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Aspergillicins A–E: five novel depsipeptides from the marine-derived fungus *Aspergillus carneus*

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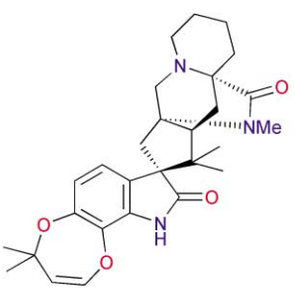
A search for new antiparasitic agents from a strain of the fungus *Aspergillus carneus* isolated from an estuarine sediment collected in Tasmania, Australia, yielded the known terrestrial fungal metabolite marcfortine A (**1**) as an exceptionally potent antiparasitic agent. This study also yielded a series of new depsipeptides, aspergillicins A–E (**2**–**6**) and the known terrestrial fungal metabolite acyl aszonalenin (**7**). Marcfortine A (**1**) and acyl aszonalenin (**7**) were identified by spectroscopic analysis, with comparison to literature data. Complete stereostructures were assigned to aspergillicins A–E (**2**–**6**) on the basis of detailed spectroscopic analysis, together with ESIMS analysis of the free amino acids generated by acid hydrolysis, and HPLC analysis of Marfey derivatives prepared from the acid hydrolysate. The peptide amino acid sequence for all aspergillicins was unambiguously assigned by MS^n ion-trap ESI mass spectrometry.

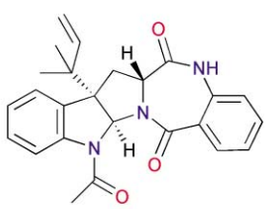
Introduction

Terrestrial microbes are well recognised as a source of novel metabolites, many of which possess valuable biological properties. Indeed, much of the pharmaceutical, agrochemical and crop protection industry is based on the exploitation of microbial natural products. Over the last decade the search for new drugs has expanded to include those produced by microbes obtained from marine sources. Bacteria and fungi isolated from such substrates as marine plants (algae, seagrasses), invertebrates (sponges, tunicates), vertebrates (fish), sands, sediments and seawater itself, have been found to yield novel bioactive natural products. While sourced from marine substrates, the marine character of such microbes is not absolute. A commonly held view[1] is that marine *derived* microbes (those isolated from marine ecosystems or substrates) can be described as either (a) *marine obligate*: growing and reproducing exclusively in marine ecosystems (*i.e.* require salt), or (b) *marine facultative*: tolerant of marine ecosystems, but also found growing and reproducing in terrestrial ecosystems (*i.e.* not requiring salt). This overlap between marine and terrestrial microbiology is hardly unexpected given the common boundaries shared by these environments. Whether a marine *derived* microbe is obligate, facultative, or its status remains unknown, these organisms are quite capable of contributing to bioprospecting and the discovery of new generation drugs.

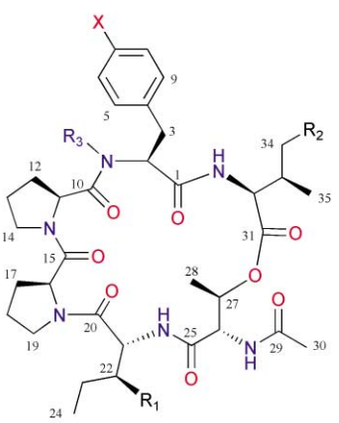
During the course of our investigations into new agrochemical agents from marine sources we generated an extensive library of marine derived microbes, comprising >100,000 bacteria and fungal isolates. This library has been chemically profiled, and screened for metabolites with agrochemical potential. One activity targeted is the ability to combat the commercially significant livestock parasite *Haemonchus contortus*. While many cultures in this isolate library are the subject of ongoing investigation, the present report describes a study into one such marine fungus, *Aspergillus carneus*, isolated from a Tasmanian saline sediment. This study resulted in the isolation of the known terrestrial fungal metabolites marcfortine A (**1**)[2,3] and acyl aszonalenin (**7**),[4,5] as well as five new depsipeptides, aspergillicins A–E (**2**–**6**).



(**1**)



(**7**)



(**2**) R_1 and R_3 = Me, R_2 = H, X = OMe
(**3**) R_1 and R_2 = H, R_3 = Me, X = OMe
(**4**) R_1 and R_3 = Me, R_2 and X = H
(**5**) R_1 = Me, R_2 , R_3 and X = H
(**6**) R_1 , R_2 and R_3 = Me, X = OMe

Results and discussion

Primary biological screening revealed that an isolate of *A. carneus* displayed potent activity against the commercially significant livestock parasite *H. contortus*. Large scale fermen-

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tation of *A. carneus* was achieved in solid media, which on processing returned MeOH extractables (7 g) with an LD_{99} against *H. contortus* of 166 μg mL^{-1}. Bioassay directed fractionation (solvent partitioning, Sephadex LH-20, and C_{18} HPLC) led to the successful isolation of the active agent as the known terrestrial fungal metabolite marcfortine A (**1**).[2,3] While marcfortine A (**1**) belongs to a class of metabolite (*i.e.* paraherquamides) known to be active against adult parasites *in vivo*, this study revealed **1** to be a potent inhibitor of *H. contortus* motility *in vitro* (LD_{99} 0.06 μg mL^{-1}). Also co-isolated with marcfortine A (**1**) were five new depsipeptides, aspergillicins A–E (**2**–**6**) (see Fig. 1), and the known fungal metabolite acyl aszonalenin (**7**).[4,5] The structures assigned to marcfortine A (**1**)[2,3] and acyl aszonalenin (**7**)[4,5] were confirmed by detailed spectroscopic analysis and comparison to published literature data. Preliminary analysis of the NMR data for the aspergillicins strongly suggested that they were peptides. Of particular note were characteristic amide carbonyl resonances in the ^{13}C NMR spectrum, along with amide NH and α-amino acid methine resonances in the ^{1}H NMR spectrum. Further analysis revealed the aspergillicins to be new natural products.

