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Aspergillamides A and B: Modified Cytotoxic Tripeptides Produced by a Marine Fungus of the Genus *Aspergillus*.

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Abstract: Two isomeric linear peptides, aspergillamides A and B (**1, 2**), were isolated from the mycelium of a cultured marine fungus of the genus *Aspergillus*. The producing strain (designated CNC-120), was obtained from a saline lake sediment sample collected from Acklins Island, the Bahamas. The structures of the new peptides were elucidated using comprehensive 2D NMR methods. At 25°C, in both acetone and dimethylsulfoxide, aspergillamide A exists as a 1:1 mixture of *trans*- and *cis*-amide rotational isomers (**1a** and **1b**). Under identical conditions, aspergillamide B is predominantly in the *cis*-amide form. The absolute stereochemistries of the amino acids in aspergillamide A were assigned as L by hydrolysis and comparison with commercial standards. Aspergillamide A showed modest *in vitro* cytotoxicity [IC_{50} = 16 μg/ml) toward the human colon carcinoma cell line HCT-116.

Keywords: Antitumor compounds; Marine metabolites; Microorganisms; Natural products; Peptides

In recent years, chemical studies of culturable marine microorganisms have led to the discovery of numerous, structurally novel, biologically active secondary metabolites.[1,2,3,4] Although most of these compounds were isolated marine bacteria, a growing number of new structures have been reported from filamentous marine fungi. The fungi producing these novel compounds have been collected from diverse marine sources including fish[5], algae[6,7], invertebrates[8], and sediments.[9] Although the distributions and ecological roles of marine fungi are not well understood, these reports suggest that fungi from diverse marine substrates are a good source of new secondary metabolites. As part of our broad interest in the ecology and secondary metabolite chemistry of marine fungi, we have been investigating isolates from marine sediments, including sediments from shallow-water marine lakes, where environmental pressures are very different from those in the open ocean. This paper describes the isolation, structure elucidation, and biological activity of aspergillamides A and B, two new peptides produced by an *Aspergillus* sp. isolated from a sediment sample collected from a saline lake on Acklins Island, the Bahamas. Aspergillamide A displayed modest *in vitro* cytotoxicity against the HCT-116 human colon tumor cell line.

The producing fungus (strain CNC-120), identified as an *Aspergillus* sp., was grown in static culture in a seawater-based fermentation medium. Following fermentation, the mycelium was collected by filtration, freeze-dried, and extracted with 1:1 dichloromethane:methanol yielding a brown, cytotoxic solid. The active material was separated using reversed-phase (C-18) column chromatography and the fractions containing impure

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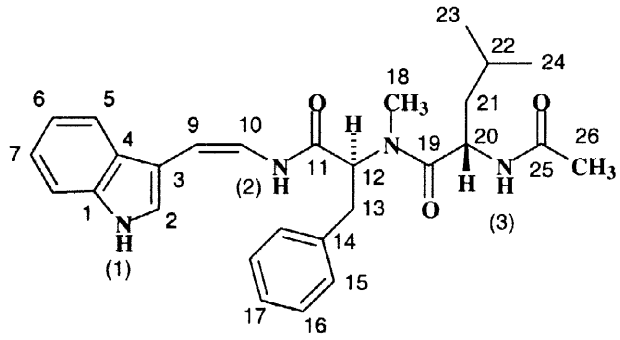
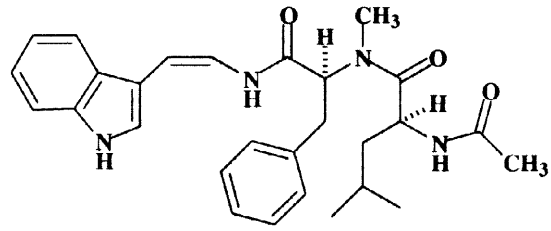
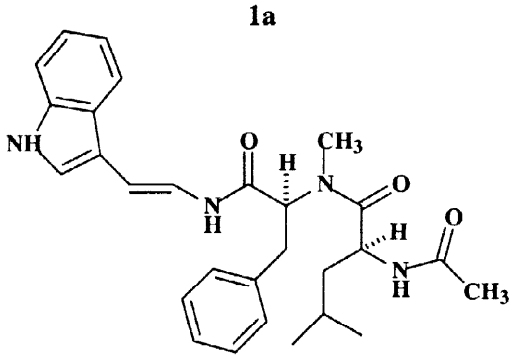
aspergillamides combined. Final purification using reversed-phase HPLC (C-18) yielded aspergillamide A (**1**, 39.1 mg) and aspergillamide B (**2**, 3 mg). The previously reported[10], but poorly defined metabolite *l*-phenylalaninamide (**3**) (12 mg), was also isolated from the mycelium extract. The peptides could not be detected in the ethyl acetate extract of the culture broth.



