6	0.73 (d, 5.5)	0.84 (d, 6.6)	0.73 (d, 6.7)	0.84 (d, 5.8)
OH-3	not observed	not observed	4.36 (d, 6.1)	
OH-11	not observed	not observed	4.29 (d, 4.6)[c]	4.26 (d, 4.8)[c]
	L-Pro		l-Pro	
2′	4.88 (dd, 6.1, 2.6)	4.86 (dd, 8.0, 3.3)	4.88 (brd, 7.2)	4.86 (dd, 8.3, 3.5)
3a′	2.22 (m)	2.22 (m)	2.22 (m)	2.26 (m)
3b′	1.71 (m)	1.88 (m)	1.72 (m)	1.88 (m)
4a′	1.92 (m)	1.91 (m)	1.93 (m)	[b]
4b′	1.71 (m)	1.75 (m)	1.71 (m)	1.76 (m)
5a′	3.93 (ddd, 12.3, 9.8, 3.0)	3.54 (m)	3.94 (dd, 12.0, 9.6, 2.7)	3.59 (m)
5b′	3.46 (ddd, 12.3, 7.8, 7.6)	3.44 (m)	3.45 (m)	3.45 (m)
	D-*allo*-Ile		D-Val	
2″	4.25(dd, 11.1, 9.5)	4.23 (dd, 8.6, 5.2)	4.14 (dd, 10.6, 9.5)	4.20 (dd, 9.2, 4.9)
3″	1.92 (m)	2.08 (dd, m)	2.67 (m)	2.37 (m)
4a″	1.53 (m)	1.23 (m)	0.89 (d, 6.7)	0.76 (d, 7.1)
4b″	1.26 (m)			
5″	0.82 (dd, 7.5, 7.3)	0.79 (dd, 7.2, 6.7)		
2″-NH	8.13 (d, 9.4)	8.10 (d, 9.3)	8.16 (d, 9.7)	8.16 (d, 9.7)
3″-Me	0.79 (dd, 7.0, 6.0)	0.74 (dd, 6.8, 5.7)	0.83 (d, 5.7)	0.82 (d, 6.9)

[a] Assignments are based on 2D NMR correlations. [b] Extensive overlap of ^{1}H NMR signals at ~1.2 to ~2.0 prevented unambiguous assignment. [c] Assignments may be interchanged.

It remains unclear whether or not acremolide structural diversity (**4–7**), and associated prolinyl amide bond conformer bias (*cis* vs *trans*), adjusts the potency and/or selectivity of the acremolide biological/ecological response, particularly given that the ecological role of these molecules remains unknown. As noted earlier, we were alert to the modest cytotoxic potency displayed by the chaetoglobosins compared to the cytoxicity of the crude *Acremonium* extract. Although not cytotoxic in their own right, we did test each of acremolides A−D (**4–7**) in combination with each of the chaetoglobosins **1** and **2**, to establish if the former could synergize the cytotoxic properties of the latter against NS-1 cells and thereby account for the anomalous cytoxicity of the crude *Acremonium* extract. This study did not reveal any significant synergistic effect. Although the acremolides did not synergize the chaetoglobosin cytotoxicity and displayed no antibacterial (*Bacillus subtilis*), antifungal (*Candida albicans*), or cytotoxic (NS-1) properties in our hands, we note their structural similarity to the known histone deacetylase (HDAC) inhibitors FR235222,[9] apicidin A,[10] and trapoxin.[11] Our investigations into the biological properties (and possible ecological role) of the acremolides remain a work in progress.