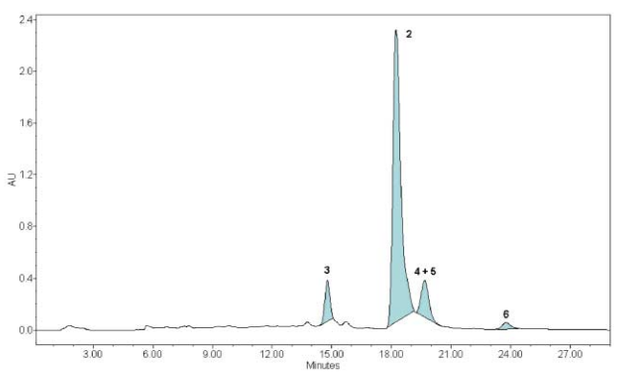

Fig. 1 C_{18} HPLC trace of aspergillicins A–E (**2**–**6**) monitored at 211 nm.

Aspergillicin A (**2**) was obtained as a colourless amorphous solid. High resolution ESI(+)MS analysis revealed a pseudo molecular ion $[M + Na]^+$ corresponding to a molecular formula ($C_{38}H_{56}N_6O_9$, Δ = +2.0 mmu) requiring 14 double-bond equivalents (DBE). The UV spectrum of aspergillicin A (**2**) exhibited absorption maxima (283, 277, 227 and 212 nm) indicative of an aromatic amino acid residue, while distinctive IR absorbances (3443 and 1550 cm^{-1}) were consistent with secondary amide moieties.

Close examination of the ^{1}H and ^{13}C NMR data revealed six isolated spin systems, attributed to six amino acid residues (see Table 1). Standard amino acids evidenced by this NMR data included Pro (×2),Val and Ile. An acetylated Thr residue was identified, while a Tyr residue was observed to be both *N*- and *O*-methylated. These residues account for 13 of the required 14 DBE, requiring aspergillicin A (**2**) to be cyclic. Mild base hydrolysis (NH_3) of **2** resulted in a molecular weight increase of 18 mass units, consistent with ring-opening of a depsipeptide where the lactone linkage was drawn from an *N*-acetylated Thr. An alternative *O*-acetylated Thr containing cyclic peptide could be excluded as it would be expected to resist ring-opening and exhibit a loss of 42 mass units on deacetylation. Examination of the 2D NMR data (see Table 1) revealed correlation sequences from; (a) the Ile 21-NH (δ 6.66) to the *N*-Ac-Thr C-25 (168.3 ppm); (b) the *N*-Ac-Thr oxymethine H-27 (δ 5.58) to the Val C-31 (170.5 ppm); (c) the Val 32-NH (δ 8.24) to the *N*-Me-Tyr-*O*-Me C-1 (169.8 ppm); and (d) the *N*-Me-Tyr-*O*-Me H-2 (δ 5.00) to the Pro C-10 (173.2 ppm). Given that the remaining residue was Pro, and that aspergillicin A (**2**) is cyclic, the planar structure must be as indicated.

Acid hydrolysis (HCl) of aspergillicin A (**2**) yielded a mixture of free amino acids. ESI(+)MS analysis of this mixture revealed M + H ions at *m/z* 116 (Pro), 118 (Val), 120 (Thr) [note that the acetate group is lost during hydrolysis], 132 (Ile) and 196 (*N*-Me-Tyr). Cleavage of the tyrosine methyl ether during hydrolysis was confirmed by subjecting authentic *N*,*O*-dimethyl tyrosine to the hydrolysis conditions, followed by ESI(+)MS and ^{1}H NMR analysis. Assignment of absolute stereochemistry to aspergillicin A (**2**), was achieved by HPLC analysis of the Marfey's derivatised[6,7] acid hydrolysate, with comparison (co-injection) to both L and D authentic standards. Hence, aspergillicin A (**2**) is comprised of L-Val, *N*-Ac-L-Thr, D-Ile, L-Pro (×2) and *N*-Me-L-Tyr-*O*-Me. Note that while Marfey's analysis can differentiate L from D isomers, including D-Thr from D-*allo*-Thr and L-Thr from L-*allo*-Thr, in our hands this technique was not able to differentiate D-Ile from D-*allo*-Ile, or L-Ile from L-*allo*-Ile. This determination was successfully achieved by analysis of the Marfey's derivatives on a chiral HPLC column. In this way aspergillicin A was found to incorporate D-*allo*-Ile. The two proline residues in aspergillicin A were determined to adopt a *trans* configuration about their respective amide bonds in deuterochloroform, on the basis of the difference in ^{13}C NMR shifts between C-12 and C-13 (Δ 3.1 ppm), and C-17 and C-18 (Δ 3.2 ppm).[8,9]

It is worthwhile noting that the interpretation of 2D NMR correlations detailed above, which led to assignment of an amino acid sequence for aspergillicin A (**2**), was heavily dependent on the correct assignment of ^{13}C NMR carbonyl carbon resonances to individual amino acids. As can be observed in Table 1, two of these carbonyl resonances overlap at δ 169.8, while two others have very similar resonance frequencies (δ 171.1 and 171.2). To ensure that our assignment of the ^{13}C NMR data and subsequent determination of the peptide sequence was correct, the ^{13}C NMR and 2D NMR data was reacquired in CD_3CN. Although analysis of this new data set supported the initial assignment, we felt that this issue was of sufficient importance to warrant determination of the amino acid sequence by another independent method (see below).

