Isolation and Structure Determination of Cryptophycins 38, 326, and 327 from the Terrestrial Cyanobacterium *Nostoc* sp. GSV 224†

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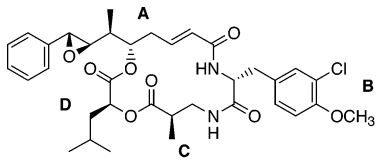
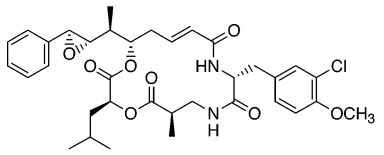
Cryptophycin-38 (**2**), -326 (**3**), and -327 (**4**) are three new trace constituents of the terrestrial cyanobacterium *Nostoc* sp. GSV 224. Cryptophycin-38 is a stereoisomer of cryptophycin-1 (**1**) and to date is the only naturally occurring analogue that possesses a *S*,*S* epoxide group in unit A. Cryptophycin-327 is a geometric isomer that differs from **1** in having a cis Δ^2-double bond in unit A. Cryptophycin-326 is related to cryptophycin-21, but has two chlorines ortho to the methoxy group in unit B. The relative and absolute stereochemistries of **2** have been related to known cryptophycins by semisynthesis and/or spectral analysis.

Cryptophycins are potent antitumor and antifungal peptolides associated with the terrestrial cyanobacteria *Nostoc* sp. GSV 224 and ATCC53789 (Nostocaceae).[1−5] The most abundant member of this class of cyclic depsipeptides is cryptophycin-1 (**1**). In addition to **1**, 24 other analogues have been isolated from the GSV 224 cyanobacterium.[4,5] Interestingly HPLC analysis shows that each cyanobacterium exhibits the same array of cryptophycins.[5,6] Nevertheless, the two cyanobacteria are significantly different genetically and biochemically. The 16S rDNAs differ by 2.8% in homology (GenBank Accession AF062637 for GSV224 and AF062638 for ATCC53789), and the other secondary metabolites that are found in GSV224 appear to be different from the ones found in ATCC53789. For example, GSV224 produces a class of peptolides, called nostopeptolides, that are totally absent in the ATCC53789 cyanobacterium.[6] Furthermore, the genes that encode for the biosynthesis of the nostopeptolides in GSV224 (GenBank Accession AF204805) are not found in ATCC53789.[7] Conversely the ATCC53789 species produces an unusual class of cyclic peptides (nostocyclopeptides) that are not present in GSV224.[8] Similarly, the genes assigned to the biosynthesis of the nostocyclopeptides in ATCC53789 (GenBank Accession AY167420) are absent in GSV224.[9]

In this paper we report the isolation and identification of three more trace cryptophycins (**2**−**4**) from GSV 224. Lyophilized GSV 224 was extracted with 4:1 CH_3CN/CH_2Cl_2, and the concentrated extract was fractionated by reversed-phase flash column chromatography with mixtures of H_2O/CH_3CN as previously described.[2] All of the cryptophycins were eluted with 35% H_2O/CH_3CN, and this mixture was further separated by reversed-phase HPLC into a number of fractions. One of the fractions, a peak having a relative retention time of 1.16 compared with 1.00 for **1**, contained the three new analogues, viz., cryptophycin-38 (**2**), cryptophycin-326 (**3**), and cryptophycin-327 (**4**). Further fractionation led to pure **3** and two subfractions. Repeated chromatography of the two subfractions by normal-phase and reversed-phase HPLC led to pure **2** and **4**. The total structures of **2**−**4** were established in a straightforward manner using a combination of spectral and chemical (semisynthesis) techniques. The same analogues appear to be present in the ATCC53789 *Nostoc* by HPLC analysis.[10]



