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Veraguamides A−G, Cyclic Hexadepsipeptides from a Dolastatin 16-Producing Cyanobacterium *Symploca* cf. *hydnoides* from Guam

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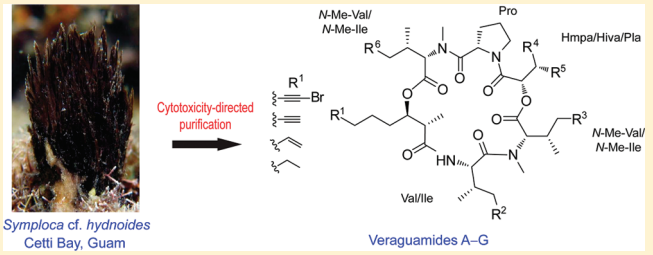
Supporting Information

ABSTRACT: Cytotoxicity-directed purification of a *Symploca* cf. *hydnoides* sample from Cetti Bay, Guam, afforded seven new cyclic depsipeptides, veraguamides A−G (**1**−**7**), together with the known compound dolastatin 16. The planar structures of **1**−**7** were elucidated using NMR and MS experiments, while enantioselective HPLC and Mosher's analysis of acid and base hydrolysates, respectively, were utilized to assign the absolute configurations of the stereocenters. Veraguamides A−G (**1**−**7**) are characterized by the presence of an invariant proline residue, multiple *N*-methylated amino acids, an α-hydroxy acid, and a C_8-polyketide-derived β-hydroxy acid moiety with a characteristic terminus as either an alkynyl bromide, alkyne, or vinyl group. These compounds and a semisynthetic analogue (**8**) showed moderate to weak cytotoxic activity against HT29 colorectal adenocarcinoma and HeLa cervical carcinoma cell lines. Preliminary structure−activity relationship analysis identified several sensitive positions in the veraguamide scaffold that affect the cytotoxic activity of this compound class. Dolastatin 16 showed only weak cytotoxic activity on both cell lines tested. The complete stereostructure of dolastatin 16 was proposed for the first time through degradation followed by a combination of advanced Marfey's analysis and modified Mosher's analysis using phenylglycine methyl ester as a chiral anisotropic reagent.



Marine cyanobacteria have provided both structurally diverse and potent cytotoxic compounds with varying mechanisms of action as lead structures for drug discovery. Most of these natural products are peptide−polyketide hybrids and are derived from the genus *Lyngbya*, marked by *N*-methylation, modified amino acids, α- and β-hydroxy acids, and fatty acid-type moieties.[1,2] We have had particular success with our drug discovery efforts based on the genus *Symploca*. From Micronesian collections of *Symploca* spp. we previously isolated the linear peptides dolastatin 10 and its analogue symplostatin 1.[3,4] These compounds are microtubule-disrupting agents that interfere with mitosis and have pico- to nanomolar IC_{50} values against cancer cells.[5] From a Floridian *Symploca* sp. collection we recently discovered the HDAC inhibitor largazole, which showed strong cytotoxic activity against cancer cells with superior selectivity over nontransformed cells.[6,7] In this light, we screened several *Symploca* sp. collections from Guam and Florida to find new cytotoxic agents. In this paper, we present the cytotoxicity-directed fractionation of a *S.* cf. *hydnoides* collection from Cetti Bay, Guam, which afforded the known compound dolastatin 16,[8] together with seven new cyclic depsipeptides, given the trivial names veraguamides A−G (**1**−**7**).[9]

RESULTS AND DISCUSSION

The freeze-dried cyanobacterium was extracted with EtOAc−MeOH (1:1) and subsequently solvent-partitioned into hexanes-, *n*-BuOH-, and H_2O-soluble fractions. The *n*-BuOH-soluble fraction showed biological activity at 10 μg/mL in an initial cytotoxicity screen using the HT29 colorectal adenocarcinoma cell line. This fraction was further purified by silica column chromatography, with the cytotoxic activity concentrated in the fraction eluting with 20% *i*-PrOH in CH_2Cl_2. Reversed-phase HPLC purification of this silica fraction yielded dolastatin 16 and veraguamides A−G (**1**−**7**).

HRESIMS of the major compound in this series, veraguamide A (**1**), showed the distinctive 1:1 isotopic cluster for a Br-containing compound for the $[M + H]^+$ peak at *m*/*z* 767.3675/769.3660, suggesting a molecular formula of $C_{37}H_{59}BrN_4O_8$. The ^1H NMR spectrum of **1** displayed characteristic peptide resonances for a secondary amide proton (δ_H 6.25), two tertiary amide *N*-CH_3's (δ_H 3.00, δ_H 2.94), and several α-protons (δ_H 3.85−4.95). 2D NMR analysis (Table 1) in $CDCl_3$ using HSQC, COSY, TOCSY, and HMBC established the presence of four amino acids (Pro, Val, 2 × *N*-Me-Val) and an α-hydroxy acid [(2-hydroxy-3-methylpentanoic acid (Hmpa)]. The last spin system consisted of a CH_3 doublet (δ_H 1.25) that showed a COSY correlation to a methine (δ_H 3.11) and HMBC correlations to a carbonyl (δ_C 170.8) and an oxymethine (δ_C 76.4). Further extension of this unit using HMBC and COSY

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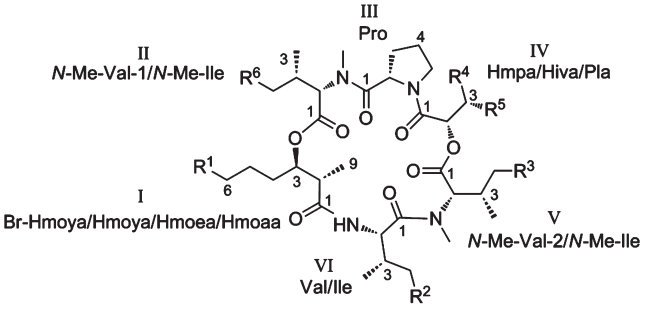
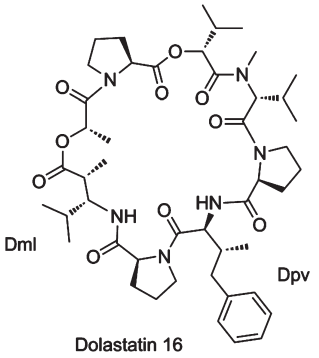


Veraguamides		R^1	R^2	R^3	R^4	R^5	R^6
A	(1)	Br	H	H	Et	Me	H
B	(2)	Br	H	H	Me	Me	H
C	(3)		H	H	Et	Me	H
D	(4)		H	H	Et	Me	Me
E	(5)		Me	Me	Et	Me	H
F	(6)		H	H	Ph	H	H
G	(7)		H	H	Et	Me	H
semisynthetic	(8)		H	H	Et	Me	H



Dolastatin 16

established the presence of a 8-bromo-3-hydroxy-2-methyl-7-octynoic acid (Br-Hmoya) moiety in **1**. This was supported by HMBC correlations of the methylene ($\delta_{C\text{-}6}$ 19.2/$\delta_{H_2\text{-}6}$ 2.23) with two quaternary carbons at δ_C 38.4 and 79.3 and by the large difference in chemical shifts between these quaternary carbons characteristic for an alkynyl bromide.[10] The linear sequence of *N*-Me-Val-1−Pro−Hmpa−*N*-Me-Val-2−Val−Br-Hmoya was established on the basis of HMBC correlations between α-protons and carbonyl groups (Table 1) and was verified by MS/MS fragmentation (Figure 1). The deshielded C-3 methine of the Br-Hmoya unit suggested acylation with the carbonyl of *N*-Me-Val-1 to form a cyclic hexadepsipeptide, corroborated by HMBC and consistent with the molecular formula requirements based on HRESIMS.

Veraguamide B (**2**) showed a 1:1 isotopic pattern for the pseudomolecular ion $[M + H]^+$ at *m/z* 753.3517/755.3508, suggesting the presence of a Br as in **1** with a negative difference of 14 amu corresponding to one less CH_2 unit and thus a molecular formula of $C_{36}H_{57}BrN_4O_8$. Comparison of the ^{1}H NMR spectrum of **1** and **2** showed differences in the splitting pattern in the CH_3 region at δ_H 0.93 ppm and the chemical shift of the α-proton (δ_H 4.85) of the α-hydroxy acid (Table 2). The vicinal methine (δ_H 2.17) of the α-hydroxy acid showed COSY correlations to two methyl groups (δ_H 0.93, δ_H 1.02) instead of COSY correlations to methylene and methyl protons in **1**. Therefore, **2** possesses a 2-hydroxyisovaleric acid (Hiva) instead of the Hmpa unit as in **1**.

