

Halogenated Fatty Acid Amides and Cyclic Depsipeptides from an Eastern Caribbean Collection of the Cyanobacterium *Lyngbya majuscula*[†]

Jorge I. Jiménez,^{‡,§} Tifanie Vansach,^{‡,⊥} Wesley Y. Yoshida,^{||} Bryan Sakamoto,[∇] Peter Pörzgen,[⊥] and F. David Horgen^{⊥,*}

AgraQuest, Inc., Davis, California 95618, College of Natural Sciences, Hawaii Pacific University, Kaneohe, Hawaii 96744, Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822, and School of Medicine, St. George's University, Grenada, West Indies

Received March 17, 2009

A lipophilic extract of an eastern Caribbean collection of *Lyngbya majuscula* yielded two new halogenated fatty acid amides, grenadamides B (**1**) and C (**2**), and two new depsipeptides, itralamides A (**3**) and B (**4**), along with the known compounds hectochlorin and deacetylhectochlorin. The recently reported depsipeptide carriebowmide (**5**) was also present in the extract and isolated as its sulfone artifact (**6**). Compounds **1–4** were identified by spectroscopic methods. The configurations of the amino acid residues of **3**, **4**, and **6** were determined by LC-MS analyses of diastereomeric derivatives of the acid hydrolysates (advanced Marfey's method). Based on the configurational analysis of **6**, in direct comparison with authentic carriebowmide (**5**), a minor structural revision of **5** is proposed. Compounds **1** and **2** displayed marginal activity against the beet armyworm (*Spodoptera exigua*). Compounds **1–4** and **6** were assessed for general cell toxicity in human embryonic kidney (HEK293) cells. Only itralamide B (**4**) displayed significant cytotoxicity, showing an IC₅₀ value of 6 ± 1 μM.

Cyanobacteria are a rich source of novel secondary metabolites. This chemical diversity is best represented in the genus *Lyngbya* (and especially *L. majuscula*), which is a prolific source of halogenated and nonhalogenated fatty acid amides, lipopeptides, and nonribosomal peptides, many of which possess significant bioactivity against a number of mammalian pharmacological targets. Recent reviews¹ and databases of natural products² have cataloged hundreds of secondary metabolites from this pantropically distributed marine genus, with isolation efforts often showing significant overlap of constituents between study populations but also yielding a remarkable, and a seemingly unending, range of unique and frequently bioactive compounds. Our current interest in identifying novel cyanobacterial metabolites with insecticidal and ion channel inhibitory activity led us to investigate a lipophilic extract of *L. majuscula* collected in waters of the eastern Caribbean island-country of Grenada. We are aware of only one previous report of cyanobacterial compounds from Grenada,³ which describes unique cyclopropyl-containing polyketides, including the dienyl fatty acid esters grenadiene and debromogrenadiene and the fatty acid amide grenadamide, from *L. majuscula*. In the current study, a *L. majuscula* extract demonstrated a unique chemical profile, yielding two new fatty acid amides, grenadamides B (**1**) and C (**2**), and two novel depsipeptides, itralamides A (**3**) and B (**4**).⁴

Results and Discussion

A sample of *L. majuscula* was collected in True Blue Bay, Grenada. The sample was air-dried, extracted with 2-PrOH/CH₂Cl₂ (1:1), and subjected to reversed-phase column chromatography (CC) using a MeOH/H₂O step gradient mobile phase. HPLC of the MeOH/H₂O (80:20) fraction yielded the known *Lyngbya* metabolites hectochlorin⁵ and deacetylhectochlorin,⁶ while repeated reversed-phase HPLC of the 100% MeOH eluate led to the isolation of fatty acid amides **1** and **2**, cyclic depsipeptides **3** and **4**, and the sulfone of the known depsipeptide carriebowmide (**5**).⁷

[†] This work was presented in part at the 48th Annual Meeting of the American Society of Pharmacognosy, Portland, ME, July 14–18, 2007. Poster No. P141S.