1 cryptophycin-1



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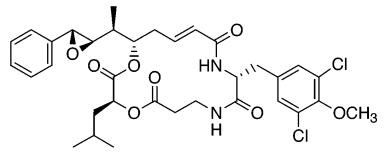
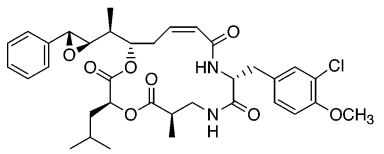


3 cryptophycin-326



4 cryptophycin-327

Cryptophycin-38 (**2**) has the molecular formula $C_{35}H_{43}ClN_2O_8$ based on its HREIMS. The fragmentation pattern in the EIMS of **2** is essentially identical with that of **1**. Diagnostic fragment ion peaks at *m*/*z* 412/414 (ion a), 280/282 (ion b), 227 (ion c), and 195/197 (ion d)[11] strongly suggested that the elemental compositions, but not necessarily the gross structures, of the four amino/hydroxy acid units of **2** and **1** are the same. Although the ^{1}H NMR spectra of **2** (Table 1) and **1**[2] were comparable, the chemical shifts of some of the proton signals were significantly different, particularly those for H-7 and H-8 in unit A. These NMR differences suggested that the epoxide group in unit A of **2** has a different stereochemistry. The proposed structure, including the absolute stereochemistry, was rigorously established by comparing the physical (optical rotations, HPLC retention times) and spectral (EIMS, ^{1}H NMR, and ^{13}C NMR) data for natural **2** with the data for the semisynthetic 7*S*,8*S* (**2**), 7*R*,8*S* (cryptophycin-39, **5**), and 7*S*,8*R* (cryptophycin-77, **6**) epoxides. The data fit

† Dedicated to the late Dr. D. John Faulkner (Scripps) and the late Dr. Paul J. Scheuer (Hawaii) for their pioneering work on bioactive marine natural products.

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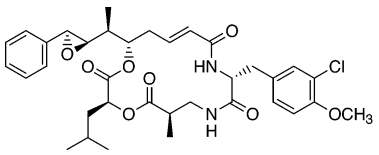
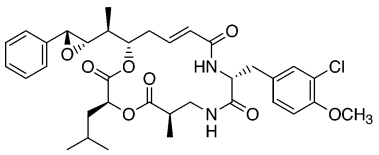
Table 1. ^{1}H NMR Data for Cryptophycin-38, -326, and -327 in $CDCl_3$