The HRESIMS spectrum of veraguamide C (**3**) showed a negative deviation of 79 amu compared with **1**, which indicated the lack of Br and a molecular formula of $C_{37}H_{60}N_4O_8$. This was supported by the absence of the 1:1 isotopic pattern for the $[M + H]^+$ peak when compared to **1**. The ^{1}H NMR spectrum of **3** showed an additional triplet at δ_H 1.93 with $J_{H,H}$ = 2.5 Hz (Table 2); otherwise it was virtually identical to that of **1**. This proton correlated to a methine at δ_C 68.8 and a quaternary C (δ_C 83.6) in the HSQC and HMBC spectra, respectively. These signals are indicative of a terminal alkyne; hence **3** had to bear a 3-hydroxy-2-methyl-7-octynoic acid (Hmoya) moiety in lieu of the Br-Hmoya present in **1** and **2**.

Veraguamide D (**4**) appeared closely related to **3**, as its ^{1}H NMR spectrum showed the acetylenic proton at δ_H 1.93 (Table 3). In comparison to **3**, the HRESIMS spectrum of **4** showed a positive difference of 14 amu, corresponding to an additional CH_2 unit and in agreement with a molecular formula of $C_{38}H_{62}N_4O_8$. The ^{1}H and ^{13}C NMR spectra of **4** showed a high-field CH_3 at δ_C 11.4/δ_H 0.93, characteristic of an Ile- or Ile-derived moiety. COSY (δ_H/δ_H 0.93/1.46, 1.46/2.02, 2.02/4.01) and (δ_C/δ_H 15.8/4.01, 28.7/4.01) correlations established that the *N*-Me-Val-1 residue is replaced by an *N*-Me-Ile in veraguamide D (**4**).

Compound **5** ($C_{39}H_{64}N_4O_8$) exhibited a close relationship to both **3** and **4**, showing a positive deviation of 28 and 14 amu, respectively, and also having the Hmoya moiety. The NMR data of **5** (Table 3) indicated the presence of two high-field methyl (δ_C 11.7/δ_H 0.96, δ_C 11.5/δ_H 0.85) and additional methylene (δ_C 26.6/δ_H 1.54, 1.06; δ_C 23.9/δ_H 1.47, 1.05) groups in comparison with **3**, which suggested that two isopropyl groups in the latter are replaced with *sec*-butyl groups in the former. This is further corroborated by HMBC and COSY correlations, which established the replacement of Val and *N*-Me-Val-2 moieties with Ile and *N*-Me-Ile, respectively, in veraguamide E (**5**). The *N*-Me-Val residue that was replaced by *N*-Me-Ile was located at different positions in **4** and **5**, with *N*-Me-Val-1 replaced in the former and *N*-Me-Val-2 in the latter. This NMR result was verified by MS/MS fragmentation of both **4** and **5** (Figure 1).

The ^{1}H NMR spectrum of veraguamide F (**6**) (Table 4) showed additional resonances for aromatic protons at δ_H 7.2−7.4 ppm, upfield-shifted *N*-Me protons to δ_H 2.60 ppm presumably due to shielding by the aromatic ring, and a low-field α-proton of the hydroxy acid (δ_H 5.47), with the acetylenic proton still present (δ_H 1.93). COSY correlations of δ_H 5.47 to diastereotopic CH_2 protons at δ_H 3.17/δ_H 2.91, together with HMBC correlations of the latter to aromatic carbons at δ_C 136.2 and 129.3 (Table 4), established the presence of phenyllactic acid (Pla) as the α-hydroxy acid in **6**. These NMR-derived conclusions fulfilled the molecular formula requirements for $C_{40}H_{58}N_4O_8$ based on HRESIMS of **6**.

Veraguamide G (**7**) lacked the acetylenic signal (δ_C 68.8/δ_H 1.93) observed for **3**−**6** and instead showed downfield resonances of a terminal methylene (δ_C 114.9/δ_H 4.97) and a methine (δ_C 138.2/δ_H 5.74). These signals indicated that the terminal alkyne group of the C_8-polyketide-derived moiety is replaced by a terminal vinyl group. This conclusion was further supported by the positive deviation of 2 amu compared to **3** and a molecular formula of $C_{37}H_{62}N_4O_8$. Hence, the Hmoya unit present in **3**−**6** was replaced by 3-hydroxy-2-methyl-7-octenoic acid (Hmoea) in **7**.

Enantioselective HPLC analysis coupled with mass spectrometry or UV detection of the acid hydrolysates of **1**−**7** allowed us

Table 1. NMR Data for Veraguamide A (1) in $CDCl_3$

unit	C/H no	δ_C[a]	δ_H (J in Hz)[b]	COSY[b]	HMBC[b]
Br-Hmoya	1	170.8, C			
	2	42.4, CH	3.11, br q (7.4)	H-3,H_3-9	1, 3, 4, 9
	3	76.4, CH	4.85, dt (10.2, 2.5)	H-2, H-4a, H-4b	1, 1 (*N*-Me-Val-1)
	4a	27.4, CH_2	2.06, m	H-3, H-4b, H-5a, H-5b	5, 6
	4b		1.59, m	H-3, H-4a, H-5a, H-5b	
	5a	25.0, CH_2	1.60, m	H-4a, H-4b, H-5b, H_2-6	7, 8
	5b		1.41, m	H-4a, H-4b, H-5a, H_2-6	6, 8
	6	19.2, CH_2	2.23, m	H-5a, H-5b	7, 8
	7	38.4, C			
	8	79.3, C			
	9	14.1, CH_3	1.25, d (7.4)	H-2	1, 2, 3
N-Me-Val-1	1	170.6, C			
	2	65.0, CH	3.93, d (10.3)	H-3	1, 3, 4, *N*-Me, 1 (Pro)
	3	28.3, CH	2.30, m	H-2, H_3-4, H_3-5	2, 4, 5
	4	19.56, CH_3	0.98, d (6.6)	H-3	2
	5	19.51, CH_3	0.91, d (6.6)	H-3	2
	N-Me	28.6, CH_3	3.00, s		2, 1 (Pro)
Pro	1	172.1, C			
	2	57.3, CH	4.94 dd (8.9, 4.5)	H-3a, H-3b	1, 3, 4, 1 (Hmpa)
	3a	29.5, CH_2	2.30, m	H-2, H-3b, H-4a	1, 2, 5
	3b		1.79, m	H-2, H-3a, H-4a	1, 2, 5
	4a	24.9, CH_2	2.03, m	H-3a, H-3b, H-4b, H-5a, H-5b	2, 3, 5
	4b		1.98, m	H-4a, H-5a, H-5b	2, 3, 5
	5a	47.3, CH_2	3.84, dt (−17.0, 7.1)	H-4a, H-4b, H-5b	2, 3, 4
	5b		3.61, dt (−17.0, 7.1)	H-4a, H-4b, H-5a	3, 4
Hmpa	1	165.9, C			
	2	76.1, CH	4.90, d (9.1)	H-3	1, 3, 4, 5, 1 (*N*-Me-Val-2)
	3	35.7, CH	1.97, m	H-2, H_3-6	
	4a	24.81, CH_2	1.54, m	H-4b, H_3-5	
	4b		1.13, m	H-4a, H_3-5	
	5	10.5, CH_3	0.87, t (7.3)	H-4a, H-4b	3, 4
	6	13.8, CH_3	1.01, d (6.8)	H-3	2, 3, 4
N-Me-Val-2	1	169.6, C			
	2	66.0, CH	4.15, d (9.5)	H-3	1, 3, 5, *N*-Me, 1 (Val)
	3	28.5, CH	2.27, m	H-2, H_3-4, H_3-5	1
	4	20.4, CH_3	1.00, d (7.0)	H-3	2
	5	20.2, CH_3	1.11, d (7.0)	H-3	2, 3
	N-Me	30.0, CH_3	2.94, s		2, 1 (Val)
Val	1	173.4, C			
	2	52.8, CH	4.70, dd (8.7, 6.5)	H-3, NH	1, 3, 4, 5, 1 (Br-Hmoya)
	3	32.1, CH	1.96, m	H-2, H_3-4, H_3-5	5
	4	20.3, CH_3	0.95, d (6.7)	H-3	2, 3, 5
	5	17.5, CH_3	0.88, d (6.7)	H-3	2, 3
	NH		6.25, d (8.7)	H-2	1, 1 (Br-Hmoya)

[a] 100 MHz. [b] 600 MHz.

to assign the absolute configuration of all the amino acids and α-hydroxy acid components as L and *S*, respectively. To determine the absolute configuration at C-2 and C-3 of the Br-Hmoya unit, veraguamide A (**1**) was subjected to methanolysis to yield the linear fragment **9** (Figure 2). The observed coupling constant of 3.2 Hz was characteristic for a *syn* configuration, whereas a coupling constant near 6.3 Hz would have been expected for the *anti* configuration.[11] The absolute configuration at C-3 and consequently for C-2 of the Br-Hmoya unit of **9** was determined using Mosher's analysis. The derived $\Delta\delta$ values (Figure 2) predicted an *R* configuration at C-3, and hence from the relative configuration, C-2 should have an *S* configuration. Of note, comparison of the $^3J_{H,H}$ values of H-2 and H-3 with a model system to assign the relative configuration could only be applied when C-3 bears a free hydroxy group. This moiety is involved in intramolecular H-bonding with the adjacent carbonyl group, thus hindering free bond rotation across C-2 and C-3.[12,13] Accordingly, the corresponding MTPA esters (**10**, **11**) did not show the