Experimental Section

General Experimental Procedures. Chiroptical measurements ([α]$_D$) were obtained on a JASCO P-1010 intelligent remote module polarimeter in a 100 by 2 mm cell. Ultraviolet (UV) absorption spectra were obtained using a CARY3 UV–visible spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were performed on either a Bruker Avance 500 or 600 spectrometer, in the solvents indicated and referenced to residual ^{1}H signals in the deuterated solvents. Electrospray ionization mass spectra (ESIMS) were acquired using an Agilent 1100 Series separations module equipped with an Agilent 1100 Series LC/MSD mass detector in both positive and negative ion modes. High-resolution (HR) ESIMS measurements were obtained on a Finnigan MAT 900 XL trap instrument with a Finnigan API III source. Initial high-performance liquid chromatography (HPLC) was carried out on a system consisting of two Shimadzu LC-8A preparative liquid chromatographs with static mixer, Shimadzu SPD-M10AVP diode array detector, and Shimadzu SCL-10AVP system controller. Subsequent HPLC was performed using an Agilent 1100 Series separations module equipped with Agilent 1100 Series diode array and/or multiple wavelength detectors, and Agilent 1100 Series fraction collector, controlled using ChemStation Rev.9.03A and Purify version A.1.2 software.

Fungal Material. The fungal strain *Acremonium* sp. (MST-MF558a) was isolated from an estuarine sediment sample collected from the Huon River near Franklin in Tasmania, Australia. Microscopic examination of the strain grown on malt extract agar showed the presence of lateral subulate unbranched conidiophores once-septate near the base, each bearing at the tip a ball of conidia. The spores were more or less globose, hyaline, and aseptate and measured approximately 3–3.5 × 2.5–3 μm. The morphology is considered typical of the genus *Acremonium*. Examination of the rDNA primers for ITS4 sequence led to a 513 base pair sequence. A Blast search failed to show any correlation to a known species within this genus. The strain is considered to represent a novel species, named *Acremonium nov.* sp. (MST-558a).

Extraction and Isolation. The mycelia from a solid fermentation (115.6 g, 21 days at 24 °C) were extracted with MeOH (2 L) for 24 h at 28 °C, decanted, concentrated *in vacuo* to an aqueous residue (400 mL), and then extracted with EtOAc (3 × 400 mL), after which the combined organic phase was concentrated *in vacuo* to afford a brown syrup (19.85 g). After adjusting to pH 7, the remaining aqueous phase (400 mL) was adsorbed onto C_{18} Bond Elute SPE cartridges (2 × 10 g) and eluted with H_2O (400 mL), then 50% MeOH/H_2O, and finally

MeOH. The latter two fractions were concentrated *in vacuo* to yield 1.23 and 0.065 g of residue, respectively. The aqueous eluent was subsequently adjusted to pH 4 and the suspension readsorbed onto the same C_{18} Bond Elute SPE cartridges and eluted with 50% MeOH/H_2O followed by MeOH. The latter two fractions were concentrated *in vacuo* to yield residues of 1.22 and 0.264 g, respectively. All SPE fractions from the mycelial extracts along with the crude MeOH and EtOAc extracts were subjected to a broad range of biological screens. Both the crude MeOH extract and EtOAc-soluble fraction from the mycelia showed significant cytotoxic activity (LD_{99} 16 and 1.6 μg/mL, respectively), prompting further investigation of the EtOAc fraction.

A portion of the EtOAc fraction (7.2 g) was subjected to solvent partitioning between combinations of CH_2Cl_2, MeOH, and H_2O, to yield a CH_2Cl_2 (1.97 g)-soluble fraction and a quantity of closely related material (0.77 g) that precipitated from aqueous MeOH. The precipitate was also CH_2Cl_2 soluble. These two CH_2Cl_2-soluble fractions displayed comparable cytotoxicity (LD_{99} 1.8 and 1.2 μg/mL, respectively) and similar HPLC-DAD-ELSD and ^{1}H NMR metabolite profiles. Further investigations were directed at the larger CH_2Cl_2-soluble fraction.