unit	position	δ_H (mult.; *J* in Hz cryptophycin-38	cryptophycin-326	cryptophycin-327
A	2	5.80 (d; 15.4)	5.75 (dd; 15.3, 1.1)	5.81 (d; 11.3)
	3	6.68 (ddd; 15.4, 9.9, 5.4)	6.69 (ddd; 15.3, 10.4, 5.0)	6.00 (ddd; 11.3, 9.6, 5.1
	4 (*proS*)	2.65 (dt; −14.5, 9.9−11.2)	2.45 (ddd; −14.0, 11.3, 10.4)	3.88 (ddd; −15.5, 9.6, 5.1)
	4 (*proR*)	2.55 (brdd; −14.5, 5.4)	2.58 (dddd; −14.0, 5.0, 1.7, 1.1)	2.50 (dt; −15.5, 5.1)
	5	5.12 (ddd; 11.2, 5.0, 1.6)	5.18 (ddd; 11.3, 5.0, 1.7)	5.05 (dt; 8.1, 5.1)
	6	1.76 (pd; 7−7.8, 5.0)	1.81 (dqd; 7.5, 6.9, 5.0)	1.93 (dp; 8.1, 7.4)
	6-Me	1.03 (d; 7.0)	1.15 (d; 6.9)	1.15 (d; 7.4)
	7	2.88 (dd; 7.8, 2.0)	2.93 (dd; 7.5, 1.9)	2.92 (dd; 7.4, 1.9)
	8	3.58 (d; 2.0)	3.70 (d; 1.9)	3.71 (d; 1.9)
	10/14	7.22 (m)	7.25 (brd; 8.4)	7.25 (m)
	11/13	7.28−7.36 (m)	7.36 (m)	7.34 (m)
	12	7.28−7.36 (m)	7.37 (m)	7.34 (m)
B	2	4.81 (m)	4.76 (ddd; 8.7, 7.6, 6.1)	4.68 (ddd; 8.3, 7.3, 6.5)
	2-NH	5.70 (d; 8.7)	5.80 (d; 8.7)	5.72 (d; 8.3)
	3*proR*	3.03 (dd; −14.4, 7.4)	2.93 (dd; −14.4, 7.6)	2.94 (dd; −14.4, 7.3)
	3*proS*	3.13 (dd; −14.4, 5.5)	3.15 (dd; −14.4, 6.1)	3.18 (dd; −14.4, 6.5)
	5	7.22 (d; 2.1)	7.16 (s)	7.24 (d; 2.0)
	7-OMe	3.86 (s)	3.86 (s)	3.87 (s)
	8	6.83 (d; 8.4)		6.84 (d; 8.6)
	9	7.07 (dd; 8.4, 2.1)	7.16 (s)	7.10 (dd; 8.6, 2.0)
C	2	2.71 (pd; 6.7−7.3, 4.0)	2.60 (m)	2.78 (dqd; 9.4, 6.9, 4.5)
	2′		2.55 (m)	
	2-Me	1.22 (d; 7.3)		1.17 (d; 6.9)
	3*proS*	3.29 (dt; −13.5, 6.7)	3.35 (dddd; −13.9, 5.5, 5.0, 4.2)	3.11 (ddd; −13.4, 9.4, 5.6)
	3*proR*	3.49 (dt; −13.5, 4−5)	3.63 (dddd; −13.9, 8.9, 5.0, 4.0)	3.60 (ddd; −13.4, 6.9, 4.5)
	3-NH	6.96 (brt; 5−6.7)	6.86 (t; 5.0)	6.99 (dd; 6.9, 5.6)
D	2	4.90 (dd; 10.0, 3.3)	4.88 (dd; 10.2, 3.3)	4.88 (dd; 10.5, 3.2)
	3	1.72 (m)	1.68 (m)	1.56 (m)
	3′	1.49 (m)	1.31 (m)	1.21 (m)
	4	1.69 (m)	1.68 (m)	1.60 (m)
	5	0.86 (d; 6.7)	0.85 (d; 6.3)	0.75 (d; 6.5)
	5′	0.89 (d; 6.5)	0.84 (d; 6.3)	0.76 (d; 6.5)

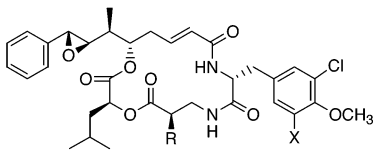
perfectly with the data for semisynthetic **2**. It was clear from the vicinal ^{1}H−^{1}H coupling constants that **2** was a *trans*-epoxide ($J_{7,8}$ = 2.0 Hz for **2** and 2.0 Hz for **1**), not a *cis*-epoxide ($J_{7,8}$ = 3.7 Hz for **5** and 4.4 Hz for **6**); moreover, chemical shifts also indicated that **2** was a *trans*-epoxide (δ_{H7} = 2.88 and δ_{H8} = 3.58 for **2** vs δ_{H7} = 2.92 and δ_{H8} = 3.69 for **1**), not a *cis*-epoxide (δ_{H7} = 3.18 and δ_{H8} = 4.15 for **5** and δ_{H7} = 3.12 and δ_{H8} = 4.07 for **6**).