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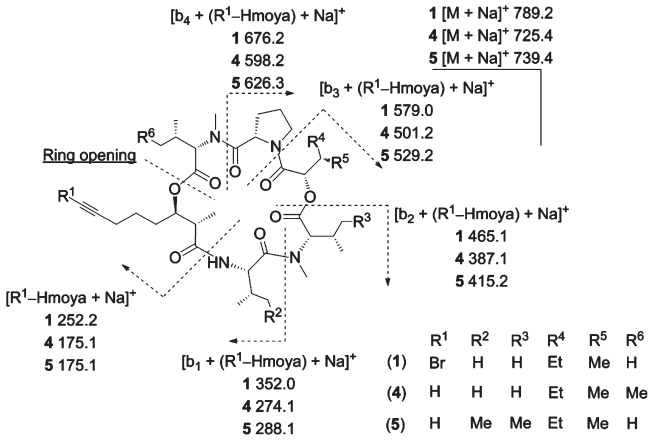

Figure 1. MS/MS fragmentation of veraguamide A (**1**), veraguamide D (**4**), and veraguamide E (**5**).

same $^3J_{H,H}$ values for H-2 and H-3 as that of **9**. The same absolute configuration at C-2 and C-3 for Hmoya, Hmoea, and 3-hydroxy-2-methyloctanoic acid (Hmoaa) is expected on the basis of virtually identical ^{13}C NMR shifts and specific rotations observed for **1**−**8**.

To gain insight into structure−activity relationships, veraguamide A (**1**) was partially (Lindlar catalyst, H_2) and fully (Pd/C, H_2) hydrogenated to yield the semisynthetic veraguamide G (**7**) and tetrahydroveraguamide A (**8**), respectively. The cytotoxic activities of **1**−**8**, semisynthetic veraguamide G (**7**), and dolastatin 16 were evaluated for effects on viability of HT29 colorectal and HeLa cervical adenocarcinoma cells (Table 6). The IC_{50} values of the natural and semisynthetic veraguamide G (**7**) were comparable, suggesting the activities of these compounds were not likely due to traces of highly biologically active impurities. The most active in this series of compounds are veraguamides D (**4**) and E (**5**), with IC_{50} values more than 5-fold lower than those for their related congener veraguamide C (**3**). This suggested that increased hydrophobicity of specific units (II, IV, V, VI) increased the cytotoxicity of this compound class, with the position having minimal effect on the bioactivity, as **4** and **5** showed comparable IC_{50}'s. However, modification with bulkier groups is detrimental to the activity, as exemplified by a close to 10-fold decrease in the cytotoxic activity of **6** (Table 6) compared to **3**, where a phenyllactic acid (Pla) moiety is introduced at position IV of the former instead of the Hmpa unit. The C_8-polyketide-derived moiety also plays a role in the cytotoxicity of these compounds. Comparing the biological activities of related compounds **1**, **3**, **7**, and **8**, weaker cytotoxicity was observed for compounds with a Br-Hmoya or Hmoaa unit, while compounds with Hmoya or Hmoea were about equally potent. This then suggests the importance of a π-system combined with the presence of acetylenic or vinylic protons in this moiety for cytotoxic activity.

The veraguamides are reminiscent of other cyanobacterial compounds such as hantupeptins,[13,14] antanapeptins,[15] and trungapeptins.[16] These compounds are also cyclic hexadepsipeptides with the characteristic C_8-polyketide-derived units as Hmoya, Hmoea, or Hmoaa. Veraguamide F (**6**) is a constitutional isomer of antanapeptin D,[15] where an *N*-Me-Phe and Hiva are present in the latter instead of Pla and *N*-Me-Val as in **6**. It is interesting that subtle changes in structure of these compounds have a profound effect on the cytotoxicity. Antanapeptins A−D (brine shrimp) as well as trungapeptin A (KB and LoVo cells) did not display cytotoxicity at the reported concentrations (10 μg/mL),[15,16] while hantupeptins A−C were cytotoxic against MOLT-4 leukemia and MCF7 breast cancer cells, with hantupeptin A being the most active in this series, with IC_{50}'s of 32 nM and 4.0 μM, respectively.[13] Trungapeptins B and C were not tested for cytotoxicity.[16]

Although dolastatin 16 has been isolated previously from sea hares[8] and cyanobacteria,[15,17] its stereostructure has been only partially assigned. Therefore we analyzed the remaining dolaphenvaline (Dpv) and dolamethylleucine (Dml) units. The absolute configuration of the Dpv unit in dolastatin 16 was determined as 2*S*,3*R* using enantioselective HPLC-MS analysis of the FDLA-derivatized acid hydrolysate in comparison with that for pitiprolamide.[18] The configuration of the Dpv unit in dolastatin 16 is the same as in pitiprolamide and kulokekahilide-1 determined from X-ray crystallography and chemical synthesis, respectively.[18,19] To determine the configuration of the stereocenters in the Dml unit, we carried out acid and base hydrolysis of dolastatin 16 to yield the Dml unit and the linear fragment **12**, respectively. The Dml unit is related to the 3-amino-2-methylpentanoic acid (Map) unit, present in dolastatin 12[20,21] and majusculamide C,[22] as well as the 3-amino-2-methylhexanoic acid (Amha) unit in kulokekahilide-1,[19] lyngbyastatin 1,[21] and the ulongamides.[23] For all these α-substituted β-amino acids, the corresponding Marfey's adducts of the two 3*R* isomers (2*S*,3*R* and 2*R*,3*R*) consistently eluted later by reversed-phase C_{18} HPLC than those of the two 3*S* isomers (2*S*,3*S* and 2*R*,3*S*), while the C-2 stereoisomers are poorly resolved and variable.[19−23] Extrapolation to the elution order we obtained for the Dml suggested the presence of 3*R* in the Dml unit of dolastatin 16.[24] To assign the configuration at C-2 of Dml, modified Mosher's analysis using phenylglycine methyl ester (PGME)[25] derivatization of **12** (Figure 3) suggested a 2*R* configuration. The applicability of this chiral derivatization technique has been demonstrated for α- and β-substituted carboxylic acids,[25] but may not have been widely used for α,β-disubstituted carboxylic acids. From the $\Delta\delta$ values that we obtained (Figure 3), it is predicted that there is some deviation from the presumed conformation of the PGME amide, but nonetheless a systematic arrangement of positive and negative $\Delta\delta$ values was obtained. Therefore, we propose a 2*R*,3*R* configuration for the Dml unit of dolastatin 16. The ^{13}C NMR chemical shifts for the Dpv and Dml unit in both dolastatin 16 and homodolastatin 16 were similar, suggesting the latter would also have (2*S*,3*R*)-Dpv and (2*R*,3*R*)-Dml units.[8,26] Dolastatin 16 showed weak cytotoxic activity, with IC_{50}'s of 78 and 58 μM for HT29 and HeLa cell lines, respectively. These values are significantly higher than those reported by Pettit et al. against a different panel of cell lines.[8]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 polarimeter. UV spectra were recorded on a SpectraMax M5 (Molecular Devices). ^{1}H and 2D NMR spectra were recorded in $CDCl_3$ on a Bruker Avance II 600 MHz spectrometer equipped with a 5 mm TXI cryogenic probe using residual solvent signals (δ_H 7.26; δ_C 77.0) as internal standards. HSQC and HMBC experiments were optimized for $^1J_{CH}$ = 145 and $^nJ_{CH}$ = 7 Hz, respectively. TOCSY experiments were done using a mixing time of 100 ms. ^{13}C NMR spectra were recorded on a Varian 400 MHz or Bruker 500 MHz NMR spectrometer. HRESIMS data were obtained