Initial fractionation of a portion of the CH_2Cl_2 solubles (458 mg) was via 10% stepwise gradient elution from H_2O to MeOH through a C_{18} SPE cartridge (5 g). HPLC fractionation of a relatively polar SPE fraction (7.5 mg) (2 mL/min, 15 min gradient elution from 90% H_2O/MeCN to 100% MeCN (with a 0.10% TFA modifier) through a Zorbax SB-C_8 5 μm 250 × 9.4 mm column) yielded RKB 3564S (**3**, 2.7 mg, 0.14%[13]). HPLC fractionation of a relatively nonpolar SPE fraction (135.5 mg) (2 mL/min, 20 min gradient elution from 60% H_2O/MeCN to 100% MeCN (with a 0.10% TFA modifier) through a Zorbax SB-C_8 5 μm 250 × 9.4 mm column) returned 19-*O*-acetylchaetoglobosin D (**1**, 3.2 mg, 0.16%[13]) and 19-*O*-acetylchaetoglobosin B (**2**, 3.3 mg, 0.17%[13]). HPLC fractionation of a third SPE fraction (95.7 mg) (2 mL/min, 20 min gradient elution from 30% H_2O/MeOH to 100% MeOH (with a 0.10% TFA modifier) through a Zorbax SB-C_8 5μm 250 × 9.4 mm column) yielded four new lipodepsipeptides, acremolides A (**4**, 27.9 mg, 1.4%[13]), B (**5**, 18.7 mg, 0.95%[13]), C (**6**, 12.3 mg, 0.62%[13]), and D (**7**, 6.8 mg, 0.34%[13]).

19-*O*-Acetylchaetoglobosin D (1): yellow solid; ^{1}H and ^{13}C NMR characteristics were as reported;[1,12] (+)-ESIMS (100 kV) *m/z* 593 [M + Na]$^+$, 571 [M + H]$^+$; (−)-ESIMS (100 kV) *m/z* 569 [M − H]$^-$.

19-*O*-Acetylchaetoglobosin B (2): yellow solid; ^{1}H and ^{13}C NMR characteristics were as reported;[1,12] (+)-ESIMS (100 kV) *m/z* 593 [M + Na]$^+$; (−)-ESIMS (100 kV) *m/z* 571 [M + H]$^+$, 569 [M − H]$^-$.

RKB 3564S (3): light yellow solid; ^{1}H NMR data (CD_3OD, 500 MHz) δ_H 7.03 (1H, dd, J = 8.0 and 7.8 Hz, H-5), 6.92 (1H, d, J = 7.8 Hz, H-4), 6.77 (1H, dd, J = 15.6 and 1.8 Hz, H-8), 6.66 (1H, dd, J = 8.0 and 1.1 Hz, H-6), 6.11 (1H, dq, J = 15.6 and 6.6 Hz, H-9), 4.75 (2H, s, H-7) 1.88 (3H, dd, J = 6.6 and 1.7 Hz, H-10); ^{13}C NMR data (CD_3OD, 125 MHz) δ_C 157.4 (C-1), 140.6 (C-3), 130.0 (C-8), 129.7 (C-5), 128.9 (C-9), 124.8 (C-2), 118.5 (C-4), 114.7 (C-6), 56.9 (C-7), 19.0 (C-10); (+)-ESIMS (100 kV) *m/z* 187 [M + Na]$^+$; (−)-ESIMS (100kV) *m/z* 199 [M + Cl]$^-$; ^{1}H and ^{13}C NMR data were in good agreement with literature data.[2]

Acremolide A (4): white solid; $[\alpha]_D$ −103 (*c* 0.02, MeOH); UV (EtOH) λ_{max} (ϵ) 205 (10 000), 258 (sh) nm; ^{1}H NMR data (d_6-DMSO, 600 MHz), see Table 1; ^{13}C NMR data (d_6-DMSO, 150 MHz), see Table 2; (+)-ESIMS (100 kV) *m/z* 525 [M + Na]$^+$, 503 [M + H]$^+$; (−)-ESIMS (100 kV) *m/z* 501 [M − H]$^-$; (+)-HRESIMS *m/z* 525.2953 ([M + Na]$^+$, $C_{28}H_{42}O_6N_2Na$ requires 525.2941).

Acremolide B (5): white solid; $[\alpha]_D$ −98 (*c* 0.02, MeOH); UV (EtOH) λ_{max} (ϵ) 210 (10 600), 260 (sh) nm; ^{1}H NMR data (d_6-DMSO, 600 MHz), see Table 3; ^{13}C NMR data (d_6-DMSO, 150 MHz), see Table 2; (+)-ESIMS (100 kV) *m/z* 523 [M + Na]$^+$, 501 [M + H]$^+$; (−)-ESIMS (100 kV) *m/z* 499 [M − H]$^-$; (+)-HRESIMS *m/z* 523.2791 ([M + Na]$^+$, $C_{28}H_{40}O_6N_2Na$ requires 523.2784).