5 cryptophycin-39



6 cryptophycin-77



7 cryptophycin-31 X = Cl; R = CH_3
8 cryptophycin-21 X = R = H

Cryptophycin-326 (**3**) has the molecular formula $C_{34}H_{40}Cl_2N_2O_8$ based on HRFABMS. In the EIMS of **3** characteristic fragment ions are observed at *m/z* 446/448/450 (ion a), 314/316 (ion b), 229/231 (ion d), and 227 (ion c), indicating that another chlorine was located in unit B. The two chlorines have to be in a symmetrical 3,5-dichloro-4-methoxyphenyl group since the ^{13}C chemical shifts for unit B of **3** (Table 2) are essentially identical with those found for unit B of cryptophycin-31 (**7**).[2] The remaining ^{13}C chemical shifts are the same as those assigned to units A, C, and D of cryptophycin-21 (**8**).[4] The optical rotation of **3** ($[\alpha]_D$ +9° in $CDCl_3$) is dextrorotatory, as are **1**, **7**, and **8** ($[\alpha]_D$ +34°, +51°, and +40° in MeOH, respectively).

Cryptophycin-327 (**4**) has the molecular composition $C_{35}H_{43}ClN_2O_8$ based on HRFABMS. Even though many of the ^{1}H chemical shifts were significantly different, analysis of the COSYspectrum led us to conclude that units A−D in **4** and **1** were similar. The major differences in the ^{1}H NMR spectra of the two compounds were the chemical shift of C-3 and the magnitude of the vicinal coupling between H-2 and H-3 in unit A. In **4** the Δ^2-double bond was clearly cis (11.3 Hz), whereas in **1** it was trans (15.5 Hz). Most of the remaining coupling constants were similar, except for the ones between H_2-4 and H-5 in unit A (11.1 and 1.9 Hz for **1**, but both 5.1 Hz for **4**) and between H-2 and H_2-3 in unit C (6.3 and 3.8 Hz for **1**, but 9.4 and 4.5 Hz for **4**).

An explanation of these coupling constant differences was obtained by a molecular modeling study of **1** and **4**. At the top of Figure 1 is shown the molecular model (CS Chem3D Pro, version 4.0) of the preferred conformation of **1** based on X-ray crystallographic and NMR studies.[2] The molecular model of **4** was generated in the following manner: First the trans NH−CO−CH=CH−CH_2 segment overlapping units A and B was highlighted and then deleted from the molecular model of **1**. Protons automatically appeared where the amide N and methylene C had previously been. Next a cis NH_2−CO−CH=CH−CH_3 molecule was constructed, energy minimized, and moved to the place where the trans segment had originally been located in the molecular model of **1**. Care was taken to position

Table 2. ^{13}C NMR Data for Cryptophycin-38, -326, and -327 in $CDCl_3$

		δ_C		
unit	carbon	cryptophycin-38	cryptophycin-326	cryptophycin-327
A	1	165.5	165.7	165.8
	2	125.3	125.4	125.4
	3	141.4	141.1	138.0
	4	36.7	36.7	29.2
	5	76.9	76.0	76.4
	6	41.0	40.6	38.99
	6-Me	13.4	13.4	13.4
	7	63.2	62.9	63.9
	8	56.1	58.9	59.4
	9	137.1	136.7	136.8
	10/14	125.4	125.3	125.9
	11/13	128.6	128.7	128.7
	12	128.6	128.5	128.52
B	1	171.0	170.9	170.9
	2	53.6	53.5	53.6
	3	35.1	35.0	34.9
	4	129.9	134.6	129.7
	5	131.0	129.6	131.1
	6	122.5	129.3	119.8
	7	154.0	151.1	154.1
	7-OMe	56.3	60.7	56.1
	8	112.3	129.3	112.2
	9	128.4	129.6	128.50
C	1	175.6	172.3	174.6
	2	38.3	32.3	39.4
	2-Me	14.1		13.8
	3	41.1	34.6	42.3
D	1	170.8	170.2	170.7
	2	71.5	71.1	70.8
	3	39.3	39.5	39.02
	4	24.7	24.4	24.5
	5	21.4	21.2	22.8
	5′	23.1	22.8	21.1

