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NEW DIKETOPIPERAZINE METABOLITES FROM THE SCLEROTIA OF *ASPERGILLUS OCHRACEUS*

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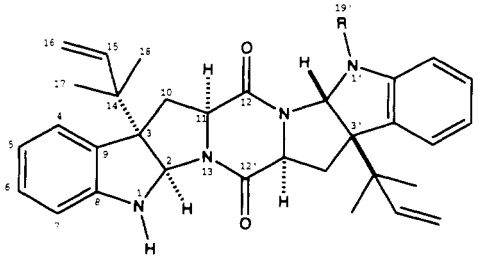
ABSTRACT.—Three new diketopiperazine-containing metabolites **1–3** have been isolated from the sclerotia of *Aspergillus ochraceus* (NRRL 3519) by chromatography on Sephadex LH-20 and reversed-phase hplc. The structures of these compounds were established using extensive high-field 1D and 2D nmr experiments. All three compounds cause moderate reduction in weight gain in assays against the lepidopteran crop pest *Helicoverpa zea*.

Chemical studies of the sclerotia of *Aspergillus* spp. have led to the discovery of a variety of new natural products with anti-insectan and other biological activities (1–6). Most of these compounds are indole diterpenoids, and several of them contain previously undescribed or rare ring systems. Initial studies have focused on members of the *Aspergillus flavus* taxonomic group, and investigations of sclerotial metabolites from other groups remain limited. Analysis of anti-insectan extracts from the sclerotia of an isolate of *Aspergillus ochraceus* Wilhelm (Aspergillaceae) (NRRL 3519) have led to the isolation of three new diketopiperazine-containing compounds **1–3**, which exhibit moderate activity against the corn earworm *Helicoverpa zea*. Details of these studies are the subjects of this report.

The most abundant metabolite **1** from the hexane and $CHCl_3$ extracts of the intact sclerotia had the molecular formula $C_{33}H_{38}O_2N_4$ based on the hreims data $[M]^+$ at *m/z* 522.3010, Δ −1.6 mmu). The presence of an amine NH group was deduced from the broad ir absorption at 3380 cm^{-1} and the exchangeable proton signal at 5.37 ppm in the ^{1}H-nmr spectrum. Two tertiary amide groups were inferred from the ^{13}C-nmr spectrum (165.3 and 168.3 ppm), the broad ir absorption at 1665 cm^{-1}, and the lack of further heteroatom-bonded protons as indicated by a DEPT experiment.

The presence of three pairs of very similar spin systems, consisting of two ortho-disubstituted benzenoid rings, two isolated CH-CH_2 units, and two vinyl groups, was deduced from ^{1}H-^{1}H COSY and HETCOR (7) data. Results of selective INEPT (8) experiments (Table 1) allowed extension of the benzenoid moieties to dihydroindole and *N*-methyldihydroindole substructures (A and A′, Figure 1). Selective INEPT irradia-



1 R=Me
2 R=H

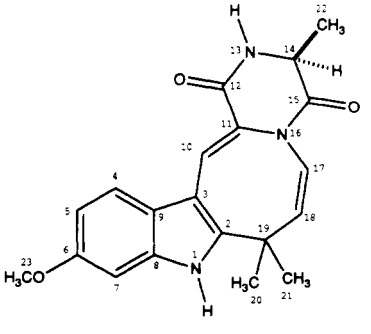


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TABLE 1. Nmr Data for *N*-Methylepiamauromine [**1**] and Epiamauromine [**2**].