Aspergillicin B (**3**) was isolated in pure form ($C_{37}H_{54}N_6O_9$, Δ = −1.3 mmu), and found to differ from aspergillicin A (**2**) by replacement of the D-*allo*-Ile residue by a D-*nor*-Val residue. This amino acid assignment was consistent with the ^{1}H NMR (see Table 2) and ESIMS data, and was confirmed by ESI(+)MS analysis of the acid hydrolysate which revealed M + H ions at *m/z* 116 (Pro), 118 (Val and/or *nor*-Val), 120 (Thr) [note that the acetate group is lost during hydrolysis], and 196 (*N*-Me-Tyr) [note that the methyl ether is cleaved during hydrolysis], and HPLC analysis of the Marfey's derivatised acid hydrolysate. Confirmation of the amino acid peptide sequence was achieved by mass spectral analysis (see below).

Aspergillicins C (**4**) ($C_{37}H_{54}N_6O_8$, Δ = −0.3 mmu) and *D* (**5**) ($C_{36}H_{52}N_6O_8$, Δ = −0.4 mmu) were minor components that were only isolated as a mixture. Despite this, it was possible to interpret the ^{1}H NMR (see Table 2) and ESIMS data and propose that **4** and **5** differed from **2** in replacement of the *N*-Me-L-Tyr-*O*-Me residue by *N*-Me-L-Phe and L-Phe respectively. This amino acid assignment was supported by ESI(+)MS analysis of the **4/5** acid hydrolysate which revealed M + H ions at *m/z* 116 (Pro), 118 (Val and/or *nor*-Val), 120 (Thr) [note that the acetate group is lost during hydrolysis], 132 (Ile), 166 (Phe) and 180 (*N*-Me-Phe) and HPLC analysis of the Marfey's derivatised acid hydrolysate. Confirmation of the amino acid peptide sequence was achieved by mass spectral analysis (see below).

Aspergillicin E (**6**) ($C_{39}H_{58}N_6O_9$, Δ = 0.0 mmu) was a very minor co-metabolite of aspergillicin A–D, with spectral data very similar to that of aspergillicin A (**2**). Indeed, the only obvious differences between spectral data for **6** and **2** was that the former was 14 atomic mass units heavier, and the ^{1}H NMR shift for H-32 in **6** (δ 4.68, dd, see Table 2) was shifted slightly downfield compared to that in **2** (δ 4.39, dd, see Table 1). Given the

Table 1 NMR ($CDCl_3$, 400 MHz) data for aspergillicin A (**2**)

No.	^{13}C δ	^{1}H δ (m, *J*/Hz)	DQFCOSY	gHMBC
N-Me-L-Tyr-*O*-Me				
1	169.8			
2	62.5	5.00 (dd, 11.6, 3.4)	H-3a, H-3b	C-1, C-10, *N*-Me
3a	33.4	3.16 (dd, 14.7, 3.4)	H-2, H-3b	C-1, C-2, C-4 or C5/9
3b		2.97 (dd, 14.7, 11.6)	H-2, H-3a	C-1, C-2, C-4 or C5/9
4	129.7			
5/9	130.5	7.04 (d, 8.6)	H-6/8	C-3, C-7, C-9/5
6/8	114.4	6.84 (d, 8.6)	H-5/9	C-4, C-7, C-8/6
7	158.7			
O-Me	55.4	3.77 (s)		C-7
N-Me	29.4	2.82 (s)		C-2, C-10
L-Pro				
10	173.2			
11	55.2	4.32 (dd, 8.2, 5.5)	H-12a, H-12b	C-12, C-13
12a	28.5	1.05 (m)	H-11, H-12b, H-13a, H-13b	C-10, C-11, C-13, C-14
12b		0.85 (m)	H-11, H-12a, H-13b	C-10, C-14
13a	25.4	2.08 (m)	H-12a, H-13b, H-14a, H-14b	C-11, C-12, C-14
13b		1.72 (m)	H-12a, H-12b, H-13a, H-14a, H-14b	C-11, C-12, C-14
14a	47.6[a]	3.52 (m)	H-13a, H-13b, H-14b	C-12, C-13
14b		3.60 (m)	H-13a, H-13b, H-14a	C-12, C-13
L-Pro				
15	169.8			
16	58.3	4.50 (dd, 8.6, 4.9)	H-17a, H-17b	C-15, C-17, C-18
17a	27.8	2.20 (m)	H-16, H-17b, H-18a, H-18b	C-16, C-18, C-19
17b		1.85 (m)	H-16, H-17a, H-18a, H-18b	C-18, C-19
18a	24.6	1.98 (m)	H-17a, H-17b, H-18b, H-19a, H-19b	C-16, C-17
18b		1.92 (m)	H-17a, H-17b, H-18a, H-19a, H-19b	C-16, C-17
19a	47.3[a]	3.57 (m)	H-18a, H-18b, H-19b	C-17, C-18
19b		3.64 (m)	H-18a, H-18b, H-19a	C-17, C-18
D-*allo*-Ile				
20	171.2			
21	54.6	4.64 (dd, 9.5, 7.3)	H-22, 21-NH	C-20, C-22, 22-Me, C-23, C-25
22	38.0	1.65 (m)	H-21, 22-Me, H-23a, H-23b, H-24	C-23, C-24
22-Me	14.4	0.86 (m)	H-22, H-23a	C-21, C-22, C-23
23a	26.1	1.38 (m)	H-22, 22-Me, H-23b, H-24	C-21, C-22, 22-Me, C-24
23b		1.12 (m)	H-22, H-23a, H-24	C-22, C-24
24	11.7	0.91 (m)	H-22, H-23a, H-23b	C-22
21-NH		6.66 (d, 9.5)	H-21	C-25
O-Ac-L-Thr				
25	168.3			
26	55.9	4.88 (dd, 10.1, 2.8)	H-27, 26-NH	C-25, C-29
27	71.9	5.58 (dq, 6.7, 2.8)	H-26, H-28	C-31
28	16.5	1.28 (d, 6.7)	H-27	C-26, C-27
29	171.1			
30	23.1	2.17 (s)		C-29
26-NH		7.17 (d, 10.1)	H-26	C-26, C-29
L-Val				
31	170.5			
32	58.9	4.39 (dd, 8.2, 6.1)	H-33, 32-NH	C-31, C-33, C-34, C-35
33	30.4	2.18 (m)	H-32, H-34, H-35	C-32, C-34, C-35
34	19.6	0.92 (m)	H-33, H-35	C-32, C-33, C-35
35	18.2	0.85 (m)	H-33, H-34	C-32, C-33, C-34
32-NH		8.24 (d, 8.2)	H-32	C-1, C-32