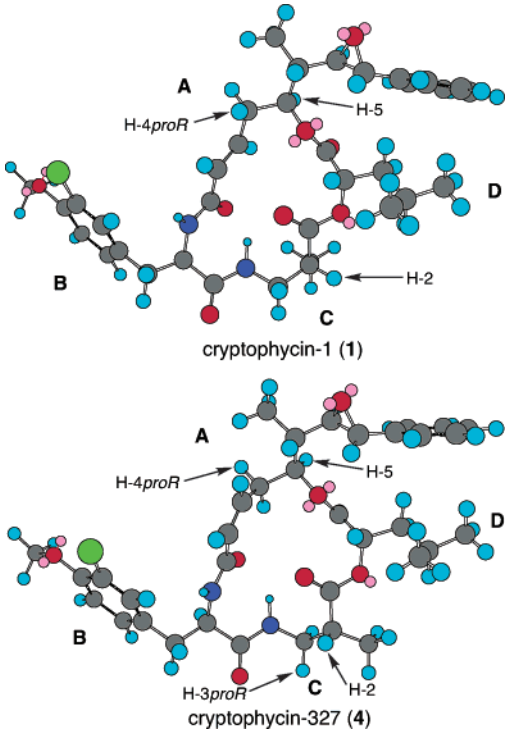


Figure 1. Molecular models depicting the preferred conformations of cryptophycin-1 (**1**) and cryptophycin-327 (**4**). The absolute stereochemistries of the two molecules are assumed to be identical. In the model for **1** H-5 in unit A is anti to H-4*proR* and gauche to H-4*proS* and in unit C H-2 is gauche to both protons on C-3. In the model for **4** H-5 in unit A is gauche to both protons on C-4 and H-2 in unit C is anti to H-3*proS* and gauche to H-3*proR*.

the amide nitrogen and methylene carbon so that they were coincidental with the positions of the two protons that had appeared on C-2 of unit B and C-5 of unit A, respectively, after deletion of the trans NH−CO−CH=CH−CH_2 segment. MM2 energy minimization led to a molecular model (not shown) in which the conformation of the cis NH−CO−CH=CH−CH_2 segment in unit A was essentially identical with the one shown in the bottom drawing of Figure 1. The 5.1 Hz coupling constants between H_2-4 and H-5 were entirely consistent with the model, as H-5 was gauche to both of the protons on C-4. ROESY data also supported the structure and conformation. The H-5 signal (δ 5.05) showed NOE cross-peaks to the signals at δ 1.15 (Me on C-6, 2.7 Å), 1.93 (H-6, 2.3−3.1 Å), 2.50 (H-4*proR*, 2.5 Å), 2.92 (H-7, 2.6 Å), and 3.88 (H-4*proS*, 2.4 Å). Moreover, the H-4*proR* signal showed additional NOE cross-peaks to the signals at δ 1.15 (Me on C-6, 2.7 Å) and 6.00 (H-3, 2.5 Å). The chemical shift for H-4*proS* was much further downfield compared with the chemical shifts of H-4*proR* in **4** and H_2-4 in **1** due to deshielding by the C-1 carbonyl of unit A.

In the preferred conformation of **1**, H-2 in unit C has to be gauche to both protons on C-3 to explain the medium to small sized couplings (6.3 and 3.8 Hz) that are observed. In the preferred conformation of **4**, however, H-2 has to be anti to one of the protons on C-3 (9.4 Hz) and gauche to the other one (4.5 Hz). Using the small procedure described above, the CH_2−CH−CH_3 segment in unit C (type shown in preferred conformation of **1**) was deleted and replaced with the segment in which the methine proton is anti to one of the methylene protons. MM2 energy minimization led to the conformation for **4** shown at the bottom of Figure 1.