Position	Compound							
	[**1**]				[**2**]			
	δC[a]	δH[b]	multiplicity, J_{HH}	Selective INEPT[a,c] (C)	δC[a]	δH[b]	(multiplicity, J_{HH})	HMBC[d]
1	—	5.37	s	3, 8	—	5.36	br s	3, 8, 9
2	79.3	5.32	s	8, 9, 14	79.3	5.30	s	8, 9, 14
3	61.8	—	—	—	62.1	—	—	—
4	125.6	7.16	d, 7.5	3, 6, 8	125.7	7.16	dd, 7.8, 1.2	3, 6, 8
5	118.5	6.73	dt, 0.9, 7.5	7, 9	118.6	6.74	ddd, 1.2, 4.2, 7.2	7, 9
6	128.1	7.07	dd, <1, 7.2	—	128.3	7.08	dt, 1.2, 7.8	4, 8
7	108.7	6.56	d, 7.5	5, 9	108.8	6.56	ddd, 0.6, 1.2, 7.8	5, 9
8	148.3	—	—	—	148.4	—	—	—
9	131.4	—	—	—	131.4	—	—	—
10	36.0	2.45	dd, 9.3, 13.8	—	36.0	2.45	dd, 9.6, 13.8	9, 12
		2.75	dd, 8.5, 13.8	2, 3, 9, 11, 12, 14		2.75	9, 13.3	9
11	58.6	4.12	dt, 1.8, 9.3	10, 12	60.7	4.04	dt, 1.8, 9	10, 12
12	165.3	—	—	—	166.0	—	—	—
14	41.5	—	—	—	41.7	—	—	—
15	143.4	5.88	dd, 10.8, 17.2	14, 17	143.3	5.86	dd, 10.8, 17.4	14, 17, 18
16	114.6	5.07	dd, <1, 17.1	—	114.7	5.06	dd, 1.2, 17.4	14
		5.10	dd, 0.9, 10.8	—		5.09	dd, 0.6, 10.8	14
17	22.3	1,11	s	3, 14, 15	22.9	1.10	s	14, 15
18	22.3	0.95	s	3, 14, 15	22.5	0.93	s	14, 15
1′	—	—	—	—	—	4.91	br s	3′, 9′
2′	82.2	5.41	s	3′, 8′, 9′, 14′	77.6	5.30	s	8′, 9′, 14′
3′	60.7	—	—	—	61.8	—	—	—
4′	128.9	7.06	dt, 0.9, 7.5	—	125.0	7.12	dd, 1.2, 7.2	3′, 8′, 9′
5′	117.2	6.65	dt, 0.9, 7.2	7′, 9′	118.9	6.72	ddd, 1.2, 4.2, 7.2	7′, 9′
6′	124.6	7.10	dt, 0.9, 7.5	—	128.8	7.06	dt, 1.2, 7.8	4′, 8′
7′	105.7	6.31	d, 7.8	9′, 15′	109.3	6.52	dm, 0.6, 1.2, 7.8	5′, 9′

TABLE 1. (Continued)

Position	Compound							
	[1]				[2]			
	δC[a]	δH[b]	multiplicity, J_{HH}	Selective INEPT[a,c] (C)	δC[a]	δH[b]	(multiplicity, J_{HH})	HMBC[d]
8′	151.2	—	—	—	150.0	—	—	—
9′	129.3	—	—	—	129.0	—	—	—
10′	37.0	2.48	dd, 6.3, 12.3	—	35.2	2.50	dd, 6, 12.6	9′
		2.25	dd, 11.1, 12.3	3′, 12′, 14′		2.35	dd, 10.8, 12.6	9′, 13′
11′	60.6	3.96	ddd, 1.8, 6.2, 11.1	10′, 12′	62.0	3.90	ddd, 1.8, 6, 11.4	10′, 13′
12′	168.3	—	—	—	168.0	—	—	—
14′	40.7	—	—	—	40.8	—	—	—
15′	143.4	5.80	dd, 10.8, 17.4	14′	143.4	5.90	dd, 10.8, 17.4	14′, 17′, 18′
16′	114.3	4.98	dd, 0.9, 17.4	—	114.4	5.01	dd, 1.2, 17.4	14′
		5.03	dd, 0.9, 9.6	—	—	5.07	dd, 0.6, 10.8	14′
17′	22.2	1.00	s	3′, 15′, 17′	22.9	1.05	s	14′, 15′
18′	22.9	0.89	s	3′, 14′, 15′, 18′	22.5	0.95	s	14′, 15′
19′	33.0	2.98	s	2′, 8′	—	—	—	—