Table 2. NMR Data for Veraguamides B (2) and C (3) in $CDCl_3$

		2		3	
unit	C/H no.	δ_C[a]	δ_H (J in Hz)[b]	δ_C[a]	δ_H (J in Hz)[b]
Br-Hmoya[c]/	1	170.8, C		170.8, C	
Hmoya[d]	2	42.3, CH	3.13, br q (7.4)	42.4, CH	3.10, br q (7.2)
	3	76.4, CH	4.85, d (8.7)	76.4, CH	4.86, dt (10.4, 2.5)
	4a	27.5, CH_2	2.07, m	27.4, CH_2	2.07, m
	4b		1.60, m		1.62, m
	5a	24.93, CH_2	1.61, m	25.2, CH_2	1.62, m
	5b		1.42, m		1.44, m
	6	19.2, CH_2	2.21, m	18.0, CH_2	2.19, m
	7	38.4, C		83.6, C	
	8	79.4, C		68.8, CH	1.93 t (2.5)
	9	14.6, CH_3	1.25, d (7.4)	14.5, CH_3	1.25, d (7.2)
N-Me-Val-1	1	170.7, C		170.7, C	
	2	65.0, CH	3.94, d (10.4)	65.0, CH	3.93, d (11.0)
	3	28.26, CH	2.29, m	28.3, CH	2.28, m
	4	19.57, CH_3	0.98, d (6.5)	19.58, CH_3	0.98, d (6.8)
	5	19.55, CH_3	0.92, d (6.5)	19.56, CH_3	0.91, d (6.8)
	N-Me	28.7, CH_3	3.00, s	28.7, CH_3	3.00, s
Pro	1	172.1, C		172.2, C	
	2	57.2, CH	4.95 dd (8.6, 4.8)	57.3, CH	4.94 dd (8.4, 5.0)
	3a	29.4, CH_2	2.28, m	29.5, CH_2	2.28, m
	3b		1.79, m		1.79, m
	4a	24.99, CH_2	2.04, m	24.89, CH_2	2.03, m
	4b		1.98, m		1.99, m
	5a	47.3, CH_2	3.80, dt (−16.4, 6.9)	47.3, CH_2	3.84, dt (−16.8, 7.1)
	5b		3.60, dt (−16.4, 6.9)		3.60, dt (−16.8, 7.1)
Hiva[c]/Hmpa[d]	1	165.8, C		165.9, C	
	2	77.2, CH	4.85, d (8.7)	76.0, CH	4.89, d (9.4)
	3	29.6, CH	2.17, m	35.7, CH	1.98, m
	4	18.1, CH_3	1.02, t (6.6)	24.81, CH_2	1.54, m
					1.13, m
	5	18.5, CH_3	0.93, d (6.6)	10.5, CH_3	0.86, t (7.3)
	6			13.8, CH_3	1.01, d (6.7)
N-Me-Val-2	1	169.6, C		169.6, C	
	2	66.1, CH	4.15, d (10.2)	66.0, CH	4.13, d (10.0)
	3	28.34, CH	2.28, m	28.5, CH	2.28, m
	4	20.3, CH_3	0.99, d (6.8)	20.4, CH_3	0.99, d (6.4)
	5	20.1, CH_3	1.11, d (6.8)	20.2, CH_3	1.10, d (6.4)
	N-Me	30.0, CH_3	2.94, s	30.0, CH_3	2.93, s
Val	1	173.5, C		173.4, C	
	2	52.8, CH	4.71, dd (8.6, 6.4)	52.8, CH	4.70, dd (8.6, 6.2)
	3	32.2, CH	1.98, m	32.1, CH	1.96, m
	4	20.3, CH_3	0.94, d (6.4)	20.3, CH_3	0.94, d (6.7)
	5	17.5, CH_3	0.88, d (6.4)	17.6, CH_3	0.87, d (6.7)
	NH		6.26, d (8.6)		6.26, d (8.6)

[a] 100 MHz. [b] 600 MHz. [c] Refers to compound **2**. [d] Refers to compound **3**.

using an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector. LRESIMS measurements and MS/MS fragmentation were done on an ABI 3200Q TRAP.

Biological Material. The *Symploca* cf. *hydnoides* cyanobacterium was collected by hand while snorkeling in the shallow waters of the southern fore-reef (1−3 m) of Cetti Bay, Guam, on April 17, 2009. A voucher specimen, which is preserved in 100% EtOH, is deposited in the University of Guam Herbarium (accession no. GUAM-GH11446). A voucher specimen is also retained at the Smithsonian Marine Station, Fort Pierce, FL.

Extraction and Isolation. The freeze-dried cyanobacterium (142.0 g) was extracted with EtOAc−MeOH (1:1) to yield the nonpolar extract (11.6 g). This was partitioned between hexanes and 20% aqueous MeOH, the latter concentrated under reduced pressure and further

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Table 3. NMR Data for Veraguamides D (4) and E (5) in $CDCl_3$

		4		5	
unit	C/H no.	δ_C[a]	δ_H (J in Hz)[b]	δ_C[a]	δ_H (J in Hz)[b]
Hmoya	1	170.8, C		170.71, C	
	2	42.4, CH	3.13, br q (7.2)	42.4, CH	3.08, br q (7.4)
	3	76.4, CH	4.86, dt (10.8, 2.6)	76.5, CH	4.85, d (9.0)
	4a	27.5, CH_2	2.07, m	27.5, CH_2	2.06, m
	4b		1.63, m		1.62, m
	5a	25.2, CH_2	1.63, m	25.2, CH_2	1.61, m
	5b		1.47, m		1.43, m
	6	17.5, CH_2	2.18, m	18.0, CH_2	2.18, m
	7	83.6, C		83.6, C	
	8	68.8, CH	1.93, t (2.5)	68.8, CH	1.93, t (2.3)
	9	14.4, CH_3	1.24, d (7.2)	14.5, CH_3	1.23, d (7.4)
N-Me-Ile[c]/	1	170.7, C		170.69, C	
N-Me-Val[d]	2	64.0, CH	4.01, d (10.6)	64.9, CH	3.93, d (10.0)
	3	34.6, CH	2.02, m	28.3, CH	2.29, m
	4	25.7, CH_2	1.46, m	19.59, CH_3	0.91, d (6.4)
	5	11.4, CH_3	0.93, t (6.5)	19.56, CH_3	0.98, d (6.4)
	6	15.8, CH_3	0.94, d (6.8)		
	N-Me	28.7, CH_3	2.99, s	28.6, CH_3	3.00, s
Pro	1	172.7, C		172.2, C	
	2	57.2, CH	4.94 dd (8.9, 4.8)	57.3, CH	4.94 dd (9.0, 5.3)
	3a	28.8, CH_2	2.26, m	29.5, CH_2	2.29, m
	3b		1.78, m		1.78, m
	4a	24.9, CH_2	2.03, m	24.89, CH_2	2.01, m
	4b		1.97, m		1.99, m
	5a	47.2, CH_2	3.82, dt (−17.0, 7.3)	47.3, CH_2	3.86, dt (−17.0, 7.0)
	5b		3.60, dt (−17.0, 7.3)		3.60, dt (−17.0, 7.0)
Hmpa	1	165.9, C		166.0, C	
	2	76.0, CH	4.90, d (9.2)	76.1, CH	4.85, d (9.0)
	3	35.7, CH	1.98, m	35.1, CH	1.98, m
	4	24.8, CH_2	1.53, m	24.86, CH_2	1.54, m
			1.12, m		1.13, m
	5	10.5, CH_3	0.86, t (7.6)	10.5, CH_3	0.86, t (7.0)
	6	13.9, CH_3	0.99, d (6.9)	13.8, CH_3	1.01, d (6.8)
N-Me-Val[c]/	1	169.6, C		169.7, C	
N-Me-Ile[d]	2	66.0, CH	4.15, d (9.4)	65.2, CH	4.22, d (9.6)
	3	28.4, CH	2.28, m	35.7, CH	1.98, m
	4	20.3, CH_3	1.10, d (6.8)	26.6, CH_2	1.54, m
					1.06, m
	5	20.2, CH_3	0.99, d (6.8)	11.7, CH_3	0.96, t (7.2)
	6			16.5, CH_3	1.04, d (6.9)
	N-Me	30.0, CH_3	2.92, s	30.1, CH_3	2.93, s
Val[c]/Ile[d]	1	173.4, C		173.5, C	
	2	52.8, CH	4.70, dd (8.6, 6.6)	52.4, CH	4.70, dd (8.4, 6.7)
	3	32.1, CH	1.98, m	38.6, CH	1.69, m
	4	19.0, CH_3	0.94, d (6.6)	23.9, CH_2	1.47, m
	5	17.6, CH_3	0.87, d (6.6)		1.05, m
				11.5, CH_3	0.85, d (6.6)
	6			16.3, CH_3	0.91, d (6.6)
	NH		6.24, d (8.6)		6.26, d (8.4)

[a] 125 MHz. [b] 600 MHz. [c] Refers to compound **4**. [d] Refers to compound **5**.