The three new analogues are weaker cytotoxins than **1** against the human tumor cell line KB (nasopharyngeal). (i) Cryptophycin-38 (**2**) is weaker because the configuration of the epoxide has to be *R,R* for maximum activity. Changing it to *S,S* as it is in **2** results in a several hundred-fold loss in cytotoxicity. Cryptophycin-38 (**2**) shows IC_{50} values averaging 54.7 nM compared with 0.0092 nM for **1**.[2] Similarly the semisynthetic *S,R* (**5**) and *R,S* (**6**) analogues show IC_{50} values of 72 and 654 nM, respectively. (ii) Cryptophycin-326 (**3**) is weaker (IC_{50} 1160 nM) than **1** because addition of a second chlorine to the aromatic ring in unit B results in at least a 10-fold to 100-fold loss of cytotoxicity.[2] (iii) An *E* Δ^2-double bond in unit A of **1** appears to be important for optimum activity. Changing it to Z as it is in cryptophycin 327 (**4**) results in virtual loss of cytotoxicity (IC_{50} 14 100 nM)

Experimental Section

NMR Analysis. NMR spectra were determined on an 11.75-T instrument operating at 500 MHz for ^1H and 125 MHz for ^{13}C. ^1H chemical shifts are referenced in $CDCl_3$ to residual $CHCl_3$ (7.26 ppm); ^{13}C chemical shifts are referenced to the solvent ($CDCl_3$, 77.0 ppm).

Biological Material. The cyanobacterium was grown in mass culture as previously described.[2]

Extraction and Isolation. Lyophilized *Nostoc* sp. GSV 224 (660 g) was extracted with 4:1 MeCN/CH_2Cl_2, and the extract (15.0 g) was subjected to reversed-phase flash column chromatography to give 6.0 g of material that contained only cryptophycins. Reversed-phase HPLC of the mixture of cryptophycins on a 250 × 10 mm Econosil C18 column (10 μm) with 30:70 H_2O/MeCN (flow rate 6.0 mL/min, detection at 254 nm) led to a fraction (t_R 44 min, 105 mg) that consisted of three new cryptophycins (by comparison cryptophycin-1 (**1**) eluted at t_R 38 min on this column). Normal-phase HPLC of this fraction on an Econosil silica column (250 × 10 mm, 5 μm) with 1:1 EtOAc/hexanes (4.0 mL/min) gave cryptophycin-326 (**3**) (t_R 11.5 min, 0.8 mg) and two subfractions eluting at 7 min

(A) and 20 min (B). HPLC of subfraction A (2 mg) on a smaller Econosil C18 column (250 × 4.6 mm, 5 μm, 35:65 H_2O/MeCN, 1.5 mL/min) yielded cryptophycin-327 (**4**) (t_R 13 min, 0.5 mg). HPLC of subfraction B on a 250 × 22 mm column (Econosil C18, 10 μm) with 32.5:67.5 H_2O/MeCN (6.0 mL/min) produced cryptophycin-38 (**2**) (t_R 57 min, 0.6 mg).

Cryptophycin-38 (2): $[\alpha]_D$ −15° ($CHCl_3$, *c* 0.4); UV (MeOH) λ_{max} (ϵ) 202 (131 250), 218 (71 417), 280 (6192) nm; IR (film) ν_{max} 3409, 2959, 1746, 1666, 1537, 1257 cm^{-1}; ^1H NMR data, Table 1; ^{13}C NMR data, Table 2; EIMS *m*/*z* (rel int) 654/656 (11/4), 412/414 (29/7, ion a), 280/282 (14/5, ion b), 253 (6), 227 (43, ion c), 195/197 (68/27, ion d), 184 (25), 155 (51), 141 (35), 91 (100); HREIMS *m*/*z* 654.2725 (calcd for $C_{35}H_{43}{}^{35}ClN_2O_8$, *m*/*z* 654.2708).

Cryptophycin-326 (3): $[\alpha]_D$ +9° ($CHCl_3$, *c* 0.4); UV (MeOH) λ_{max} (ϵ) 208 (39 659), 218 (25 255), 280 (1368) nm; IR (film) ν_{max} 3401, 3280, 2958, 2932, 2872, 1738, 1667, 1537, 1268, 1174 cm^{-1}; ^1H NMR data, Table 1; ^{13}C NMR data, Table 2; FABMS *m*/*z* (rel int) 675/677/679 (16/11/3, MH^+); HRFABMS *m*/*z* 675.2250 (calcd for $C_{34}H_{41}{}^{35}Cl_2N_2O_8$, *m*/*z* 675.2240).