[a]75 MHz, in $CDCl_3$.
[b]300 MHz, in $CDCl_3$.
[c]$J = 7$ Hz.
[d]600 MHz, in $CDCl_3$.

tions of the methyl proton singlets at 1.11 and 0.95 ppm, as well as the vinylic resonance at 5.88 ppm, all showed a quaternary carbon signal at 41.5 ppm, giving rise to the dimethylpropenyl unit B. Analogous results were obtained to give unit B′. Selective INEPT irradiation of both Me proton signals of dimethylpropenyl group B also showed the carbon signal corresponding to C-3 of dihydroindole A, indicating that this dimethylpropenyl unit must be attached to the 3 position of A. Analogous correlations permitted linkage of dimethylpropenyl group B′ and the *N*-methyldihydroindole A′ (Figure 1).

One of the $CHCH_2$ units can also be linked to dihydroindole A at the 3 position and to the amide carbon at 165.3 ppm, as shown by selective INEPT correlations, to give partial structure C (Figure 1). A parallel set of selective INEPT results showed that the other $CHCH_2$ group, the *N*-methyldihydroindole unit, and the amide carbon at 168.3 ppm can be similarly linked to give partial structure C′. The two partial structures C and C′ account for all of the atoms of compound **1**, leaving only three degrees of unsaturation to assign.

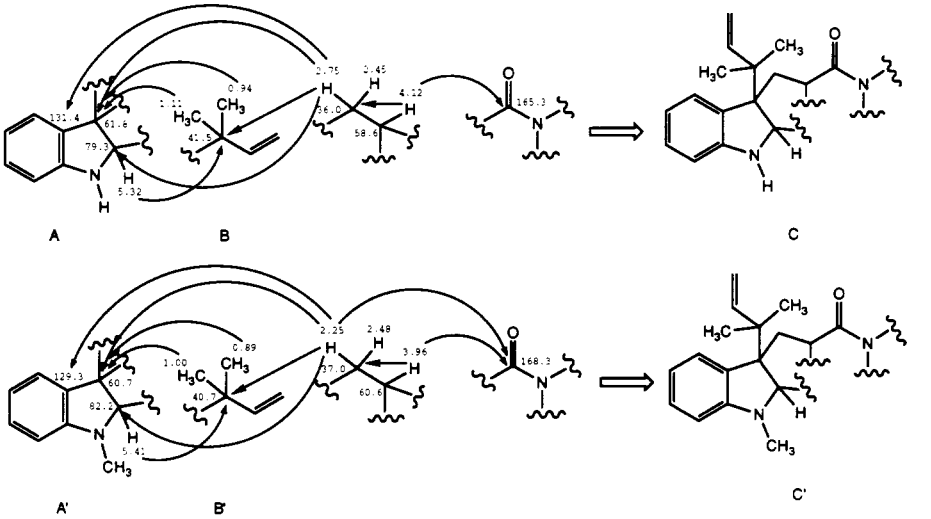
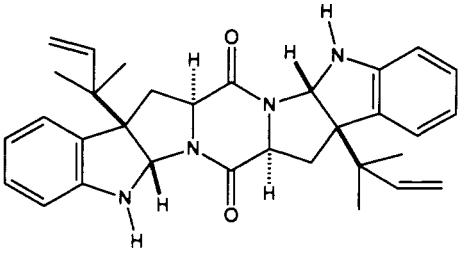


FIGURE 1. Correlations shown by selective INEPT experiments on *N*-methylepiamauromine [**1**].

Irradiation of the methine proton at 5.41 ppm in a selective INEPT experiment (H-2′ of substructure C′) showed the amide carbon resonance of substructure C (165.3 ppm). This result, together with the downfield shift of C-2 of the *N*-methyldihydroindole (82.2 ppm), suggested that C-2′ of partial structure C′ must be linked to partial structure C through the amide nitrogen of C. The downfield shift of C-2 (79.3 ppm) in the dihydroindole subunit C suggested that it might also be attached to the amide nitrogen of C′ in a similar manner, despite the absence of the corresponding correlation in selective INEPT experiments. The downfield shifts of the methine carbons (and protons) alpha to the amide carbonyls also suggested that these carbons are linked to nitrogen. Accordingly, connection of the remaining positions to form a diketopiperazine accounts for the remaining degrees of unsaturation, giving structure **1**.