[a] Interchangeable. Note: ^{13}C NMR assignments are supported by a DEPT experiment.

multiplicity of the signal had not changed, these observations could be explained if **6** was identical to **2** with the single exception being that **6** incorporated an Ile rather than a Val amino acid residue. Marfey's analysis (including the use of chiral HPLC to differentiate Ile from *allo*-Ile), confirmed that Val had been replaced by an L-*allo*-Ile and supported the amino acid composition of aspergillicin E (**6**). Confirmation of the amino acid peptide sequence was achieved by mass spectral analysis (see below).

The amino acid sequences in aspergillicin B (**3**), C (**4**), D (**5**) and E (**6**) were initially assumed to be the same as that in aspergillicin A (**2**), by comparison of ^{1}H NMR data and on biosynthetic grounds. However, given the concerns raised above for aspergillicin A (**2**) we were particularly keen to secure an independent experimental assessment of this sequence for each of the aspergillicins.

Depsipeptides are well known in the field of marine natural products chemistry. Techniques for assigning peptide sequences in such compounds typically rely on either X-ray crystallography or NMR spectroscopy, and less frequently mass spectrometry. While X-ray crystallography is clearly limited by the availability of suitable crystals, and NMR spectroscopy can be constrained by the degree of dispersion (or not) of key resonances, mass spectrometry offers great potential. With soft

Table 2 Comparison of the ^{1}H NMR ($CDCl_3$, 400 MHz) data for aspergillicins B–E (**3**–**6**)

	^{1}H δ (m, *J*/Hz)			
No.	Aspergillicin B (**3**)	Aspergillicin C (**4**)	Aspergillicin D (**5**)	Aspergillicin E (**6**)
N-Me-L-Tyr-*O*-Me				
2	5.01 (dd, 11.3, 3.4)			4.97 (dd, 11.2, 3.6)
3a	3.16 (dd, 14.7, 3.4)			3.17 (dd, 14.6, 3.4)
3b	2.97 (dd, 14.7, 11.3)			2.98 (dd, 14.6, 11.4)
5,9	6.85 (d, 8.6)			6.84 (d, 8.8)
6,8	7.05 (d, 8.6)			7.03 (d, 8.8)
O-Me	3.78 (s)			3.78 (s)
N-Me	2.83 (s)			2.83 (s)
N-Me-L-Phe				
2		5.03 (dd, 11.3, 3.4)		
3a		3.22 (dd, 14.3, 3.4)		
3b		3.03 (dd, 14.3, 11.3)		
5,9		7.30 (m)		
6,8		7.32 (m)		
7		7.26 (m)		
N-Me		2.84 (s)		
L-Phe				
2			4.81 (m)	
3a			3.15 (dd, 13.7, 5.2)	
3b			2.97 (dd, 13.7, 11.6)	
5,9			7.15 (m)	
6,8			7.16 (m)	
7			7.12 (m)	
2-NH			5.99 (d, 9.8)	
L-Pro				
11	4.33 (dd, 7.9, 4.6)	4.27 (dd, 7.6, 5.5)	4.10 (t, 7.6)	4.30 (dd, 7.8, 5.0)
12a	1.04 (m)	1.08 (m)	1.06 (m)	1.0–1.8 (m)
12b	0.97 (m)	0.96 (m)	0.95 (m)	1.0–1.8 (m)
13a	2.11 (m)	2.05 (m)	2.07 (m)	1.8–2.3 (m)
13b	1.73 (m)	1.72 (m)	1.69 (m)	1.0–1.8 (m)
14a	3.68 (m)	3.65 (m)	3.67 (m)	3.6–3.7 (m)
14b	3.61 (m)	3.59 (m)	3.62 (m)	3.6–3.7 (m)
L-Pro				
16	4.52 (dd, 8.6, 4.9)	4.51 (dd, 8.6, 4.0)	4.48 (dd, 8.6, 4.0)	4.50 (dd, 8.6, 5.0)
17a	2.22 (m)	2.19 (m)	2.21 (m)	1.8–2.3 (m)
17b	1.87 (m)	1.88 (m)	1.84 (m)	1.8–2.3 (m)
18a	1.98 (m)	1.97 (m)	1.96 (m)	1.8–2.3 (m)
18b	1.94 (m)	1.94 (m)	1.92 (m)	1.8–2.3 (m)
19a	3.58 (m)	3.53 (m)	3.55 (m)	3.49 (m)
19b	3.52 (m)	3.47 (m)	3.50 (m)	3.49 (m)
D-*allo*-Ile				
21		4.64 (dd, 9.8, 7.6)	4.56 (m)	4.61 (dd, 9.6, 7.6)
22		1.68 (m)	1.70 (m)	1.0–1.8 (m)
22-Me		0.88 (m)	0.89 (m)	0.83–0.95 (m)
23a		1.39 (m)	1.35 (m)	1.0–1.8 (m)
23b		1.16 (m)	1.14 (m)	1.0–1.8 (m)
24		0.91 (m)	0.92 (m)	0.83–0.95 (m)
21-NH		6.65 (d, 9.8)	6.64 (d, 9.8)	6.61 (d, 9.6)
D-*nor*-Val				
21	4.56 (dd, 9.8, 8.9)			
22a	1.84 (m)			
22b	1.68 (m)			
23a	1.43 (m)			
23b	1.32 (m)			
24	0.93 (t, 6.7)			
21-NH	6.65 (d, 9.8)			
O-Ac-L-Thr				
26	4.88 (dd, 10.1, 2.8)	4.88 (dd, 10.1, 2.8)	4.87 (dd, 10.1, 2.8)	4.86 (dd, 10.0, 2.4)
27	5.58 (dq, 6.7, 2.8)	5.57 (dq, 6.7, 2.8)	5.45 (dq, 6.7, 2.8)	5.60 (dq, 6.5, 2.4)
28	1.29 (d, 6.7)	1.29 (d, 6.7)	1.23 (d, 6.7)	1.28 (d, 6.4)
30	2.17 (s)	2.18 (s)	2.15 (s)	2.16 (s)
26-NH	See note	7.08 (d, 10.1)	6.98 (d, 10.1)	6.81 (d, 10.0)