* To whom correspondence should be addressed. Tel: (808) 236-5864. Fax: (808) 236-5880. E-mail: dhorgen@hpu.edu.

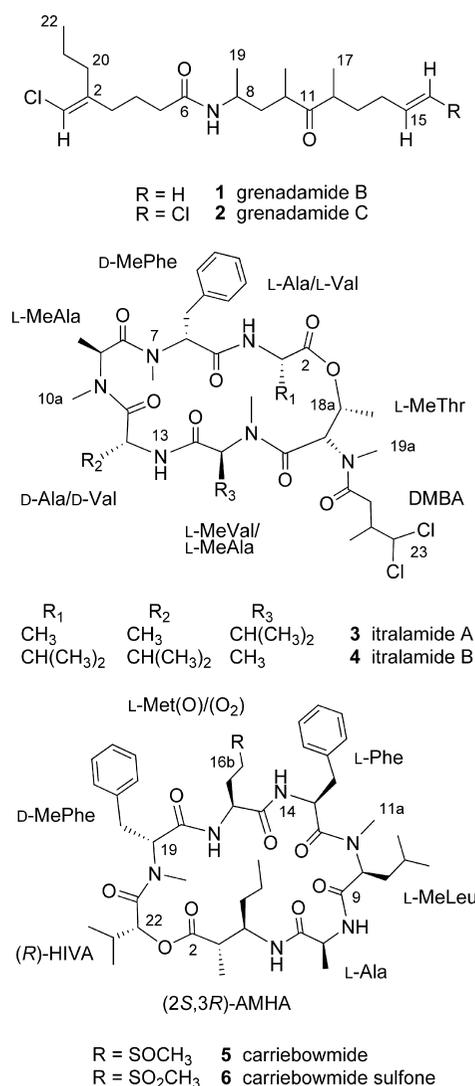
[‡] Contributed equally to the research.

[§] AgraQuest, Inc.

[⊥] Hawaii Pacific University.

^{||} University of Hawaii at Manoa.

[∇] St. George's University.



Grenadamide B (**1**) was obtained as a colorless oil. Analysis of the ¹³C NMR and accurate MS data indicated a molecular formula of C₂₁H₃₆ClNO₂, which requires four degrees of unsaturation. The presence of a chlorine was supported by a 3:1 peak cluster ([M +

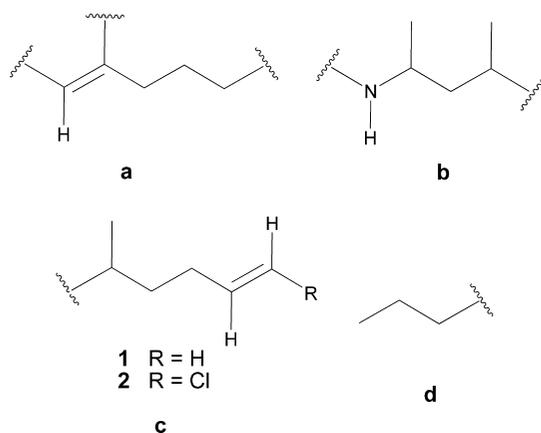


Figure 1. Partial structures of **1** and **2**.

HJ⁺) at *m/z* 370/372 in the ESIMS. The ¹³C NMR spectrum of **1** contained two carbonyl signals at δ_C 171.6 and 217.5 along with resonances at δ_C 112.6, 115.0, 138.1, and 141.8, characteristic of substituted carbon–carbon double bonds. These signals account for all 4 degrees of unsaturation in the molecule.

Four ¹H spin systems were assembled by COSY (Figure 1), and HMBC data indicated the connection of the carbonyl at δ_{C-6} 171.6 to segments **a** and **b** (cross-peaks from δ_{H-5} 2.08 and δ_{NH-7} 5.49 to C-6) and the linkage of the ketone carbonyl at δ_{C-11} 217.5 to segments **b** and **c** (cross-peaks from δ_{H-10} 2.73 and δ_{H-12} 2.67 to C-11). Segment **a** was connected to the C-3 propyl side chain (segment **d**) through an HMBC cross-peak between δ_{H-1} 5.78 and C-20 and δ_{H-21} 1.41 and C-2. Attachment of the vinyl chloride to C-1 of segment **a** completed the planar structure of **1**. The C-1/C-2 olefin geometry was assigned as *E* on the basis of a ROESY cross-peak between H-1 and H-3. The relative and absolute configurations at C-8, C-10, and C-12 were not assigned.