This gross structure is nearly identical to that of amauromine [**4**] (9,10) [=nigrifortine (11)], a vasodilator originally isolated from the fungus *Amauroascus* sp., differing only in the presence of an N-Me group in compound **1**. However, NOESY results



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(Table 2) showed that the relative stereochemistry of **1** (*N*-methylepiamauromine) is different from that of amauromine. The signal for H-2 was correlated with H-11 and with both methyls of the C-3 dimethylpropenyl group. Thus, H-2, H-11, and the dimethylpropenyl group must be on the same face of the ring system. H-2′ showed correlations with both methyls of the C-3′ dimethyl propenyl group, indicating that these substituents are on the same face of the corresponding rings. H-11′ was correlated to H-11, but not to H-2′. Acid hydrolysis (9,12,13) of *N*-methylepiamauromine, derivatization using 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (Marfey's reagent) (14), and hplc comparison to tryptophan standards indicated that the tryptophan obtained as a degradation product (9,13) had the L configuration. These results permitted proposal of the absolute stereochemistry of *N*-methylepiamauromine as shown in **1**. This assignment was supported by investigation of structure **2**.

The ^{1}H- and ^{13}C-nmr spectra of compound **2** (epiamauromine), as well as the one-bond correlations obtained from an HMQC (15) experiment (Table 1), were very similar to those of *N*-methylepiamauromine, except for the absence of the N-Me signal and the appearance of an exchangeable proton resonance at 4.91 ppm, suggesting that this compound is the demethylated analogue of *N*-methylepiamauromine. This assumption was consistent with the mass spectrum of **2**, which gave a molecular ion at *m/z* 508.2846, corresponding to the molecular formula $C_{32}H_{36}O_2N_4$ (Δ −0.8). The results of an HMBC (16) experiment (Table 1) showed correlations analogous to the selective INEPT correlations for *N*-methylepiamauromine, further supporting proposal of this structure. Epiamauromine has the same gross structure as amauromine, but these two compounds differ stereochemically. Amauromine is a symmetrical dimer showing only half the expected signals in the ^{1}H- and ^{13}C-nmr spectra (9), while epiamauromine

TABLE 2. Some Pertinent NOESY[a] Correlations for *N*-Methylepiamauromine [**1**] and Epiamauromine [**2**].

Proton	**1**	**2**
	(ppm) Correlations (H)	(ppm) Correlations (H)
H-2	(5.32) 11, 15, 17, 18	(5.30) 11, 15, 17, 18
H-10α	(2.75) 10β, 11, 15, 17, 18	(2.75) 10β, 11
H-10β	(2.45) 10α, 11	(2.45) 4, 10α, 11
H-11	(4.12) 2, 10α, 10β, 11′, 17, 18	(4.04) 2, 10α, 10β, 11′
H-2′	(5.41) 10′β, 15′, 17′, 18′, 19′	(5.30) 15′, 17′, 18′
H-10′α	(2.48) 10′β, 11′	(2.5) 4′, 10′β, 11′
H-10′β	(2.25) 2′, 10′α, 11′, 17′, 18′	(2.35) 10′α, 11′
H-11′	(3.96) 10′α, 10′β, 11	(3.90) 10′α, 10′β, 11

[a]600 MHz, in $CDCl_3$.

showed different sets of signals for each half of the molecule using the same nmr solvent. NOESY results (Table 2) indicated that epiamauromine has the same relative stereochemistry as *N*-methylepiamauromine. The NOESY data for **2** do not conclusively rule out the alternative structure in which H-2′, H-11′, and the C-3′ dimethylpropenyl group are all on the same face of the system. However, the corresponding structure would have an axis of symmetry analogous to that of **4**, resulting in a comparable simplification of the nmr data that was not observed. This also provides further support for the proposed stereochemistry of **1**. The proposed difference in stereochemistry between **1–2**, and amauromine is also consistent with the difference in $[\alpha]_D$ values for epiamauromine (−50°, $c = 0.18$ g/dl, $CHCl_3$) and amauromine (−583°, $c = 1.0$, $CHCl_3$) (9).