Acremolide C (6): white solid; $[\alpha]_D$ −77 (*c* 0.02, MeOH); UV (EtOH) λ_{max} (ϵ) 208 (9400) nm; ^{1}H NMR data (d_6-DMSO, 600 MHz), see Table 4; ^{13}C NMR data (d_6-DMSO, 150 MHz), see Table 2; (+)-ESIMS (100 kV) *m/z* 491 [M + Na]$^+$, 469 [M + H]$^+$, (−)-ESIMS (100 kV) *m/z* 513 [M + HCO_2]$^-$; (+)-HRESIMS *m/z* 491.3101 ([M + Na]$^+$, $C_{25}H_{44}O_6N_2Na$ requires 491.3097).

Acremolide D (7): white solid; $[\alpha]_D$ −79 (*c* 0.02, MeOH); UV (EtOH) λ_{max} (ϵ) 206 (7700) nm; ^{1}H NMR data (d_6-DMSO, 600 MHz), see Table 4; ^{13}C NMR data (d_6-DMSO, 150 MHz), see Table 2; (+)-ESIMS (100 kV) *m/z* 477 [M + Na]$^+$, 455 [M + H]$^+$, (−)-ESIMS (100 kV) *m/z* 499 [M + HCO_2]$^-$; (+)-HRESIMS *m/z* 477.2955 ([M + Na]$^+$, $C_{24}H_{42}O_6N_2Na$ requires 477.2941).

Marfey's Analysis. Individual samples of acremolides A−D (**4–7**) (50 μg) in 6 M HCl (200 μL) were heated at 100 °C overnight. The resulting hydrolysates were treated with 1 M $NaHCO_3$ (20 μL) and 1% FDAA/acetone (100 μL) at 37 °C for 1 h, then neutralized with 1 M HCl (20 μL) and diluted with MeCN (810 μL) prior to HPLC analysis. DNP derivatives of amino acid standards were prepared in a similar fashion, by reacting directly with 1 M $NaHCO_3$ (20 μL) and 1% FDAA/acetone (100 μL) at 37 °C for 1 h, then neutralizing with 1 M HCl (20 μL) and diluting with MeCN (810 μL) prior to HPLC analysis. Marfey's DNP derivatives were analyzed by HPLC using a C_3 Marfey's HPLC method (a 1 mL/min, 55 min linear gradient elution from 85:15:5 to 35:60:5 solvent A:solvent B:solvent C through a Zorbax StableBond C_3 5 μm 150 × 4.6 mm HPLC column, maintained at 50 °C where solvent A is H_2O; solvent B is MeOH; and solvent C is acetonitrile with 1% (v/v) formic acid, and with diode array detection monitoring at 340 nm and ESI mass detection under both +ve and −ve ion modes).[5]

Oxidation of 4. A suspension of acremolide A (**4**) (5.7 mg) and pyridinium dichromate (3.8 mg) in dry CH_2Cl_2 (2 mL) was stirred for 24 h, after which the reaction was quenched with H_2O, filtered through Celite, and washed with CH_2Cl_2 to remove chromium salts. The combined CH_2Cl_2 washings were concentrated *in vacuo*, and the crude product (5.7 mg) was purified by HPLC (2 mL/min, 20 min gradient elution from 50% H_2O/MeCN to 100% MeCN (with a 0.01% TFA modifier) through a Zorbax SB-C_8 5 μm 250 × 9.4 mm column) to yield the diketone 3,11-dioxoacremolide A (**8**) (3.2 mg, 57%) as a white solid: ^{1}H NMR data (d_6-DMSO, 600 MHz) see Table 3; ^{13}C NMR data (d_6-DMSO, 150 MHz) see Table 2; (+)-ESIMS (100 kV) *m/z* 499 [M + H]$^+$; (−)-ESIMS (100 kV) *m/z* 497 [M − H]$^-$; (+)-HRESIMS *m/z* 521.2645 ([M + Na]$^+$, $C_{28}H_{38}O_6N_2Na$ requires 521.2628).

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