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Table 4. NMR Data for Veraguamide F (6) in $CDCl_3$

unit	C/H no.	δ_C[a]	δ_H (J in Hz)[b]
Hmoya	1	170.9, C	
	2	42.2, CH	3.24 br q (7.3)
	3	76.6, CH	4.89, dt (10.6, 2.4)
	4a	27.5, CH_2	2.08, m
	4b		1.64, m
	5a	25.2, CH_2	1.65, m
	5b		1.45, m
	6	18.0, CH_2	2.20, m
	7	83.5, C	
	8	68.9, CH	1.93, t (2.5)
	9	14.6, CH_3	1.29, d (7.3)
N-Me-Val-1	1	170.7, C	
	2	65.3, CH	3.95, d (10.4)
	3	28.3, CH	2.32, m
	4	19.6, CH_3	1.00, d (6.5)
	5	19.7, CH_3	0.93, d (6.5)
	N-Me	28.7, CH_3	3.06, s
Pro	1	172.3, C	
	2	57.1, CH	4.97, dd (9.0, 4.5)
	3a	29.1, CH_2	2.27, m
	3b		1.83, m
	4a	25.0, CH_2	2.07, m
	4b		1.97, m
	5a	47.0, CH_2	3.62, m
	5b		3.56, m
Pla	1	165.4, C	
	2	72.8, CH	5.47, dd (9.8, 4.0)
	3	36.7, CH_2	3.17, m
			2.91, m
	4	136.2, C	
	5//9	129.3, CH	7.18, d (8.0)
	6/8	128.5, CH	7.28, m
	7	126.7, CH	7.20, m
N-Me-Val-2	1	168.9, C	
	2	65.8, CH	4.05, d (10.5)
	3	27.4, CH	2.08, m
	4	19.8, CH_3	0.89, d (6.8)
	5	20.0, CH_3	0.91, d (6.8)
	N-Me	29.0, CH_3	2.60, s
Val	1	173.4, C	
	2	52.7, CH	4.76, dd (8.5, 6.1)
	3	32.3, CH	1.98, m
	4	20.3, CH_3	0.91, d (6.6)
	5	17.7, CH_3	0.88, d (6.6)
	NH		6.28, d (8.5)

[a] 100 MHz. [b] 600 MHz.

partitioned between *n*-BuOH and H_2O. The *n*-BuOH fraction was concentrated to dryness (2.84 g) and chromatographed on Si gel eluting first with CH_2Cl_2, followed by increasing concentrations of *i*-PrOH; after 100% *i*-PrOH, increasing gradients of MeOH were used. The 20% *i*-PrOH fraction was subjected to a C_{18} SPE eluting with 25%, 50%, 75%, and 100% MeOH in H_2O. The 100% MeOH fraction was purified by semipreparative reversed-phase HPLC (Phenomenex Synergi-Hydro RP, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient

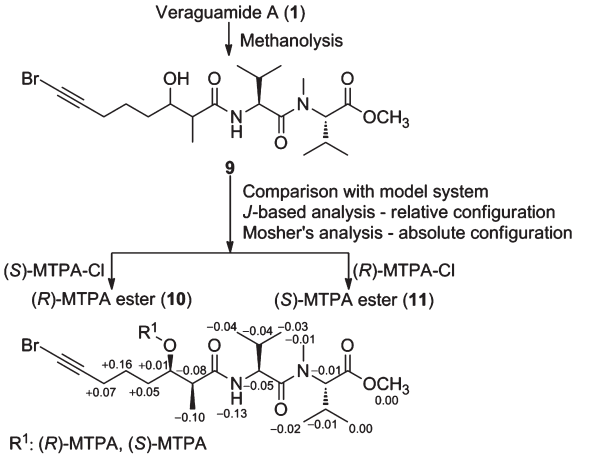

Figure 2. Assignment of absolute configuration of veraguamide A (**1**) using methanolysis and subsequent Mosher's analysis. $\Delta\delta = \delta(S\text{-MTPA ester}) - \delta(R\text{-MTPA ester})$.

of MeOH−H_2O (70%−100% MeOH in 60 min and then 100% MeOH for 10 min) to yield dolastatin 16 (t_R 32.6 min, 21.6 mg), semipure veraguamide C (t_R 36.4 min, 15.1 mg), semipure veraguamide F (t_R 37.4 min, 10.0 mg), a mixture of veraguamides B and D (t_R 40.0 min, 25.0 mg), veraguamide A (**1**) (t_R 42.6 min, 25.9 mg), and a mixture of veraguamides E and G (t_R 43.7 min, 9.0 mg).

The final purification of the semipure veraguamide C (**3**) was achieved using semipreparative HPLC (Phenomenex Phenyl-Hexyl, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient of MeOH−H_2O (85%−100% MeOH in 40 min and then 100% MeOH for 5 min) to yield veraguamide C (**3**) (t_R 19.9 min, 10.7 mg). Using the same chromatographic conditions, purification of the semipure veraguamide F yielded veraguamide F (**6**) (t_R 21.7 min, 6.8 mg). The mixture of veraguamides B and D was resolved using the same chromatographic condition with a different linear gradient (70%−100% MeOH in 45 min and then 100% MeOH for 10 min) to yield veraguamide D (**4**) (t_R 36.6 min, 4.0 mg) and veraguamide B (**2**) (t_R 37.3 min, 11.5 mg). The mixture of veraguamides E and G was further purified using the same chromatographic conditions to yield veraguamide G (**7**) (t_R 39.9 min, 4.4 mg) and veraguamide E (**5**) (t_R 40.5 min, 3.6 mg).

Hydrogenation of 1. A catalytic amount of 10% Pd/C was added to a methanolic solution of **1** (1.8 mg/mL). The reaction was left to stir for 6 h under a hydrogen balloon. The catalyst was filtered through a Celite pad, and the filtrate, upon concentration, was purified by semipreparative HPLC (Phenomenex Phenyl-Hexyl, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient of MeOH−H_2O (70%−100% MeOH in 45 min and then 100% MeOH for 10 min) to yield **8** (t_R 40.9 min, 1.2 mg).

Partial hydrogenation of **1** was carried out with Lindlar catalyst, using the same reaction and chromatographic conditions stated above. This afforded the semisynthetic veraguamide G (t_R 39.9 min, 1.7 mg). The LRESIMS and ^{1}H NMR spectra of the semisynthetic veraguamide G were in good agreement with the spectra for the natural product (**7**).

*Veraguamide A (**1**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −44 (*c* 0.44, MeOH); UV (MeOH) λ_{max} (log ε) 202 (6.29); IR (film) ν_{max} 3428, 2966, 2877, 2361, 2334, 1736, 1647, 1190 cm^{-1}; ^{1}H NMR, ^{13}C NMR, COSY, and HMBC data, see Table 1; HRESIMS *m/z* 767.3675 [M + H]$^+$ (calcd for $C_{37}H_{60}{}^{79}BrN_4O_8$, 767.3594), *m/z* [M + H]$^+$ 769.3660 (calcd for $C_{37}H_{60}{}^{81}BrN_4O_8$, 769.3574) (100:100 [M + H]$^+$ ion cluster).

*Veraguamide B (**2**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −40 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.30); IR (film) ν_{max} 3378,

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Table 5. NMR Data for Veraguamide G (7) and Tetrahydroveraguamide A (8) in $CDCl_3$

		7		8	
unit	C/H no.	δ_C[a]	δ_H (J in Hz)[b]	δ_C[b,c]	δ_H (J in Hz)[b]
Hmoea[d]/	1	170.9, C		170.7, C	
Hmoaa[e]	2	42.4, CH	3.10, br q (7.4)	42.1, CH	3.08, br q (7.4)
	3	76.8, CH	4.85, dt (10.6, 2.4)	76.8, CH	4.86, dt (10.1, 2.1)
	4a	27.9, CH_2	1.98, m	31.0, CH_2	1.21, m
	4b		1.45, m		1.26, m
	5a	25.5, CH_2	1.48, m	28.2, CH_2	1.39, m
	5b		1.30, m		
	6a	33.2, CH_2	2.05, m	25.8, CH_2	1.39, m
	6b				1.20, m
	7	138.2, CH	5.74, m	22.2, CH_2	1.26, m
	8	114.9, CH_2	4.97, m	13.6, CH_3	0.85, t (6.9)
	9	14.4, CH_3	1.22, d (7.4)	14.0, CH_3	1.23, d (7.4)
N-Me-Val-1	1	170.7, C		170.7, C	
	2	65.0, CH	3.93, d (9.8)	64.9, CH	3.93, d (10.7)
	3	28.3, CH	2.28, m	28.3, CH	2.28, m
	4	19.59, CH_3	0.98, d (6.4)	19.2, CH_3	0.98, d (6.5)
	5	19.54, CH_3	0.92, d (6.4)	19.3, CH_3	0.91, d (6.5)
	N-Me	28.6, CH_3	3.01, s	28.4, CH_3	3.00, s
Pro	1	172.2, C		172.0, C	
	2	57.3, CH	4.95, dd (8.7, 5.0)	57.1, CH	4.94, dd (9.0, 5.0)
	3a	29.4, CH_2	2.29, m	29.1, CH_2	2.28, m
	3b		1.79, m		1.79, m
	4a	24.9, CH_2	2.03, m	24.6, CH_2	2.03, m
	4b		1.98, m		1.99, m
	5a	47.3, CH_2	3.84, dt (−16.7, 7.1)	47.0, CH_2	3.84, dd (−17.0, 7.3)
	5b		3.61, dt (−16.7, 7.1)		3.60, dd (−17.0, 7.3)
Hmpa	1	165.9, C		165.7, C	
	2	76.0, CH	4.90, d (8.7)	76.6, CH	4.90, d (8.8)
	3	35.7, CH	1.98, m	35.4, CH	1.98, m
	4	24.8, CH_2	1.54, m	24.5, CH_2	1.54, m
			1.13, m		1.13, m
	5	10.5, CH_3	0.86, t (7.3)	10.2, CH_3	0.86, t (7.1)
	6	13.8, CH_3	1.00, d (6.0)	13.5, CH_3	1.00, d (6.4)
N-Me-Val-2	1	169.6, C		169.5, C	
	2	66.0, CH	4.15, d (10.2)	65.8 CH	4.14, d (9.6)
	3	28.6, CH	2.28, m	28.1, CH	2.28, m
	4	20.4, CH_3	1.00, d (6.1)	20.0, CH_3	0.99, d (6.6)
	5	20.2, CH_3	1.10, d (6.1)	19.9, CH_3	1.10, d (6.6)
	N-Me	30.0, CH_3	2.93, s	29.7, CH_3	2.93, s
Val	1	173.4, C		173.3, C	
	2	52.7, CH	4.70, dd (8.6, 6.7)	52.5, CH	4.70, dd (8.6, 6.2)
	3	32.1, CH	1.98, m	31.7, CH	1.96, m
	4	20.3, CH_3	0.93, d (6.8)	19.3, CH_3	0.93, d (6.3)
	5	17.6, CH_3	0.86, d (6.8)	17.2, CH_3	0.87, d (6.3)
	NH		6.23, d (8.6)		6.26, d (8.6)

[a] 125 MHz. [b] 600 MHz. [c] Based on HSQC and HMBC. [d] Refers to compound **7**. [e] Refers to compound **8**.