The third compound **3** ($C_{20}H_{21}N_3O_3$; hr fabms $[M + H]^+$ ion at m/z 352.1670, Δ −1.0 mmu), was significantly different from **1** and **2**. Compound **3** contains an indole subunit (partial structure D in Figure 2) substituted at the 6 position with an MeO group, as revealed by ^1H decoupling, uv, HMQC, and HMBC data (Table 3). The presence of an alanyl moiety (E) and subunit F (Figure 2) were also inferred from these data. The Me protons of subunit F were found to be correlated to C-2 of the indole ring system in the HMBC experiment (Table 3), indicating linkage of subunit F to the 2 position of the indole. An isolated vinylic proton signal at 7.57 ppm was correlated to C-2, C-3, and C-9 of the indole, indicating linkage of the corresponding double bond to C-3 of the indole as shown in Figure 2.

The amide proton at 6.37 ppm was found to correlate with an amide carbon at 165.6 ppm which, in turn, was correlated to the vinylic proton at 7.57 ppm, thus giving rise to partial structure G. Insertion of the remaining tertiary amide nitrogen atom to form a diketopiperazine structure and an 8-membered ring afforded structure **3**. Confirmation of this structure was provided by irradiation of the vinylic proton at 5.82 ppm in a selective INEPT experiment, which showed the carbon signals at 125.0 (C-

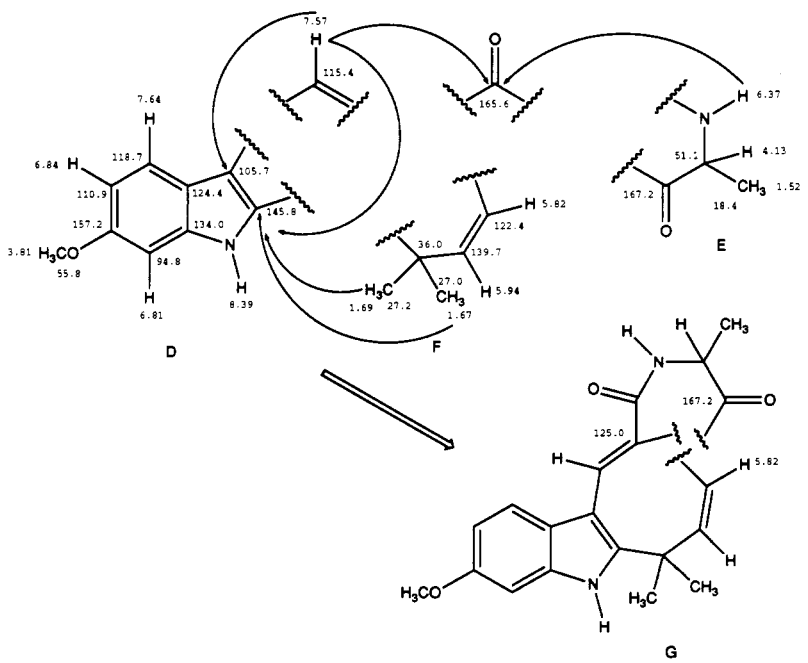

FIGURE 2. Correlations shown by the HMBC experiment on cycloechinulin [3].

TABLE 3. ^{1}H- and ^{13}C-nmr Data for Cycloechinulin [**3**].