The 1D NMR and ESIMS spectra of grenadamide C (**2**) were consistent with a structurally related analogue of **1** having an additional chlorine atom, as indicated by a 10:6:1 isotope peak cluster ([M + H]⁺) at *m/z* 404/406/408. Analysis of the ¹³C NMR and accurate MS data provided a molecular formula of C₂₁H₃₅Cl₂NO₂, which requires 4 degrees of unsaturation. The ¹H NMR of **2** revealed the absence of the signals at δ_H 4.92–5.00 observed in **1** and the appearance of new signals at δ_H 5.79 and 5.88 attributed to two methines located at C-15/C-16. A downfield shift of C-16 in the ¹³C NMR spectrum of **1** from δ_C 115.0 to 117.3 in **2** is indicative of the electron-withdrawing effect of the chlorine atom assigned to C-16, which explains the reduced magnitude of the vicinal coupling of H-15/H-16 from 17 to 13 Hz. This provided evidence for an *E*-geometry for the C-15/C-16 carbon double bond, which was further supported by a strong ROESY cross-peak between H-16 and H-14.

Itralamides A (**3**) and B (**4**) were initially obtained as an 8:3 mixture (220 μ g). Due to the exceptionally low yield of the two closely related compounds, the mixture of **3** and **4** was subjected to NMR analyses prior to further separation. Ittralamide A (**3**) displayed a protonated molecular ion in the ESI-TOF-MS that indicated a molecular formula for **3** of C₃₆H₅₄Cl₂N₆O₈, which requires 12 degrees of unsaturation. Analysis of ¹H, COSY, 1D-TOCSY, HSQC, and HMBC NMR spectra of the mixture of **3** and **4** in CDCl₃ (Table 2) indicated the presence of the following amino acid residues in **3**: alanine (Ala) ($\times 2$), *N*-methylalanine (MeAla), *N*-methylphenylalanine (MePhe), *N*-methylthreonine (MeThr), and *N*-methylvaline (MeVal), as well as 4,4-dichloro-3-methylbutanoic acid (DMBA), accounting for 11 of the 12 degrees of unsaturation.

The residue sequence of **3** was determined by HMBC and ROESY NMR correlations as indicated in Figure 2. The NMe or NH hydrogens of each amino acid residue gave HMBC correlations to the carbonyl of its adjacent *N*-terminal residue, allowing the

assignment of the partial sequence DMBA-MeThr-MeVal-Ala2-MeAla-MePhe. As an exception, the attachment of Ala1 to the C-terminal of MePhe was indicated by a ROESY correlation between the NH of Ala (δ_{H-4} 7.53) and the α -hydrogen of MePhe (δ_{H-6} 5.74). At the same time, the ester bond between the Ala and the β -OH group of MeThr fulfilled the last required double-bond equivalent and was supported by a ROESY correlation between the NMe group of MeThr (δ_{H-19a} 3.42) and the Me side chain of Ala (δ_{H-3a} 1.57).

Itralamide B (**4**) displayed a [M + H]⁺ in the ESI-TOF-MS that indicated a molecular formula for **4** of C₃₈H₅₈Cl₂N₆O₈. Analysis of the **3** and **4** mixture NMR data set indicated that **4** was structurally related to **3** and attributed the 28 Da mass difference between **3** and **4** to a replacement of the two Ala residues and one MeVal residue in **3** with two valine (Val) residues and one MeAla residue in **4**. The determination of the residue sequence of **4** was made similarly to that of **3** through interpretation of HMBC and ROESY correlations to give the following sequence: DMBA-MeThr-MeAla2-Val2-MeAla1-MePhe-Val1, and the ester bond between β -OH of MeThr and Val is indicated by the ROESY correlation between the NMe group of MeThr (δ_{H-19a} 3.41) and the β -position of Val (δ_{H-3a} 2.35).