2966, 2886, 1738, 1657, 1190 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 2; HRESIMS m/z 753.3517 $[M + H]^+$ (calcd for $C_{36}H_{58}{}^{79}BrN_4O_8$, 753.3438), m/z $[M + H]^+$ 755.3508 (calcd for $C_{36}H_{58}{}^{81}BrN_4O_8$, 755.3418) (100:100 $[M + H]^+$ ion cluster).

*Veraguamide C (**3**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −44 (*c* 0.31, MeOH); UV (MeOH); λ_{max} (log ε) 202 (4.17); IR (film) ν_{max} 3429, 2965, 2871, 1732, 1650, 1190 cm^{-1};^1H NMR and ^{13}C NMR data, see Table 2; HRESIMS m/z 689.4486 $[M + H]^+$ (calcd for $C_{37}H_{61}N_4O_8$, 689.4490).

*Veraguamide D (**4**):* colorless, amorphous solid; $[\alpha]^{20}_D$ −57 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.30); IR (film) ν_{max} 3378, 2958, 2878, 1732, 1647 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 3;

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Table 6. Cytotoxic Activity (IC_{50}, μM) of Natural and Semisynthetic Veraguamides

compound	HT29	HeLa
veraguamide A (**1**)	26	21
veraguamide B (**2**)	30	17
veraguamide C (**3**)	5.8	6.1
veraguamide D (**4**)	0.84	0.54
veraguamide E (**5**)	1.5	0.83
veraguamide F (**6**)	49	49
veraguamide G (**7**)	2.7	2.3
tetrahydroveraguamide A (**8**)	33	48
dolastatin 16	78	58

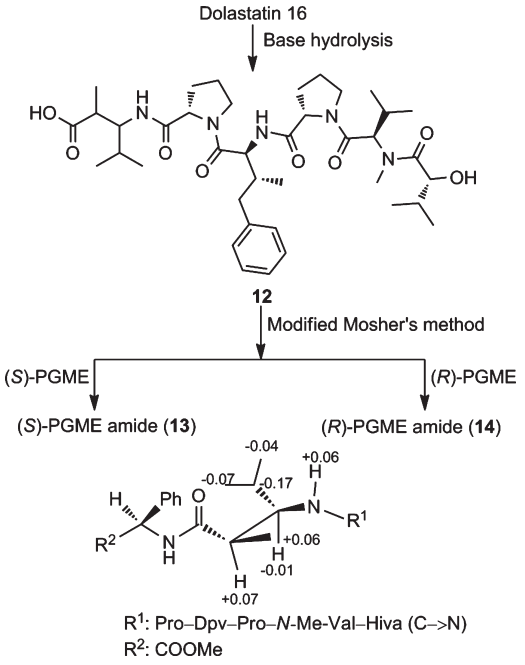

Figure 3. Assignment of absolute configuration of dolastatin 16 by base hydrolysis and modified Mosher's analysis using PGME. $\Delta\delta = \delta(S\text{-PGME amide}) - \delta(R\text{-PGME amide})$.

HRESIMS m/z 703.4639 $[M + H]^+$ (calcd for $C_{38}H_{63}N_4O_8$, 703.4646).

Veraguamide E (***5***): colorless, amorphous solid; $[\alpha]^{20}_D$ −56 (*c* 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.30); IR (film) ν_{max} 3320, 2958, 2877, 1733, 1647 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 3; HRESIMS m/z 717.4799 $[M + H]^+$ (calcd for $C_{39}H_{65}N_4O_8$, 717.4802).

Veraguamide F (***6***): colorless, amorphous solid; $[\alpha]^{20}_D$ −41 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.33); IR (film) ν_{max} 3450, 3306, 2965, 2871, 1737, 1661, 1647, 1490 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 4; HRESIMS m/z 723.4411 $[M + H]^+$ (calcd for $C_{40}H_{59}N_4O_8$, 723.4333).

Veraguamide G (***7***): colorless, amorphous solid; $[\alpha]^{20}_D$ −48 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.26); IR (film) ν_{max} 3436, 2965, 2879, 1740, 1661, 1646 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 5; HRESIMS m/z 691.4649 $[M + H]^+$ (calcd for $C_{37}H_{63}N_4O_8$, 691.4646).

Tetrahydroveraguamide A (***8***): colorless, amorphous solid; $[\alpha]^{20}_D$ −43 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.33); ^1H NMR and ^{13}C NMR data, see Table 5; HRESIMS m/z 693.4791 $[M + H]^+$ (calcd for $C_{37}H_{65}N_4O_8$, 693.4802).

Dolastatin ***16***: colorless, amorphous solid; $[\alpha]^{20}_D$ +22 (*c* 0.18, MeOH) {lit.[8] $[\alpha]^{20}_D$ +15.5 (*c* 0.20, MeOH)}; UV (MeOH) λ_{max} (log ε) 202 (4.51); IR (film) ν_{max} 3407, 2965, 2871, 1733, 1647 cm^{-1}; ^1H NMR and ^{13}C NMR data are identical with literature values[8] (Supporting Information); LRESIMS m/z 879.6 $[M + H]^+$, 901.3 $[M + Na]^+$.

Acid Hydrolysis of Veraguamides and Enantioselective Analysis. Portions of **1**−**7** (100 μg) were acid-hydrolyzed (200 μL of 6 N HCl, 110 °C, 20 h), and the product mixtures dried, reconstituted in 100 μL of H_2O, and analyzed by enantioselective HPLC-UV and enantioselective HPLC-MS. The absolute configurations of the amino acids *N*-Me-Ile, Ile, *N*-Me-Val, Val, and Pro were determined by enantioselective HPLC-MS [column, Chirobiotic TAG (250 × 4.6 mm), Supelco; solvent, MeOH−10 mM NH_4OAc (40:60, pH 5.30); flow rate, 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan)]. The acid hydrolysates of **1**−**4**, **6**, and **7** showed retention times at 7.8, 11.6, and 13.6 min corresponding to L-Val, *N*-Me-L-Val, and L-Pro, respectively. The acid hydrolysate of **4** in addition showed a retention time at 12.4 min, corresponding to *N*-Me-L-Ile. The acid hydrolysate of **5** showed retention times at 8.4, 11.6, 12.4, and 13.6 min, corresponding to L-Ile, *N*-Me-L-Val, *N*-Me-L-Ile, and L-Pro, respectively. The retention times (t_R, min; MRM ion pair) of the authentic amino acids were as follows: *N*-Me-L-Val (11.6; 132→86), *N*-Me-D-Val (34.3), L-Val (7.8; 118→72), D-Val (13.7), *N*-Me-L-Ile (12.4; 146→100), *N*-Me-L-*allo*-Ile (15.0), *N*-Me-D-Ile (49.0), *N*-Me-D-*allo*-Ile (51.0), L-Ile (8.4; 132→86), L-*allo*-Ile (8.6), D-*allo*-Ile (17.6), D-Ile (20.2), L-Pro (13.6; 116→70), D-Pro (36.0). Compound-dependent parameters used were as follows: *N*-Me-Val: DP 29.4, EP 4.2, CE 17.4, CXP 2.7, CEP 10.6; Val: DP 5.7, EP 9.0, CE 40.0, CXP 8.0, CEP 10.0; *N*-Me-Ile: DP 35.0, EP 7.0, CE 17.0, CXP 2.0, CEP 10.0; Ile: DP 40.0, EP 9.0, CE 15.0, CXP 3.0, CEP 8.0; Pro: DP 35.0, EP 7.7, CE 22.7, CXP 5.0, CEP 10.3. Source gas parameters used were as follows: CUR 40, CAD Medium, IS 4500, TEM 750, GS1 65, GS2 65.