Table 2 (*Cont.*)

No.	^{1}H δ (m, *J*/Hz) Aspergillicin B (**3**)	Aspergillicin C (**4**)	Aspergillicin D (**5**)	Aspergillicin E (**6**)
L-Val				
32	4.40 (dd, 7.9, 5.8)	4.40 (dd, 8.2, 5.8)	4.46 (dd, 7.9, 5.2)	
33	2.21 (m)	2.20 (m)	2.23 (m)	
34	0.92 (d, 7.0)	0.98 (d, 6.7)	0.92 (d, 7.0)	
35	0.87 (d, 7.0)	0.87 (d, 6.7)	0.82 (d, 7.0)	
32-NH	8.23 (d, 7.9)	8.26 (d, 8.2)	8.27 (d, 7.9)	
L-*allo*-Ile				
32				4.68 (dd, 9.2, 4.8)
33				1.8–2.3 (m)
34				1.0–1.8 (m)
35				0.83–0.95 (m)
32-NH				8.16 (d, 9.2)
34-Me				0.83–0.95 (m)

Note: obscured by H-5, 9.

Table 3 MS^n data for aspergillicins A–E (**2**–**5**)

	2		**3**		**4**		**5**		**6**	
MS^n	[M + Na]$^+$ *m*/*z* 763		[M + Na]$^+$ *m*/*z* 749		[M + Na]$^+$ *m*/*z* 733		[M + Na]$^+$ *m*/*z* 719		[M + Na]$^+$ *m*/*z* 777	
n	Mass loss	Residue	Mass loss	Residue	Mass loss	Residue	Mass loss	Residue	Mass loss	Residue
2	99[a]	Val	99[b]	Val	99[c]	Val	99[d]	Val	113[e]	Ile
3	191	*N*-Me-*O*-Me-Tyr	191	*N*-Me-*O*-Me-Tyr	161	*N*-Me-Phe	147	Phe	191	*N*-Me-*O*-Me-Tyr
4	97	Pro	97	Pro	97	Pro	97	Pro	97	Pro
5	97	Pro	97	Pro	97	Pro	97	Pro	97	Pro
6	113	Ile	99	*nor*-Val	113	Ile	113	Ile[(g)]	113	Ile

Note: Mass losses of *m*/*z* 99 and 113 cannot by themselves distinguish between Val or *nor*-Val, and Ile and Leu. In all cases after MS^6 the dominant ion observed was *m*/*z* 166 for *N*-Ac-Thr. Other ions observed:[a] *m*/*z* 719, 646; [b] *m*/*z* 705, 632; [c] *m*/*z* 689, 616; [d] *m*/*z* 675; and [e] *m*/*z* 733, 646.

ionisation techniques (ESI, APCI) and ion traps, it is possible to select molecular ions (even from mixtures) and monitor collision induced dissociation (CID) to diagnostic fragment ions. Specifically in the case of depsipeptides, Gross has reported[10] a method for peptide sequencing based on the initial selective ring-opening of the lactone during collision induced dissociation of sodiated molecular ions. Using this methodology the aspergillicins (**2**–**6**) were subjected to a series of MS^{n}CID experiments using an ion-trap mass spectrometer to confirm the peptide sequence, the results of which are given in Table 3. In all cases, the initial MS/MS spectrum yielded a b_5* ion[11] with sufficient intensity to fully sequence the depsipeptide. In the case of aspergillicin D the b_5* ion was the major ion observed prior to CID, whereas for all other aspergillicins the major ion was [b_5* − H_2O]. The only other significant ions observed were due to loss of 44 amu. MS^n studies of these gave the same peptide sequence, suggesting that the loss arises from elimination of CO_2. It was satisfying to note that the aspergillicins A–E (**2**–**6**) sequence data determined by MS^{n}CID experiments was in full agreement with that established by 2D NMR.