The advanced Marfey's method was used to determine the absolute configurations of the amino acid residues of purified **3** and **4**. The reaction products of the hydrolysate of purified ittralamide A (**3**) showed the presence of L-FDLA derivatives of both D- and L-Ala, L-MeAla, D-MePhe, L-MeThr, and L-MeVal on the basis of reconstructed selected ion chromatograms (SIC) (see Supporting Information). The integration of both D- and L-Ala derivative peaks (monitored ion *m/z* 384.1) was stoichiometric, indicating the presence of an equal number of L- and D-Ala residues, but their respective positions could not be initially assigned. However, LC-ESI-MS of the acid hydrolysate of **3** indicated the presence of a partially hydrolyzed product, the dipeptide MePhe-Ala1, which was isolated and subjected to extended acid hydrolysis (72 h). Analysis of the dipeptide hydrolysate by the advanced Marfey's method confirmed the presence of D-MePhe and identified Ala1 as the L-configured enantiomer. Consequently, Ala2 was assigned as the D-configured enantiomer, although we were not successful at isolating any partially hydrolyzed Ala2-containing dipeptide, nor did the scarcity of the sample allow for additional analyses.

Lastly, advanced Marfey's method was applied to the acid hydrolysate of purified ittralamide B (**4**) and showed the presence of L-MeAla, D-MePhe, L-MeThr, and both D- and L-Val (roughly a 1:3 ratio by LC-ESIMS) in the L-FDLA product mixture (see Supporting Information). This nonstoichiometric ratio of Val enantiomers might be explained by incomplete hydrolysis of the Val-containing dipeptides, including MePhe-Val1, MeAla-Val2, and Val2-MeAla, which are expected to be relatively resistant to hydrolysis, as suggested by the rule of six for predicting rates of dipeptide hydrolysis.⁸ Both MePhe-Val1 and MeAla1-Val2 were detected in the acid hydrolysate of **4** and were isolated and subjected to extended acid hydrolysis (72 h). Marfey analysis of MeAla-Val2 was unsuccessful, but analysis of the MePhe-Val1 dipeptide hydrolysate confirmed the presence of D-MePhe and identified Val1 as the L-configured enantiomer. This supported the assignment of L-Val1 and D-Val2, which placed the respective D- and L-enantiomers in the analogous positions compared with the Ala residues in **3**. The absolute configuration of DMBA in both **3** and **4** was not determined.

The recently reported caribowmid (**5**)⁷ was isolated from the 100% MeOH CC fraction as its sulfone (**6**), following repeated conversion of **5** to **6** during isolation. Despite close similarities between **5** and **6** in the NMR chemical shifts for their respective 3-amino-2-methylhexanoic acid (AMHA) residue (in CD₃OD as reported for **5**), we determined a configuration for the AMHA residue in **6** (2*S*,3*R*) that differed from that reported for **5** (2*R*,3*R*),