1a



1b



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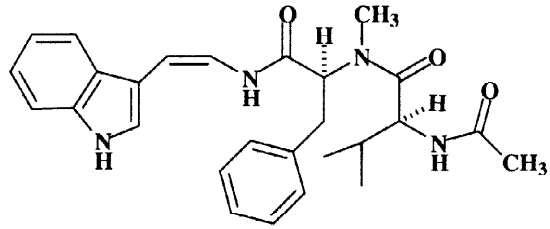

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Aspergillamides A and B (**1**, **2**), obtained as single peaks during HPLC separation, were isolated as amorphous white solids. Aspergillamides A and B were isomeric, as each was analyzed for $C_{28}H_{34}N_4O_3$ by HRFABMS methods. The NMR data for **1**, however, were much more complex than expected. The ^1H NMR spectrum showed highly complex overlapping signals, and the ^{13}C spectrum indicated more than 50 resolved signals. The ^{13}C spectrum of aspergillamide B, however, showed the same fundamental pattern as aspergillamide A but showed the 28 carbon signals expected based on the mass spectral data. The ^1H NMR spectrum of aspergillamide B was much less complex and integrations were consistent with the formula. The UV spectrum of aspergillamide A showed absorption maxima at 286, 221, and 200 nm indicating an aromatic chromophore or extended conjugation.[11] Broad IR absorptions at 3285 and 1630 cm^{-1} indicated the presence of an amine or amide NH and an amide carbonyl, respectively. Since these data indicated that the aspergillamides were small peptides, we interpreted the complex NMR spectra from aspergillamide A to indicate that this compound existed as a mixture of amide rotational isomers. It was also determined that the relatively simple NMR spectra obtained for aspergillamide B were due to this compound existing predominately in one conformation.

The structure assignment of aspergillamide A involved comprehensive 2D NMR analysis of each of the amide conformers (Tables I and II). Spectral data obtained at room temperature in acetone or dimethylsulfoxide indicated that aspergillamide A existed as a 1:1 mixture of *trans*- and *cis*-amides. Proton NMR COSY experiments illustrated a set of phenylalanine spin systems with α-proton signals at δ 5.38 for the *trans*-amide (**1a**) and δ 5.21 for the *cis*-amide (**1b**). Likewise, a pair of leucine signals were assigned with the corresponding α-protons at δ 4.91 for **1a** and δ 4.46 for **1b**. The methylene protons at C-13 in the phenylalanine unit of **1b** exhibited a unique diastereotopic relationship with proton assignments of δ 1.38 and δ 0.15. Other interesting features of the ^{1}H NMR spectrum were a very complex aromatic region due to the indole and phenyl rings, along with three exchangeable NH protons dispersed between 8 and 10 ppm.

The ^{13}C spectrum of aspergillamide A showed complex doubling for all carbon assignments except for C-1, C-5, and C-6 which were not resolved at 50 MHz. At 125 MHz, all carbons were assigned for **1a** and **1b** using a combination of HMQC and HMBC analyses. The HMBC data, recorded in acetone-d_6, clearly defined the phenylalanine and leucine units, while the dehydrotryptamine unit was secured using HMBC data in both acetone-d_6 and dimethylsulfoxide-d_6 (Tables I and II). In dimethylsulfoxide-d_6 , the indole nitrogen proton and the amide protons showed additional HMBC correlations not observed in acetone-d_6. The sequence of the peptide units, and confirmation of the dehydrotryptamine functionality, were also established by HMBC data. For example, the olefin proton at C-10 showed a 2-bond correlation to C-11, the α-proton at C-12 showed correlations to C-11 and C-19, and the α-proton at C-20 correlated to C-19 and C-25. Support for the 3-substituted indole were found in correlations from the proton at C-10 to C-3 and from the proton at C-9 to C-2 and C-4. Further evidence for the sequence were 3-bond correlations for the protons at C-13 to C-11, the N(CH3) at C-18 showed to C-19, and the diastereotopic protons of C-21 both correlated to C-19. The stereochemistry of the dehydrotryptamine double bond at C-9 - C-10 was assigned as *Z* on the basis of the observed *cis*-coupling constants (J = 10.0 Hz for **1b** and J =9.5 Hz for **1a**). These results were also confirmed by nOe experiments.