In summary, the analyses and arguments presented above allowed for the assignment of complete stereostructures to the aspergillicins, even in the case of aspergillicins C (**4**) and D (**5**) which were isolated as an inseparable mixture, and aspergillicin E (**6**) which was only available in sub milligram quantities. The use of MS^n ion trap experiments was particularly effective in achieving this outcome.

With regards to biological activity it is worthwhile noting that the original goal for this investigation was the discovery of a new antiparasitic agent(s). In this respect marcfortine A (**1**) is an exceptionally potent *in vitro* nematocidal agent (LD_{99} 0.06 μg mL^{-1}), which displays a noteworthy paralytic mode of action. Although known from terrestrial sources, this is the first account of marcfortine A (**1**) from a marine-derived fungus and the first account of its ability to paralyse nematodes *in vitro*.

As regards new chemistry, the aspergillicins are new depsipeptides some of which incorporate the unusual *N*-Me-L-Tyr-*O*-Me residue. This residue is rare among marine natural products but is found in the didemnins, anticancer cyclic peptides obtained from marine tunicates.[12,13] Although the aspergillicins do not exhibit antiparasitic activity against *H. contortus* they do exhibit modest cytotoxic activity (LD_{99} 25–50 μg mL^{-1}). A more detailed evaluation of the biological potential of the aspergillicins is currently under review.

Finally, in light of the discussion presented in this report it is useful to consider whether our marine derived *A. carneus* is unique, or merely a reisolation of an otherwise *known* terrestrial microbe. In an attempt to address this issue we compared the marine isolate with a selection of commercially available cultures of *A. carneus*. Notably, our marine isolate can be compared with MST-FP1367 (NRRL 1926, Fig. 2), a strain classified by Raper who proposed[14] the original description of *A. carneus.* MST-FP1367 exhibits the typical white mycelial growth with pink to pale red pigmentation in the edges of growing mycelia. HPLC analysis of MST-FP1367 extracts (with ELSD and PDA detection) revealed citrinin and related metabolites, as previously reported.[15] These gross morphologies and metabolite profiles provide a generic measure of the defining characteristics of *A. carneus*. In contrast to MST-FP1367, MST-MF156 exhibits a uniform brown mycelia with extracts devoid of citrinin or related metabolites. These observations, together with similar comparisons to other commercial strains of *A. carneus*, suggest that while *A. carneus* may inhabit both marine and terrestrial environs, significant strain divergence has emerged. A particularly noteworthy outcome of this divergence is the variation in metabolite profiles encountered in marine *vs.*

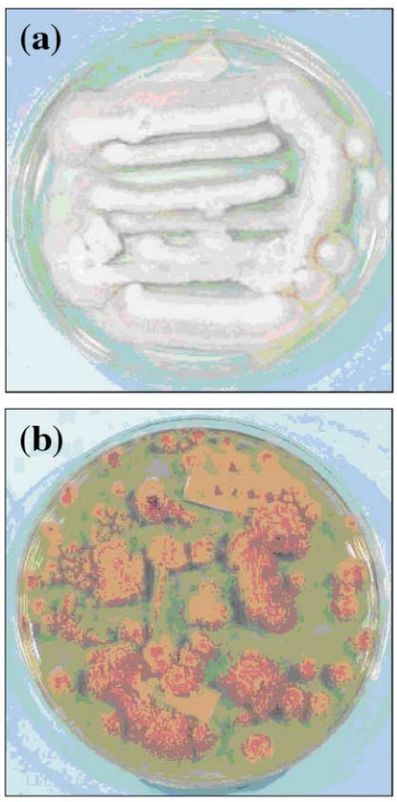

Fig. 2 Image of an agar plate culture of *Aspergillus carneus* (a) type strain (MST-FP1367), (b) marine strain (MST-MF156).

terrestrial strains. Quite apart from chemotaxonomic significance, such variation brings with it the potential for discovering novel and useful bioactive natural products.

Experimental

General procedures

Size exclusion chromatography was performed using sealed columns connected to an ISCO Retriever 500 fraction collector, an ISCO Tris peristaltic pump and ISCO UA 60 UV detector. The columns were packed with either Sephadex LH-20 or Sephadex G-10 eluted with methanol and water respectively. High-performance liquid chromatography (HPLC) was performed using either a Waters 600 solvent delivery system equipped with a Waters 2700 sample manager and Waters 996 photodiode array detector, or a Waters 2790 separations module equipped with a Waters 996 photodiode array detector, Alltech 500 evaporative light scattering detector with low temperature adapter, and Waters Fraction Collector II. Both systems operated under PC control running Waters Millennium[32] software. Chiroptical measurements ($[\alpha]_D$) were obtained on a Jasco Dip-1000 digital polarimeter in a 100 × 3 mm cell. Ultraviolet (UV) absorption spectra were obtained using a Hitachi Model 150–20 double beam spectrophotometer, while infrared (IR) spectra were acquired using a Bio-rad FTS 165 FT-IR spectrometer under PC control running Bio-rad Win-IR software. ^{1}H and ^{13}C NMR spectra were performed on either a Varian Inova 400, Varian Unity 300, or Varian Unity 400 plus spectrometer, in the solvents indicated and referenced to residual ^{1}H signals in the deuterated solvents. ESIMS were acquired using a Waters 2790 separations module equipped with a Micromass ZMD mass detector. High-resolution ESIMS measurements were obtained on a Bruker BioApex 47E FT mass spectrometer at a cone voltage of 100 kV. Nematocidal screening was performed using published procedures.[16] Authentic amino acids were sourced commercially from Sigma-Aldrich except for *N*-methylated amino acids which were obtained from Bachem.