Table 1. NMR Assignments for **1** and **2** in CDCl₃

position	grenadamide B (1)				grenadamide C (2)	
	δ_C	δ_H (J in Hz)	COSY	HMBC	δ_C	δ_H (J in Hz)
1	112.6	5.78, br s	H-3	H-20	112.5	5.75, br s
2	141.8			H-1, H-20, H-21	141.7	
3	34.0	2.07, dd (16, 8.0)	H-1, H-4	H-1, H-4	33.9	2.03, m
4	23.4	1.73, m	H-3, H-5	H-5	23.3	1.69, m
5	35.9	2.08, dd (16, 8.0)	H-4	H-4	35.8	2.06, m
6	171.6			H-4, H-5, H-7, H-8	171.6	
7		5.49, d (8.5)	H-8			5.63, d (8.6)
8	43.6	3.87, m	H-7, H-9, H-19	H-7, H-9, H-19	43.5	3.82, m
9	39.2	1.35, m; 1.82, m	H-8, H-9 ^a , H-10	H-8, H-10, H-18, H-19	39.1	1.31, m; 1.80, m
10	42.1	2.73, m	H-9, H-18	H-8, H-9, H-18	41.8	2.71, m
11	217.5			H-9, H-10, H-12, H-13, H-17, H-18	216.8	
12	44.1	2.67, m	H-13, H-17	H-13, H-17	43.9	2.63, m
13	31.5	1.38, m; 1.78, m	H-12, H-13 ^a , H-14	H-14, H-17	31.3	1.34, m; 1.73, m
14	31.4	1.96, m	H-14, H-15, H-16a/b	H-12, H-13, H-15, H-16a/b	28.5	1.94, m
15	138.1	5.75, dddd (17, 10, 6.6, 3.5)	H-14, H-16a/b	H-13, H-14	133.0	5.79, dt (13, 7.0)
16	115.0	<i>pro-E</i> : 4.95, ddt (10, 3.2, 3.2) <i>pro-Z</i> : 4.97, ddt (17, 3.5, 3.5)	H-14, H-15	H-14, H-15	117.3	5.88, dt (13, 1.3)
17	17.1	1.06, d (6.8)	H-12	H-12, H-13	17.2	1.02, d (7.0)
18	16.8	1.05, d (6.7)	H-10	H-9, H-10	16.7	1.02, d (7.0)
19	21.4	1.09, d (6.5)	H-8	H-8, H-9	21.4	1.05, d (6.5)
20	31.7	2.15, m	H-21	H-1, H-21, H-22	31.6	2.12, m
21	20.3	1.41, m	H-20, H-22	H-22	20.2	1.39, m
22	13.9	0.90, t (7.4)	H-21	H-21	13.8	0.87, t (7.4)

^a Geminal ¹H,¹H coupling.

using the advanced Marfey's method (see Supporting Information). This discrepancy prompted us to directly compare the (1-fluoro-2,4-dinitrophenyl)-5-L-leucinamide (L-FDLA) derivatives of the hydrolysate product of **6** with that of authentic caribowamide (**5**), which was kindly provided to us by Drs. Sarath Gunasekera and Valerie Paul. For both samples, LC-ESIMS analysis detected identical L-FDLA derivatives of AMHA that had retention times corresponding with that of the standard L-FDLA-(2*S*,3*R*)-AMHA derivative, but differing from that of L-FDLA-(2*R*,3*R*)-AMHA, D-FDLA-(2*S*,3*R*)-AMHA, and D-FDLA-(2*R*,3*R*)-AMHA standards (Supporting Information). We therefore propose a minor revision of the structure of caribowamide (**5**) to reflect a (2*S*,3*R*)-configuration for the AMHA residue.

Compounds **1**–**4** demonstrate structural features that are characteristic of cyanobacterial metabolites including halogenation, *N*-methylation, and D-configured amino acids. Grenadamides B (**1**) and C (**2**) are related to pitiamide A, which was isolated from a mixed cyanobacterial assemblage of *L. majuscula* and *Microcoleus* sp. collected in Guam.⁹ Compounds **1** and **2** belong to a handful of cyanobacterial compounds containing an unusual terminal substituted vinyl chloride, including several malyngamides,^{10–13} jamaicamides,¹⁴ and taveuniamide.¹⁵ These compounds possess a range of biological activities including toxicity to crustaceans,^{12,13,15} mammalian cells,^{11,14} and fish¹² as well as fish feeding deterrent activity.⁹ Grenadamides B (**1**) and C (**2**) showed marginal activity in our insecticidal assay against beet armyworms. At 1.0 mg/mL, **1** and **2** showed 38% and 50% mortality, respectively.