The assignments of **1a** and **1b** for the *trans*- and *cis*-rotational isomers were accomplished using one dimensional difference nOe experiments (Figure 1). The *trans*-amide isomer was assigned as **1a** based on the well known N-(Me) and α-proton nOe interaction observed for the *trans*-amide relationship.[11] Irradiation of the N(Me) group at δ 3.05 (C-18) produced a significant nOe of the δ 4.91 α-proton signal at C-20 confirming the *trans*-amide conformation. In a similar fashion, nOe experiments firmly established **1b** as the *cis*-amide. Irradiation of the C-20 proton at δ 4.46 produced a strong enhancement of the C-12 proton at δ 5.38. Securing these clear reference points allowed all subsequent carbon and proton signals to be confidently assigned (Tables I and II).

The equilibrium between the *cis*- and *trans*-amide rotamers was investigated by ^{1}H NMR experiments at several temperatures. The equilibrium was 1:1 at ambient NMR temperature, but was modified to a 3:2 ratio favoring the *cis*-isomer **1b** at 5°C. Continued cooling to -15°C converted this ratio to 2:1 favoring **1b**. This same ratio was observed with cooling to -65°C. Heating the mixture from NMR ambient to 40°C modified the equilibrium to 1.2:1 favoring the *trans*-amide **1a** and increasing the temperature to 90°C modified the ratio to 2:1 in favor of **1a**. These data clearly showed the *cis*-isomer to be the more stable conformation of aspergillamide A.

Table I. NMR Data for Aspergillamide A (1a, *Trans* Isomer)

carbon #	^{13}C[a]	DEPT[b]	^{1}H[c]	COSY[c]	HMBC[d]
1	136.5	C			
2	123.2	CH	7.66 (s,1H)		136.5, 127.4, 110.7
3	110.7	C			
4	127.4	C			
5	118.8	CH	7.60 (m, 1H)	7.13, 7.08	136.5, 122.1
6	119.1	CH	7.08 (m, 1H)	7.40, 7.13	127.4, 111.7, 122.1
7	122.1	CH	7.13 (m, 1H)	7.40, 7.08	136.5
8	111.7	CH	7.40 (m, 1H)	7.08	127.4, 119.1
9	102.6	CH	5.95 (d, J = 9.5 Hz,1H)	6.80	127.4, 123.3, 119.7
10	119.7	CH	6.80 (m, 1H)	9.20, 5.95	167.5, 110.7, 102.6
11	167.5	C			
12	60.1	CH	5.21 (dd, J= 7.0, 6.5Hz, 1H)	3.36, 3.17	174.3, 167.5, 138.1, 33.5, 32.2
13	33.5	CH_2	3.36 (m, 1H), 3.17[e] (m, 1H)	5.21	167.5, 138.1, 129.5, 60.1, 32.2[e]
14	138.1	C			
15	129.5 (x2)	CH	7.27 (s, 2H)[g]	7.29	138.1, 128.8, 126.8, 33.5
16	128.8 (x2)	CH	7.29 (s, 2H)[g]	7.27	138.1, 129.5, 126.8
17	126.8	CH	7.25 (m, 1H)		128.8
18	32.2	CH_3	3.05 (s, 3H)		174.3, 60.1
19	174.3	C			
20	47.7	CH	4.91 (m, 1H)	7.05, 1.40. 1.25	174.3, 169.2, 41.5, 24.7
21	41.5	CH_2	1.40 (m, 1H), 1.25 (m, 1H)	4.91, 1.55	174.3, 47.7, 24.7, 22.6, 21.0
22	24.7	CH	1.55 (m, 1H)	1.40, 1.25, 0.82, 0.77	
23	22.6	CH_3	0.77 (d, J = 7Hz, 3H)	1.55	41.5, 24.7, 21.0
24	21.0	CH_3	0.82 (d, J = 7Hz, 3H)	1.55	41.5, 24.7, 22.6
25	169.2	C			
26	22.1	CH_3	1.84 (s, 3H)		169.2
NH(1)			10.4 (s, 1H)		C1(135.8)[f], C3(109.5)[f], C4(126.5)[f]
NH(2)			9.20 (d, J = 9.50 Hz, 1H)	6.80	C11(168.6)[f], C10(118.4)[f],C9(103.5)[f]
NH(3)			7.05 (d, J = 8.0 Hz, 1H)	4.91	C25(168.8)[f]