Multistage mass spectrometry experiments

All multistage mass spectrometry (MS^n) experiments were carried out on a Finnigan model LCQ (San Jose, CA) quadrupole ion trap mass spectrometer. Each of the aspergillicin samples were dissolved in MeOH at a concentration of 0.1 mg mL^{-1} and introduced to the electrospray ionization (ESI) source of the mass spectrometer *via* a Harvard syringe drive operating at a flow rate of 3 μL min^{-1}. The spray voltage was set at −5 kV and nitrogen sheath gas was obtained from a boiling liquid nitrogen source supplied at 30 psi. The heated capillary temperature was 200 °C and the capillary and tube lens voltages were set at 15 and 0 V respectively. Under these conditions, the dominant ionic species for all the aspergillicins corresponded to the sodiated ions (*i.e.* $[M + Na]^+$). In order to assign the sequences of these depsipeptides, we follow the methodology of Gross, whereby the pseudomolecular ion (*i.e.* $[M + Na]^+$) is mass selected and allowed to undergo collision induced dissociation (CID) with the helium bath gas.[17,18] Any product ion(s) corresponding to an amino acid residue mass (*i.e.* $[M + Na - XNCHRCO]^+$) was then mass selected in a MS^n experiment and subjected to a further stage of CID, resulting in the assignment of the second residue mass. Thus the amino acid sequence was "read out" *via* sequential amino acid residue losses. All MS^n experiments were performed on mass selected ions in the quadrupole ion trap mass spectrometer using the advanced software. Typically, collision induced dissociation (CID) of $[M + Na]^+$ ions and their b_n^+ fragment ions were: (i) mass selected with an isolation width of 2.5 Th for the $[M + Na]^+$ ions and 5 Th for the b_n^+ fragment ions; (ii) activation amplitude, 0.9–1.35 V; (iii) activation (Q), 0.25 V, and (iv) activation time 30 ms. All CID spectra were the average of 20 scans.

Collection, isolation and fermentation

The fungus *Aspergillus carneus* (MST-MF156) was isolated from an estuarine sediment sample collected under the Jordan River Bridge in Tasmania, Australia. The fungus was grown on potato dextrose agar for 168 h at 28 °C prior to extraction.

Extraction and isolation

After harvesting, the mycelium and agar were repeatedly extracted with 100% MeOH. The dried combined extract (7.0 g) was again treated with MeOH (255 mL) and left to stir overnight. The resulting suspension was filtered into MeOH soluble and insoluble portions (2.97 g; 42.3%), with the MeOH soluble fraction (filtrate) being concentrated *in vacuo* to yield a dark oil (4.05 g; 50.1%). The MeOH soluble portion was further partitioned between *n*-BuOH (125 mL) and H_2O (125 mL). The *n*-BuOH soluble material (0.48 g; 6.9%) was defatted by addition of hexane (2 × 10 mL), and the polar fraction treated with CH_2Cl_2 (10 mL) to afford a suspension, which was centrifuged in order to separate the supernatant from the insoluble residue. The supernatant was removed and the residue re-treated with additional CH_2Cl_2 (10 mL) and centrifuged. The resulting supernatants were combined and concentrated *in vacuo* to yield 243.7 mg of the CH_2Cl_2 soluble material, which was subsequently submitted to Sephadex LH-20 (MeOH) to yield 100 fractions. Fractions 23–27 (18.3 mg; 0.26%) were combined based on the ESI(+)MS data and submitted to C_{18} HPLC (2 mL min^{-1} isocratic elution with 32.5% H_2O–MeOH through a 5 μm Phenomenex C_{18} (2) 250 × 10 mm column heated to 30 °C) to yield five new depsipeptides (see Fig. 1), aspergillicin A (**2**) (5.5 mg, 0.09%), aspergillicin B (**3**) (1.7 mg, 0.028%) and a mixture of aspergillicins C (**4**), D (**5**) (3.2 mg, 0.052%) and E (**6**) (0.8 mg, 0.013%). Note that percentage yields are calculated against the total mass of crude extract.

In a separate isolation run the Sephadex fractions 34–38 (97.9 mg, 1.13%) were combined based on their antiparasitic and ESI(+)MS characteristics then subjected to silica SPE with

elution by CH_2Cl_2 with increasing concentrations of MeOH. The fractions eluted by 1–2% MeOH in CH_2Cl_2 (12.6 mg, 0.14%) were subjected to silica HPLC (1 mL min^{-1} gradient CH_2Cl_2/MeOH elution through a 5 μm Zorbax Rx-SIL 150 × 4.6 mm column) to yield the known fungal metabolite marcfortine A (**1**) (4.5 mg, 0.05%). Fractions 39–40 were combined based on the ESI(+)MS data then subjected to C_{18} HPLC (2 mL min^{-1} gradient water/MeOH elution through a 5 μm Phenomenex Luna C_{18} (2) 250 × 10 mm column) to yield the known fungal metabolite acyl aszonalenin (**7**) (7.3 mg, 0.08%).