The absolute configurations of the α-hydroxy acids [2-hydroxyisovaleric acid (Hiva), 2-hydroxy-3-methylpentanoic acid (Hmpa), and phenyllactic acid (Pla)] were determined using enantioselective HPLC [column, CHIRALPAK MA (+) (50 × 4.6 mm); solvent, CH_3CN−2 mM $CuSO_4$ (10:90); flow rate, 1.0 mL/min; detection by UV (254 nm)]. The acid hydrolysates of **1**, **3**−**5**, and **7** each showed peaks at 33.0 min, corresponding to (2*S*,3*S*)-Hmpa. The acid hydrolysate of **2** contained a component that had a retention time at 10.0 min, corresponding to (2*S*)-Hiva, while **6** gave a peak at 51.0 min, corresponding to (2*S*)-Pla. The retention times of the authentic standards were as follows: (2*R*)-Hiva (6.0), (2*S*)-Hiva (10.0), (2*R*,3*S*)-Hmpa (16.0), (2*R*,3*R*)-Hmpa (19.0), (2*S*,3*R*)-Hmpa (26.0), (2*S*,3*S*)-Hmpa (33.0), (2*R*)-Pla (33.5), (2*S*)-Pla (51.0). All other amino acid units eluted within less than 5.0 min using this chromatographic condition.

Methanolysis of 1. Compound **1** (5.0 mg) was dissolved in 2.0 mL of 5% (w/w) methanolic KOH solution and stirred for 24 h at room temperature. The solvent was evaporated, and the residue was partitioned between CH_2Cl_2 and H_2O. The organic layer was collected, dried over anhydrous $MgSO_4$, and concentrated to dryness under nitrogen. The crude methanolysis product was further purified by semipreparative reversed-phase HPLC (Phenomenex Synergi-Hydro RP, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient of MeOH−H_2O (70%−100% MeOH in 60 min and then 100% MeOH for 10 min) to yield **9** (t_R 19.3 min, 1.4 mg).

9: colorless, amorphous solid; ^1H NMR ($CDCl_3$) δ 6.30 (d, J = 8.1 Hz, 1H), 4.92 (d, J = 10.5 Hz, 1H), 4.76 (dd, J = 9.1, 6.6 Hz, 1H), 3.78 (ddd, J = 8.6, 4.4, 3.2 Hz, 1H), 3.49 (s, 3H), 3.08 (s, 3H), 2.42 (qd, J = 6.8, 3.2 Hz, 1H), 2.23 (m, 2H), 2.03 (m, 1H), 1.74 (m, 2H), 1.56 (m, 1H), 1.46 (m, 2H), 1.01 (d, J = 6.8 Hz, 3H), 0.98 (d, J = 6.8 Hz, 3H), 0.93

(d, J = 6.8 Hz, 3H), 0.85 (d, J = 6.8 Hz, 3H); HRESIMS m/z 497.1640 $[M + Na]^+$ (calcd for $C_{21}H_{35}{}^{79}BrN_2O_5Na$, 497.1627), m/z $[M + Na]^+$ 499.1616 (calcd for $C_{21}H_{35}{}^{81}Br\ N_2O_5Na$, 499.1607) (100:100 $[M + Na]^+$ ion cluster).

Preparation of MTPA Esters. The methanolysis product **9** was dissolved in 50 μL of $CDCl_3$ and was divided into two equal portions; to each was added 0.75 mL of triethylamine. To one portion was added 10 μL of (*R*)-MTPA-Cl, and to the other was added 10 μL of (*S*)-MTPA-Cl to give the (*S*)-MTPA ester (**10**) and (*R*)-MTPA ester (**11**), respectively. Each reaction was allowed to stir for 24 h, and 10 μL of *N*,*N*-dimethylaminopropylamine was added to quench the reactions. The reaction products were dried under N_2 and applied onto silica SPE eluting with EtOAc−hexanes (1:1). The semipure product was further purified by semipreparative HPLC (Phenomenex Phenyl-Hexyl, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient of MeOH−H_2O (70−100% MeOH in 45 min and then 100% MeOH for 10 min) to yield **10** (t_R 38.0 min, 0.1 mg) or **11** (t_R 37.8 min, 0.1 mg).

10: colorless, amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.57 (dd, J = 6.4, 2.7 Hz, 2H), 7.41 (m, 3H), 6.14 (d, J = 9.2 Hz, 1H), 5.28 (q, J = 6.9 Hz, 1H), 4.93 (d, J = 10.6 Hz, 1H), 4.68 (dd, J = 9.1, 7.6 Hz, 1H), 3.69 (s, 3H), 3.58 (s, 3H), 3.06 (s, 3H), 2.46 (quintet, J = 7.1 Hz, 1H), 2.21 (m, 1H), 2.15 (t, J = 6.8 Hz, 2H), 1.95 (m, 1H), 1.68 (m, 1H), 1.47 (m, 2H), 1.09 (d, J = 7.0 Hz, 3H), 1.01 (d, J = 6.6 Hz, 3H), 0.92 (d, J = 6.9 Hz, 3H), 0.86 (d, J = 6.9 Hz, 3H), 0.81 (d, J = 6.9 Hz, 3H); HRESIMS m/z 729.1746 $[M + K]^+$ (calcd for $C_{31}H_{42}{}^{79}BrF_3N_2O_7K$, 729.1759), m/z $[M + K]^+$ 731.1747 (calcd for $C_{31}H_{42}{}^{81}BrF_3N_2O_7K$, 731.1755) (100:100 $[M + K]^+$ ion cluster); LRESIMS m/z 691/693 (100:100 $[M + H]^+$ ion cluster), 713/715 (100:100 $[M + Na]^+$ ion cluster).

11: colorless, amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.56 (dd, J = 4.3, 3.6 Hz, 2H), 7.41 (m, 3H), 6.27 (d, J = 8.3 Hz, 1H), 5.27 (q, J = 6.0 Hz, 1H), 4.94 (d, J = 10.4 Hz, 1H), 4.73 (dd, J = 9.1, 7.5 Hz, 1H), 3.69 (s, 3H), 3.57 (s, 3H), 3.07 (s, 3H), 2.54 (quintet, J = 6.7 Hz, 1H), 2.22 (m, 1H), 2.08 (td, J = 7.2, 2.9 Hz, 2H), 2.00 (m, 1H), 1.63 (m, 1H), 1.31 (m, 2H), 1.19 (d, J = 6.7 Hz, 3H), 1.01 (d, J = 6.3 Hz, 3H), 0.95 (d, J = 6.8 Hz, 3H), 0.90 (d, J = 7.0 Hz, 3H), 0.83 (d, J = 6.5 Hz, 3H); HRESI/APCIMS m/z 691.2202 $[M + H]^+$ (calcd for $C_{31}H_{43}{}^{79}BrF_3N_2O_7$, 691.2206), m/z $[M + H]^+$ 693.2186 (calcd for $C_{31}H_{43}{}^{81}BrF_3N_2O_7$, 693.2186) (100:100 $[M + H]^+$ ion cluster).

Advanced Marfey's Analysis of Dpv Unit in Dolastatin 16. To liberate the authentic (2*S*,3*R*)-Dpv standard, pitiprolamide (500 μg) was hydrolyzed (400 μL of 6 N HCl, 110 °C, 20 h). The mixture was dried and reconstituted in 400 μL of H_2O. Half of this sample was used to epimerize the α-amino acids in this mixture (40 μL of Et_3N and 40 μL of Ac_2O, heating at 60 °C for 1 h)[27] to yield a mixture of (2*S*,3*R*)-Dpv and (2*R*,3*R*)-Dpv standards. These standards were derivatized with 1% (w/v) Marfey's reagent (L-FDLA and DL-FDLA).[28] Dolastatin 16 was hydrolyzed and derivatized with 1% (w/v) L-FDLA according to the procedure stated above. The absolute configuration of the Dpv unit was determined by reversed-phase HPLC-MS [column, Phenomenex Synergi Hydro-RP (150 × 4.6 mm) using a linear gradient of 0.1% HCOOH in MeOH−0.1% aqueous HCOOH (40%−100% for 45 min and then 100% MeOH for 10 min); flow rate, 0.5 mL/min; detection by ESIMS in negative ion mode (MRM scan)] of the FDLA adducts. The acid hydrolysate of dolastatin 16 showed a peak at 36.1 min, corresponding to (2*S*,3*R*)-Dpv. Co-injection of the acid hydrolysate with the DL-FDLA-derivatized epimerization product showed enrichment of the peak corresponding to (2*S*,3*R*)-Dpv. The retention times (t_R, min; MRM ion pair) of the authentic amino acid L-FDLA adducts were as follows: (2*S*,3*R*)-Dpv (36.1; 486→191), (2*S*,3*S*)-Dpv [(36.6); (= D-FDLA-derivatized (2*R*,3*R*)-Dpv)], (2*R*,3*S*)-Dpv [(43.5); (= D-FDLA-derivatized (2*S*,3*R*)-Dpv)], (2*R*,3*R*)-Dpv (44.3). Compound-dependent parameters used were as follows: DP −95.0, EP −6.0, CE −31.0, CXP −2.0, CEP −26.0. Source gas parameters used were as follows: CUR 40, CAD High, IS 4500, TEM 450, GS1 40, GS2 40.