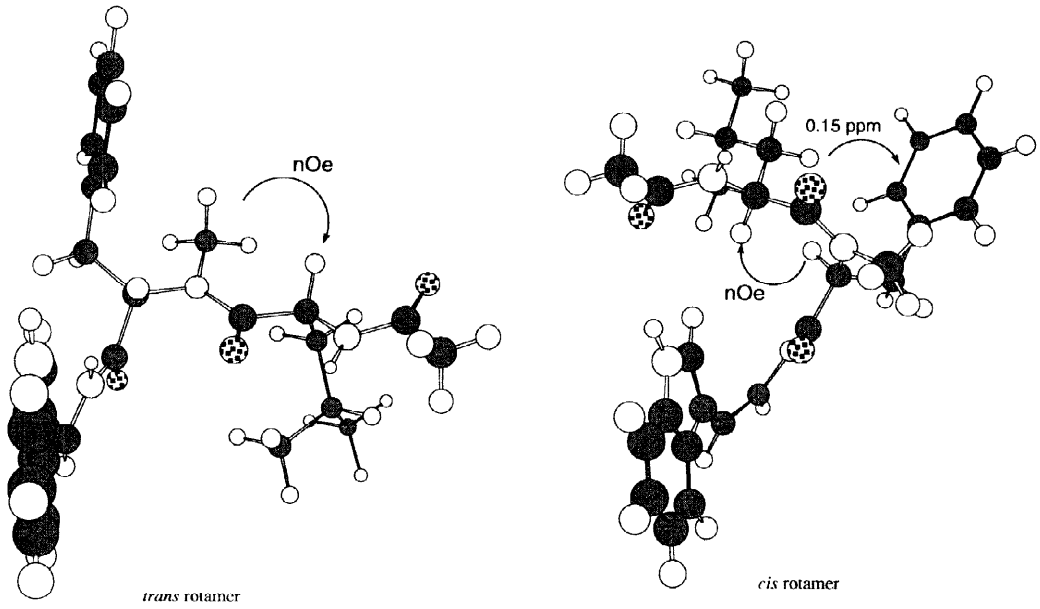
a) Recorded at 50 MHz in acetone-d_6 (assignments by the HMQC method at 500 MHz in acetone-d_6). b) Recorded at 200 MHz in acetone-d_6.
c) Recorded at 500 MHz in acetone-d_6. d) Recorded at 500 MHz in acetone-d_6 with an effective J value of 8 Hz. e) Unusual four-bond HMBC correlation
f) Recorded at 500 MHz in DMSO-d_6 with an effective J value of 8 Hz. g) Because of their chemical shifts these protons are virtually coupled and hence appear as singlets

Table II. NMR Data for Aspergillamide A (1b, *Cis* Isomer)

carbon #	^{13}C[a]	DEPT[b]	^{1}H[c]	COSY[c]	HMBC[d]
1	136.5	C			
2	124.4	CH	7.68 (s,1H)		136.5, 127.5, 110.8
3	110.8	C			
4	127.5	C			
5	118.8	CH	7.59 (m, 1H)	7.16, 7.06	136.5, 122.4
6	119.1	CH	7.06 (m, 1H)	7.45, 7.16	127.5, 111.8, 122.4
7	122.4	CH	7.16 (m, 1H)	7.45, 7.06	136.5, 122.4
8	111.8	CH	7.45 (m, 1H)	7.06	127.5, 119.1
9	104.3	CH	6.02 (d, J = 10.0 Hz,1H)	6.80	127.5, 124.4, 119.8
10	119.8	CH	6.80 (m, 1H)	8.45, 6.02	167.9, 110.8, 104.3
11	167.9	C			
12	62.0	CH	5.38 (dd, J= 10.5, 4 Hz, 1H)	3.32, 3.08	173.4, 167.9, 138.5, 35.1, 28.9
13	35.1	CH_2	3.32 (m, 1H), 3.08[e] (m, 1H)	5.38	167.9, 138.5, 130.1, 62.0, 28.9[e]
14	138.5	C			
15	130.1 (x2)	CH	7.34 (s, 2H)[g]	7.35	138.5, 129.2, 127.0, 35.1
16	129.2 (x2)	CH	7.35 (s, 2H)[g]	7.34	138.5, 130.1, 127.0
17	127.0	CH	7.20 (m, 1H)		
18	28.9	CH_3	2.95 (s, 3H)		173.4, 62.0
19	173.4	C			
20	47.0	CH	4.46 (m, 1H)	7.48, 1.38, 0.15	173.4, 171.3, 38.8, 23.9
21	38.8	CH_2	1.38 (m, 1H), 0.15 (m, 1H)	4.46, 1.45	173.4, 47.0, 23.9, 22.6, 20.1
22	23.9	CH	1.45 (m, 1H)	1.38, 0.15, 0.62, 0.69	
23	22.6	CH_3	0.69 (d, J = 6.5 Hz, 3H)	1.45	38.8, 23.9, 20.1
24	20.1	CH_3	0.62 (d, J = 6.5 Hz, 3H)	1.45	38.8, 23.9, 22.6
25	171.3	C			
26	20.9	CH_3	1.52 (s, 3H)		171.3
NH(1)			10.4 (s, 1H)		C1(135.8)[f], C3(109.6)[f], C4(126.7)[f]
NH(2)			8.45 (d, J = 11.0 Hz, 1H)	6.80	C11(167.8)f
NH(3)			7.48 (d, J = 10.0 Hz, 1H)	4.46	C25(170.4)[f]