Marcfortine A (1). Identified by spectroscopic analysis (ESI(±)MS, HRESI(±)MS, UV, $[\alpha]_D$, ^1H and ^{13}C, COSY, gHMQC, gHMBC NMR) and comparison with literature data.[2,3]

Acyl aszonalenin (7). Identified by spectroscopic analysis (ESI(±)MS, HRESI(±)MS, UV, $[\alpha]_D$, ^1H and ^{13}C, COSY, gHMQC, gHMBC NMR) and comparison with literature data.[4,5]

Aspergillicin A (2). Colourless solid; $[\alpha]_D^{20}$ −51.9° (*c* 0.03, MeOH); IR (CCl_4 solution) ν_{max}/cm^{-1} 3443 (br), 3192–3015 (br), 1550, 1253, 1006; UV (MeOH) λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 212 (9870), 227 (7530), 277 (990), 283 (870); ^1H NMR data ($CDCl_3$, 400 MHz) see Table 1; ^{13}C NMR data ($CDCl_3$, 100 MHz) see Table 1; ESI(+)MS (20 kV) *m*/*z* 763 (M + Na); HRESI(+)MS *m*/*z* 763.3986 (M + Na, $C_{38}H_{56}N_6O_9Na$ requires 763.4006).

Aspergillicin B (3). Colourless solid; $[\alpha]_D^{20}$ −46.6° (*c* 0.025, MeOH); IR (CCl_4 solution) ν_{max}/cm^{-1} 3351 (br), 2976, 2895, 1550–1220, 1050, 891; UV (MeOH) λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 210 (9800), 225 (7500), 277 (980), 283 (880); ESI(+)MS (20 kV) *m*/*z* 749 (M + Na); HRESI(+)MS *m*/*z* 749.3837 (M + Na, $C_{37}H_{54}N_6O_9Na$ requires 749.3850); ^1H NMR data ($CDCl_3$, 400 MHz) see Table 2.

Mixture of aspergillicins C (4) and D (5). Colourless solid; IR (CCl_4 solution) ν_{max}/cm^{-1} 3448 (br), 3198–3020 (br), 1551, 1253, 1006; UV (MeOH) λ_{max}/nm (ε/dm^3 mol^{-1} cm^{-1}) 212 (23020), 258 (1030), 264 (880), 283 (700); ESI(+)MS (20 kV) (**4**) *m*/*z* 733 (M + Na); HRESI(+)MS (**4**) *m*/*z* 733.3897 (M + Na, $C_{37}H_{54}N_6O_8Na$ requires 733.3900); ESI(+)MS (20 kV) (**5**) *m*/*z* 719 (M + Na); HRESI(+)MS (**5**) *m*/*z* 719.3740 (M + Na, $C_{36}H_{52}N_6O_8Na$ requires 719.3744); ^1H NMR data ($CDCl_3$, 400 MHz) see Table 2.

Aspergillicin E (6). colourless solid; ESI(+)MS (30 kV) *m*/*z* 777 (M + Na); HRESI(+)MS *m*/*z* 777.4163 (M + Na, $C_{39}H_{58}N_6O_9Na$ requires 777.4163); ^1H NMR data ($CDCl_3$, 400 MHz) see Table 2.

Base hydrolysis. Aspergillicin A (**2**) (2.0 mg) was dissolved in MeOH (2.0 mL), treated with concentrated ammonia solution (1.5 mL) and kept at room temperature for 50 h. Excess ammonia was dispelled by bubbling N_2 through the solution which was then carefully evaporated to avoid further hydrolysis. The residue was taken up in water (2 mL), extracted with EtOAc (3 × 2 mL) and the extract dried (Na_2SO_4) and evaporated to give the crude hydrolysis product (1.4 mg). ESI(+)MS (30 kV) *m*/*z* 781 (M + H_2O + Na); ESI(−)MS (40 kV) *m*/*z* 757 (M + H_2O − H).

Acid hydrolysis. To separate samples (100 μg) of aspergillicin A (**2**), aspergillicin B (**3**), aspergillicin E (**6**), and a mixture of aspergillicins C (**4**) and D (**5**), was added 6 M HCl (200 μL), and the resulting solutions stirred at 110 °C for 24 h. At this time the reaction mixtures were dried *in vacuo* and redissolved in water (50 μL). These acid hydrolysates were then subjected to ESI(±)MS analysis, followed by derivatisation with Marfey's reagent, and HPLC analysis.

ESIMS analysis of the acid hydrolysates. Individual acid hydrolysates obtained from aspergillicin A (**2**), aspergillicin B (**3**) and the mixture of aspergillicins C (**4**) and D (**5**), were submitted to ESI(±)MS analysis using direct infusion into a Micromass ZMD mass detector. Details are reported in the Results and discussion.

Marfey's analysis (refs. 6 and 7). To 50 μL of each acid hydrolysate (or authentic amino acid standard at comparable concentration) was added 1 M sodium bicarbonate (20 μL) and 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA) in acetone (100 μL). The solutions were stirred at 37 °C for 60 min, after which the mixtures were neutralised with 1 M HCl (20 μL) and the derivatised samples diluted with MeCN (810 μL). HPLC analysis involved elution of 5 μL of the derivatised solutions through a Phenomenex Luna C_{18}(2) 4.6 × 150 mm column, with a 1 mL min^{-1} 45 min linear gradient of 15–45% MeCN in solvent A (solvent A: 0.1 M aqueous ammonium acetate solution to pH 3 with the addition of TFA) with UV detection at 340 nm. Resolution of Thr and *allo*-Thr was achieved on the same HPLC column using a 1 mL min^{-1} 60 min linear gradient of 15–90% MeOH in solvent A. Resolution of Ile from *allo*-Ile required elution through a Phenomenex Chirex (urea type 3010) 3.2 × 50 mm column, with a 0.2 mL min^{-1} 50 min linear gradient of 20–75% MeCN.

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