Position	δC[a]	δH[b] (multiplicity, J_{HH})	HMBC[b]
1	—	8.39 (br s)	2, 3, 8, 9
2	145.8	—	
3	105.7	—	
4	118.7	7.63 (d, 8.7)	3, 6, 8
5	110.9	6.84 (dd, 2.2, 8.7)	7, 9
6	157.2	—	
7	94.8	6.81 (d, 2.1)	5, 6, 8, 9
8	134.0	—	
9	124.4	—	
10	115.4	7.57 (s)	2, 3, 9, 12
11	125.0	—	
12	165.6	—	
13	—	6.37 (br s)	11, 12[c], 14, 15
14	51.1	4.13 (dq, 2.3, 6.9)	12, 15, 22
15	167.2	—	
16	—	—	
17	122.4	5.82 (d, 8.2)	19, 2[c,d], 11[c], 15[c], 18[c]
18	139.7	5.94 (d, 8.2)	17, 19, 20, 21
19	36.0	—	
20	27.2	1.68 (s)	2, 18, 19, 21
21	27.0	1.67 (s)	2, 18, 19, 20
22	18.4	1.51 (d, 6.9)	14, 15
23	55.8	3.81 (s)	6

[a]75 MHz, in $CDCl_3$.

[b]600 MHz, in $CDCl_3$.

[c]Correlations observed in selective INEPT ($J = 4$ Hz) experiments, but not in the HMBC data.

[d]Denotes a 4-bond correlation.

11) and 167.2 ppm (C-15). The alanyl residue was found to possess the L configuration by acid hydrolysis of **3**, derivatization with Marfey's reagent (14), and hplc comparison with standards.

Compound **3** was assigned the name cycloechinulin because it is closely related in structure to the echinulin series of compounds, i.e., echinulin (17), neoechinulins (18,19), isoechinulins (20), preechinulin (21), and cryptoechinulin (22,23). It differs from these compounds mainly in the condensation of the isoprenyl chain with one of the nitrogen atoms of the diketopiperazine. A similar linkage has been found in only three other compounds, 10,20-dehydro[12,13-dehydroprolyl]-2-(1′,1′-dimethylallyltryptophyl)-diketopiperazine (24), austamide (24,25) and 12,13-dihydroaustamide (25).

Feeding assays using the corn earworm *Helicoverpa zea* [formerly *Heliothis zea* (26)] showed that *N*-methylepiamauromine produced 17% reduction in weight gain relative to controls after one week when incorporated into a test diet at 100 ppm dry weight. Epiamauromine and cycloechinulin at 100 ppm caused 30% and 33% reductions in weight gain, respectively.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—HMQC and HMBC data, optimized for J values of 152 and 8 Hz, respectively, were obtained using a Bruker AMX600 spectrometer. All other ^{1}H- and ^{13}C-nmr and selective INEPT (optimized for $J = 7$ or 4 Hz) spectra were obtained using a Bruker AC300 instrument. Hreims and hrfabms mass spectra were recorded on a VG ZAB-HF mass spectrometer, while low resolution eims data were recorded at 70 eV using a VG Trio I quadrupole mass spectrometer. A Beckman

Ultrasphere 5 μ 10 mm × 25 cm C_{18}-reversed phase column was used in all hplc separations. Marfey's reagent was purchased from Pierce Chemical, Rockford, Illinois. Details of other general experimental procedures have been described elsewhere (1,2,27).

EXTRACTION AND ISOLATION OF COMPOUNDS **1–3**.—Sclerotia of *A. ochraceus* were produced by solid substrate fermentation on autoclaved corn kernels and harvested as described previously (27). The intact sclerotia (21.6 g) were extracted at room temperature with hexane (200 ml, 1 day), then $CHCl_3$ (100 ml, 3 days). After removal of the solvent in vacuo, the hexane extract (22 mg) was subjected to reversed-phase hplc [MeOH-H_2O (9:1), 2 ml/min] to give *N*-methylepiamauromine [**1**] (7 mg).

The $CHCl_3$ extract (148 mg), after removal of the solvent in vacuo, was fractionated through a column of Sephadex LH-20 (25-100 μ; 2 cm × 12.5 cm) using CH_2Cl_2-hexane (1:1) (200 ml), then CH_2Cl_2-MeOH (1:1) (200 ml) as eluents. The fractions obtained with CH_2Cl_2-hexane (1:1) were combined, concentrated, and purified by hplc as above to give epiamauromine [**2**] (2.5 mg) and *N*-methylepiamauromine [**1**] (4.1 mg).