a) Recorded at 50 MHz in acetone-d_6 (assignments by the HMQC method at 500 MHz in acetone-d_6). b) Recorded at 200 MHz in acetone-d_6.
c) Recorded at 500 MHz in acetone-d_6. d) Recorded at 500 MHz in acetone-d_6 with an effective J value of 8 Hz. e) Unusual four-bond HMBC correlation.
f) Recorded at 500 MHz in DMSO-d_6 with an effective J value of 8 Hz. g) Because of their chemical shifts these protons are virtually coupled and hence appear as singlets

Figure 1. Solution Conformations of Aspergillamide A.



Aspergillamide B (**2**) was obtained in low yield (0.03%) from the mycelium crude extract. Subsequently, it was observed that aspergillamide A undergoes a room light photochemical olefin isomerization generating aspergillamide B. This conversion was complete after 6 days of room light irradiation in an NMR tube (in acetone-d_6). Evidence for the *E* configuration of the C-9 - C-10 double bond in aspergillamide B (**2**) was the coupling constant, J = 14.5 Hz, measured for the two olefinic protons. Interestingly, aspergillamide B exists in solution mainly in the *cis*-amide form. This assignment was clear because the ^{1}H NMR spectrum featured the same strongly shielded leucine proton (δ -0.10) that was seen in the *cis*-amide isomer of aspergillamide A (**1b**). Because of the facile photochemical conversion, it is possible that aspergillamide B is produced during the fermentation or subsequent isolation steps.

The absolute stereochemistries of the two amino acids present in aspergillamides A and B were secured as L by comparing the amino acids derived by hydrolysis of aspergillamide A with authentic standards. Using chiral TLC methods, the R_f values of the liberated amino acids, *N*-methylphenylalanine and leucine, clearly matched the L (natural) amino acid standards for both compounds.

The aspergillamides are unique structures possessing rare dehydrotryptamine functionalities. The sole example of this functionality from marine systems lies in the chondriamides, dehydrotrypamine amides from the red alga *Chondria* sp.[13] An exhaustive literature search failed to uncover prior observations of this unique carbon skeleton with the exception of a Japanese patent describing the structure of *l*-phenylalaninamide. This compound, which was isolated from cultures of *Aspergillus terreus* [10] and patented for the treatment of kidney disease, differs from aspergillamide B by the replacement of the leucine moiety with a valine unit. *l*-Phenylalaninamide was isolated in low yield from CNC120 and, along with aspergillamide A, displayed mild cytotoxicity against HCT-116 (IC_{50} = 16 μg/ml).

EXPERIMENTAL SECTION

Culture Conditions. *Aspergillus* sp. (strain CNC-120) was isolated from an inland salt-lake sediment collected from Acklins Island, the Bahamas. The isolation medium (A1: 1.0% starch, 0.4% yeast extract, 0.2% peptone, 1.7% agar, 75% seawater, and 25% deionized water) contained 50 μg/ml cycloheximide and rifampicin. Large-scale fermentation was performed in 20 x 2.8 L Fernbach flasks each containing 1 L of medium YPG+C (0.5% yeast extract, 0.5% peptone, 1% glucose, 0.2% crab meal, 100% seawater). The stationary fermentation proceeded for 13 days at room temperature after which time the mycelium was collected by filtration and freeze-dried.