Cryptophycin-327 (4): $[\alpha]_D$ +18° ($CHCl_3$, *c* 0.3); UV (MeOH) λ_{max} (ϵ) 208 (29 091), 218 (18 323), 280 (1815) nm; IR (film) ν_{max} 3409, 2960, 1726, 1659, 1503, 1259, 1196 cm^{-1}; ^1H NMR data, Table 1; ^{13}C NMR data, Table 2; FABMS *m*/*z* (rel int) 655/657 (5/3, MH^+); HRFABMS *m*/*z* 655.2805 (calcd for $C_{35}H_{44}{}^{35}ClN_2O_8$, *m*/*z* 655.2786).

Semisynthesis of 2. A solution of cryptophycin-3 (3 mg) and *m*-chloroperbenzoic acid (3.5 mg) in 1 mL of dry CH_2Cl_2 was stirred at 60 °C for 2 h, then washed successively with aqueous 5% sodium sulfite (1 mL) and 5% sodium carbonate. The organic layer was evaporated to obtain the reaction product, which was separated by reversed-phase HPLC (Econosil C18, 250 × 10 mm, 10 μm) using 30% H_2O/CH_3CN to give **1** (1.2 mg) and **2** (0.7 mg).

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Supporting Information Available: Experimental Section for semisynthesis of **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Trimurtulu, G.; Ohtani, I.; Patterson, G. M. L.; Moore, R. E.; Corbett, T. H.; Valeriote, F. A.; Demchik, L. *J. Am. Chem. Soc.* **1994**, *116*, 4729−4737.
(2) Golakoti, T.; Ogino, J.; Heltzel, C. E.; Husebo, T. L.; Jensen, C. M.; Larsen, L. K.; Patterson, G. M. L.; Moore, R. E.; Mooberry, S. L.; Corbett, T. H.; Valeriote, F. A. *J. Am. Chem. Soc.* **1995**, *117*, 12030−12049.
(3) Moore, R. E.; Corbett, T. H.; Patterson, G. M. L.; Valeriote, F. A. *Curr. Pharm. Des.* **1996**, *2*, 317−330.
(4) Barrow, R. A.; Hemscheidt, T.; Liang, J.; Paik, S.; Moore, R. E.; Tius, M. A. *J. Am. Chem. Soc.* **1995**, *117*, 2479−2490.
(5) Subbaaraju, G. V.; Golakoti, T.; Patterson, G. M. L.; Moore, R. E. *J. Nat. Prod.* **1997**, *60*, 302−305.
(6) Golakoti, T.; Yoshida, W. Y.; Chaganty, S.; Moore, R. E. *Tetrahedron* **2000**, *56*, 9093−9102.
(7) Golakoti, T.; Yoshida, W. Y.; Chaganty, S.; Moore, R. E. *J. Nat. Prod.* **2001**, *64*, 54−59.
(8) Hoffmann, D.; Hevel, J. M.; Moore, R. E.; Moore, B. S. *Gene* **2003**, *311*, 169−178.
(9) Becker, J. E.; Moore R. E.; Moore, B. S. *Gene* **2004**, *325*, 35−42.
(10) In the HPLC profile of the mixture of cryptophycins on a 25 cm × 22 mm column of Econosil C18 (10 μm) with 65:35 MeCN/H_2O at a flow rate of 6 mL/min (UV detection at 254 nm), the peak containing **2**, **3**, and **4** appears as a shoulder at 62 min (relative t_R = 62/53 = 1.17 where 53 is the t_R for **1** in min.
(11) For an explanation of ions a, b, c, and d, refer to Scheme 1 in our first publication.[1]

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