The fractions eluted with CH_2Cl_2-MeOH (1:1) were likewise combined, concentrated, rechromatographed on Sephadex LH-20 using $CHCl_3$-MeOH (2:1), and purified by reversed-phase hplc to yield cycloechinulin [**3**] (1.1 mg).

N-Methylepiamauromine [**1**].—Compound **1** was isolated as a white solid; hplc retention time 17.9 min; $[\alpha]_D$ −29.1° (*c* = 0.46 g/dl, $CHCl_3$); uv (MeOH) λ max 214 (log ε 4.4), 244 (log ε 4.1), 303 nm (log ε 3.8); ir (film on NaCl plate) 3380, 1665, 1605 cm^{-1}; eims (70 eV) *m/z* (rel. int.) $[M]^+$ 522 (29), 453 (59), 384 (14), 255 (17), 184 (14), 171 (58), 157 (17), 144 (100); ^1H nmr and ^{13}C nmr see Table 1; hreims found 522.3010, calcd for $C_{33}H_{38}O_2N_4$ 522.2994.

Amino acid analysis of N-methylepiamauromine [**1**].—*N*-Methylepiamauromine (1.3 mg) was hydrolyzed with 100 μl of 6 M HCl at 110° for 24 h. After the hydrolyzate was allowed to cool, the mixture was neutralized with $NaHCO_3$. Marfey's reagent (1% 1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide in Me_2CO, 1 ml) and 200 μl of 1 M $NaHCO_3$ were added to the hydrolyzate, and the mixture was heated at 40° for 1 h. Upon cooling to room temperature, 100 μl of 2 M HCl was added. The reaction mixture was then analyzed by reversed-phased hplc using 40% MeCN/0.05 M Et_3N/H_3PO_4 (pH 3). Coinjection of the resulting tryptophan derivative with D and DL standards indicated that the masked tryptophan residue in **1** has the L configuration.

Epiamauromine [**2**].—Compound **2** was isolated as a white solid: hplc retention time 12.7 min; mp 134°; $[\alpha]_D$ −50.0° (*c* = 0.18 g/dl, $CHCl_3$); uv (MeOH) λ max 215 (log ε 5.0), 243 (log ε 5.0), 300 nm (log ε 4.6); eims *m/z* (rel. int.) $[M]^+$ 508 (0.03), 199 (61), 158 (20), 150 (7), 131 (100); ^1H nmr and ^{13}C nmr see Table 1; hreims found 508.2846, calcd for $C_{32}H_{36}O_2N_4$ 508.2838.

Cycloechinulin [**3**].—Compound **3** was isolated as a yellow solid: hplc retention time 7.5 min; $[\alpha]_D$ −23.3° (*c* = 0.06 g/dl, $CHCl_3$); uv (MeOH) λ max 214 (log ε 4.3), 228 (log ε 4.2), 267 (log ε 4.0), 300 (log ε 4.1), 377 nm (log ε 3.9); eims (70 eV) *m/z* (rel. int.) $[M]^+$ 351 (77), 336 (100), 308 (20), 296 (26), 293 (37), 280 (93), 265 (34), 252 (46), 251 (47), 237 (94), 225 (82), 222 (31), 197 (64); ^1H nmr and ^{13}C nmr see Table 3; hrfabms found 352.1671, calcd for $C_{20}H_{21}O_3N_3$ + H 352.1661.

Amino acid analysis of cycloechinulin [**3**].—Cycloechinulin (0.4 mg) was dissolved in 100 μl of 6 M HCl and heated at 110° for 16 h. The resulting hydrolyzate was allowed to cool and then neutralized with $NaHCO_3$. To this mixture was added 200 μl Marfey's reagent and 40 μl of 1 M $NaHCO_3$. The mixture was then heated at 40° for 1 h. Upon cooling to room temperature, 20 μl of 2 M HCl was added. The solution was then analyzed by reversed-phase hplc as above. Coinjection with D- and L-alanine standards indicated that the alanyl residue in **3** has the L configuration.

ACKNOWLEDGMENTS

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