Advanced Marfey's Analysis of β-Amino Acid Unit in Dolastatin 12 and Dolastatin 16. Samples of dolastatin 12 and dolastatin 16 (0.2 mg each) were hydrolyzed (200 μL of 6 N HCl, 110 °C, 20 h) to liberate the β-amino acids Map and Dml, respectively. Another portion of dolastatin 12 and dolastatin 16 (0.2 mg each) was first heated with 3 N NaOH (80 °C, 3 h), and the resulting product mixture was acidified with 5% (v/v) HCl, dried under N_2, and hydrolyzed (200 μL of 6 N HCl, 110 °C, 20 h) to liberate the partially C-2-epimerized β-amino acid mixture. The reaction products were derivatized using L-FDLA and DL-FDLA.[28] The order of elution of the β-amino acids Dml and Map liberated from dolastatin 16 and dolastatin 12, respectively, was determined by reversed-phase HPLC-MS [column, Phenomenex Synergi Hydro-RP (150 × 4.6 mm) using a linear gradient of 0.1% HCOOH in MeOH− 0.1% aqueous HCOOH (25−80% for 50 min and then 100% MeOH for 5 min); flow rate, 0.5 mL/min; detection by ESIMS in negative ion mode (MRM scan)] of the FDLA adducts. The enantiomers bearing the 3*R* configuration consistently eluted later than those with the 3*S* configuration. The retention times (t_R, min; MRM ion pair) of the Dml-L-FDLA adducts were as follows: (2*S*,3*S*)-Dml [(29.3; 438.2→310.3); (= D-FDLA-derivatized (2*R*,3*R*)-Dml)], (2*R*,3*S*)-Dml [(30.8); (= D-FDLA-derivatized (2*S*,3*R*)-Dml)], (2*R*,3*R*)-Dml (33.7), (2*S*,3*R*)-Dml (35.6). The retention times (t_R, min; MRM ion pair) of the Map-L-FDLA adducts were as follows: (2*R*,3*S*)-Map [(27.5; 424.5→310.4); (= D-FDLA-derivatized (2*S*,3*R*)-Map)], (2*S*,3*S*)-Map [(29.6); (= D-FDLA-derivatized (2*R*,3*R*)-Dml)], (2*R*,3*R*)-Dml (31.0), (2*S*,3*R*)-Dml (32.5). Compound-dependent parameters used were as follows: DP −95.0, EP −6.0, CE −31.0, CXP −2.0, CEP −26.0. Source gas parameters used were as follows: CUR 40, CAD Medium, IS 4500, TEM 450, GS1 40, GS2 40.

Base Hydrolysis of Dolastatin 16. Dolastatin 16 (4.0 mg) was hydrolyzed using 2 N KOH−MeOH (1:1) for 3 h at 80 °C. The base hydrolysate was neutralized with 2 N HCl and partitioned between EtOAc and H_2O, and the organic layer was collected and dried under N_2. The crude base hydrolysate product was purified by semipreparative HPLC (Phenomenex Synergi Hydro-RP, 250 × 10 mm, 4 μm; flow rate, 2.0 mL/min) using a linear gradient of ACN−H_2O (40−100% MeOH in 45 min) to yield **12** (t_R 22.7 min, 1.0 mg).

12: colorless, amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.13−7.26 (m), 6.81 (d, J = 9.0 Hz, 1H), 6.65 (d, J = 10.4 Hz, 1H), 4.83 (d, J = 10.6 Hz, 1H), 4.73 (dd, J = 8.7, 3.8 Hz, 1H), 4.59 (m, 2H), 4.48 (m, 1H), 4.28 (m, 1H), 3.79 (m, 1H), 3.56−3.69 (m, 2H), 3.50 (m, 1H), 3.36 (m, 1H), 3.16 (s, 3H), 2.70 (m, 2H), 2.24−2.48 (m, 6H), 1.94−2.19 (m, 2H), 1.77−1.94 (m), 1.14 (d, J = 6.8 Hz, 3H), 1.03 (d, J = 7.3 Hz, 3H), 0.96 (m, 6H), 0.90 (m, 6H), 0.80 (m, 6H); HRESI/APCIMS m/z 750.4407 $[M + Na]^+$ (calcd for $C_{39}H_{61}N_5O_8Na$, 750.4418).

Modified Mosher's Analysis Using PGME. The base hydrolysate product was dissolved in 600 μL of DMF-d_7 and divided into two portions. To one portion was added 0.7 mg of (*S*)-PGME, and the solution cooled and stirred at 0 °C before the successive addition of PyBop (1.8 mg), HOBt (0.5 mg), and *N*-Me morpholine (5.0 μL). The reaction was left to stir for another 3 h at room temperature and quenched with the addition of EtOAc. The resulting solution was successively washed with 5% HCl, saturated $NaHCO_3$, and brine. The organic layer was collected and dried over anhydrous $MgSO_4$ before drying under N_2 to yield **13** (0.8 mg). The same procedure was used to prepare the (*R*)-PGME-derivatized product **14** (0.6 mg). ^{1}H NMR chemical shifts of the Dml spin system were assigned by COSY and TOCSY analyses.

13: colorless, amorphous solid; ^{1}H NMR ($CDCl_3$) δ 7.27−7.38 (m, 12H, incl. NH Dml at 7.27), 5.57 (dd, J = 14.0, 7.4 Hz, 1H), 5.02 (d, J = 10.2 Hz, 1H), 4.42 (m, 2H), 4.24 (d, J = 2.6 Hz, 1H), 3.74 (s, 3H), 3.56−3.65 (m, 3H, incl. H-3 Dml at 3.62), 3.45 (m, 2H), 3.29 (m, 1H), 2.99 (s, 3H), 2.75 (m, 2H, incl. H-2 Dml), 2.30−2.40 (m, 5H),

2.02−2.09 (m, 4H), 1.97−2.00 (m, 4H), 1.45 (m, 1H, H-5 Dml), 1.19 (d, J = 7.2 Hz, 3H, H_3-7 Dml), 1.04 (d, J = 6.6 Hz, 3H), 0.95 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.7 Hz, 3H, H_3-6 Dml), 0.85 (d, J = 6.8 Hz, 3H), 0.82 (d, J = 6.7 Hz, 3H, H_3-5 Dml), 0.80 (d, J = 6.6 Hz, 6H); HRESI/APCIMS m/z 897.5099 $[M + Na]^+$ (calcd for $C_{48}H_{70}F_3N_6O_9Na$, 897.5102).

14: colorless, amorphous solid; ^1H NMR ($CDCl_3$) δ 7.30−7.40 (m, 10H), 7.14−7.24 (m, 4H, incl. NH Dml at 7.21), 5.46 (d, J = 7.3 Hz, 1H), 4.99 (d, J = 10.2 Hz, 1H), 4.52 (dd, J = 6.4, 2.3 Hz, 1H), 4.42 (t, J = 6.4 Hz, 1H), 4.27 (d, J = 2.0 Hz, 1H), 4.01, (m, 1H), 3.72 (s, 3H), 3.56−3.67 (m, 3H, incl. H-3 Dml at 3.64), 3.02 (s, 3H), 2.65−2.68 (m, 2H, incl. H-2 Dml at 2.68), 2.30−2.40 (m, 5H), 1.89−2.02 (m, 8H), 1.62 (m, 1H, H-5 Dml), 1.13 (d, J = 6.8 Hz, 3H, H_3-7 Dml), 1.03 (d, J = 6.7 Hz, 3H), 0.98 (d, J = 6.8 Hz, 3H, H_3-6 Dml), 0.96 (d, J = 6.7 Hz, 3H), 0.89 (d, J = 6.7 Hz, 3H, H_3-5 Dml), 0.86 (d, J = 6.8 Hz, 3H), 0.81 (d, J = 6.7 Hz, 6H); HRESI/APCIMS m/z 897.5101 $[M + Na]^+$ (calcd for $C_{48}H_{70}F_3N_6O_9Na$, 897.5102).

Cell Viability Assay. HT29 colorectal adenocarcinoma and HeLa cervical carcinoma cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) under a humidified environment with 5% CO_2 at 37 °C. HeLa (3000) and HT29 (12 500) cells were seeded in 96-well plates and treated with varying concentrations of **1**−**8** and dolastatin 16, positive control (paclitaxel), and solvent control (DMSO) after 24 h of seeding. The cells were incubated for an additional 48 h before the addition of the MTT reagent. Cell viability was measured according to the manufacturer's instructions (Promega). IC_{50} values for positive control were 3.0 and 2.6 nM for HT29 and HeLa cells, respectively. IC_{50} calculations were done by GraphPad Prism 5.03 based on duplicate experiments.

■ ASSOCIATED CONTENT

Supporting Information. NMR spectra for compounds **1**−**14** and dolastatin 16. This material is available free of charge via the Internet at http://pubs.acs.org.

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