Extraction and Isolation. The freeze-dried mycelium was extracted with 1 L of 1:1 dichloromethane:methanol to produce 8.65 g of a brown solid that showed activity against the HCT-116 cell line. The extract was fractionated using reversed-phase column chromotography (Waters Sep-pak®, 35 ml, 10 g C-18 cartridge) eluting first with 70 ml of 80% aqueous methanol followed by 50 ml of 100% methanol. The active fractions (103 mg) contained impure aspergillamides that could be visualized as red spots (H_2SO_4 spray) at R_f = 0.30 to 0.35 using 80% aqueous methanol on reversed-phase C-18 TLC plates. Final purification by repeated HPLC with 80% aqueous methanol on a reversed-phase Dynamax® preparative column (C-18, 60Å, 21.4 mm x 250 mm) yielded 39.1 mg of aspergillamide A (0.45% of extract), 10.8 mg of *l*-phenylalaninamide (0.12% of extract), and 3.0 mg aspergillamide B (0.03 % extract).

Aspergillamide A (1): white amorphous powder; $[\alpha]_D$ = -26.2 (c = 3.05, MeOH), IR (neat) 3285 (br), 2957, 1630 (br), 1537, 1494, 1458, 1246, 1090, 744 cm^{-1}; ^1H and ^{13}C NMR (see Tables I and II); LRMS (FAB$^+$) 474 (M^+), 317 (56), 134 (100); HRMS, *m/z* calcd for $C_{28}H_{34}N_4O_3$ (M^+) 474.2631, found 474.2633. UV λ_{max} (MeOH): 286 nm (log e 4.44), 221 nm (log e 4.59), 200 nm (log e 4.68).

Aspergillamide B (2): white amorphous powder; ^1H NMR (200 MHz, acetone-d_6) δ 10.25 (bs, 1H), 7.80-7.77 (m, 2H), 7.60-7.15 (m, 9H), 7.08-7.05 (m, 2H), 6.64 (d, *J* = 14.5 Hz, 1H), 5.00 (m, 1H), 4.59 (m, 1H), 3.25 (m, 1H), 3.08 (m, 1H), 2.85 (s, 3H), 1.98 (s, 3H), 1.58 (s, 1H), 1.12 (m, 1H), 0.82 (t, *J* = 7.0 Hz, 6H), -0.10 (m, 1H); ^{13}C NMR (50 MHz, acetone-d_6) δ 173.9, 170.6, 166.6, 135.9, 135.6, 130.3 (X2), 129.3 (X2), 127.1, 126.8, 126.0, 123.3, 122.1, 120.8, 119.9, 119.8, 112.1, 107.3, 62.9, 47.5, 38.5, 34.2, 30.1, 24.4, 23.1, 22.2, 20.2; LRMS (FAB+) 474 (m^+), 317 (82), 134 (100); HRMS, *m/z* calcd for $C_{28}H_{34}N_4O_3$ (M^+) 474.2631, found 474.2620.

Determination of Absolute Stereochemistry of Aspergillamide A. Aspergillamide A (**1**, 12.8 mg) was dissolved in 6N HCl (2.5 ml) and heated at 90°C for 20 h. The mixture was cooled to room temperature and 1 ml of deionized water was added. A slow stream of nitrogen gas was added to the flask for 15 min to remove excess HCl. The hydrolysate was analyzed by Chirasil™ TLC (Silica gel, RP bonded, coated with Cu^{2+} and a chiral reagent). The TLC plate was heated at 100°C for 15 min to activate the surface prior to analysis. Comparison of the hydrolysate with D and L-*N*-methyl phenylalanine and leucine standards, using an optimized solvent system of 66% acetonitrile / 34% water provided adequate separation of the amino acids after visualization with 0.1% ninhydrin spray. Heating the plates after spraying allowed leucine to be visualized as

bright red spots and *N*-methyl phenyalanine spots as a light purple. *Rf* factors for the hydrolysate were: *Rf* = 0.65, (leucine standards, D-leucine = 0.55, L-leucine = 0.65) and *Rf* = 0.53, purple spot (*N*-methyl phenylalanine standards, D-*N*-methyl phenylalanine = 0.58, L-*N*-methyl phenylalanine = 0.53). The clear separation achieved allowed the amino acids to be assigned as the natural L isomers.

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