The closely related itralamides A (**3**) and B (**4**) possess a depsipeptide ring system that is common among isolates from actinomycetes and cyanobacteria;¹⁶ however, chlorination is rare among these compounds. The chlorinated DMBA side chain of itralamides A (**3**) and B (**4**) has been reported previously as a component of amides and small peptides from *Lamellodysidea*¹⁷ and *Dysidea* sponge species.^{18,19} It has been hypothesized that these and related compounds are of cyanobacterial origin.^{19,20} This hypothesis was further supported by a subsequent report by Ridley and co-workers²¹ that demonstrated an association of the cyanobacterium *Oscillatoria spongeliae* with tissues of four different Palauan sponge species and identified the cyanobacterium as the biosynthetic source of the trichloromethyl compounds isolated from the intact sponge tissues. Furthermore, *L. majuscula* has been found to produce related trichloromethyl-containing compounds.²² This

study adds further evidence that chlorinated DMBA-related compounds are biosynthesized by cyanobacteria. Other notable structural features of itralamides A (**3**) and B (**4**) include the presence of two D-configured amino acid residues and the relatively rare *N*-methylthreonine residue, which has been reported previously from a few but diverse array of organisms including a cyanobacterium,²³ sponges,²⁴ fungi,²⁵ and an actinobacterium.²⁶

Compounds **1**–**4** and **6** were assessed for general toxicity by assaying for their effects on human embryonic kidney (HEK-293) cells as measured by the cellular reduction of rezasurin to the fluorogenic metabolite resorufin²⁷ as a marker of cell viability (Table 3). Itralamide B (**4**) and deacetylhectochlorin showed moderate cell cytotoxicity, both with an IC₅₀ of 6 ± 1 μM. Interestingly, an order of magnitude lower potency was observed for itralamide A (**3**), despite the close structural relationship with itralamide B (**4**). The difference in the cytotoxicity of **3** and **4** demonstrates that subtle modifications can result in dramatically altered biological activities.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical Autopol III automatic polarimeter. The UV spectra were recorded on a PerkinElmer Lambda EZ 210 spectrophotometer or, if specified, a Thermo Finnigan Surveyor Plus photodiode array detector (PDA) coupled to an Agilent 1100 binary HPLC system. When evaporative light scattering detection (ELSD) is indicated, HPLC column eluant was split 9:1 and the low flow was directed to a Sedere Sedex 75 ELSD (coil temperature 50 °C, nebulizing air at 3.5 bar). IR spectra were recorded on a Perkin-Elmer Spectrum RX1 FTIR spectrometer. NMR experiments were performed on a Varian Unity INOVA 500 spectrometer operating at 500 and 125 MHz for ¹H and ¹³C, respectively. Accurate mass electrospray time-of-flight mass spectra (ESI-TOF-MS) were measured in positive mode on an Agilent MSD-TOF mass spectrometer, while low-accuracy and multidimensional electrospray ion trap mass spectra (ESIMS and ESIMS/MS) were measured in positive mode on a Thermo Finnigan LCQ Deca XP Max mass spectrometer.

Collection of Material. Colonies of *Lyngbya majuscula* (70 g dry wt) were collected at a depth of 1–10 m in True Blue Bay, Grenada, on January 14, 2001. The sample was air-dried. A dried voucher specimen (voucher number 011401-TBB-01) is on file at Hawaii Pacific University.

Isolation of Compounds 1–4 and 6. The air-dried material was extracted with 2-PrOH/CH₂Cl₂ (50:50, v/v). The crude organic extract

Table 2. NMR Assignments for **3** and **4** in CDCl₃

		itralamide A (3)			itralamide B (4)		
residue	position	$\delta_c^{a,b}$	δ_H (J in Hz)	HMBC	$\delta_c^{a,b}$	δ_H (J in Hz)	HMBC
Ala-1/	2	171.6		H-3a	172.1		H-3
Val-1	3	47.1	4.89, dq (9.3, 7.3)	H-3a	56.6	4.95, dd (9.3, 2.3)	H-3b, H-3c
	3a	19.4	1.57, d (7.3)		32	2.35, m	H-3b, H-3c
	3b				20.5	1.04, d (6.8)	H-3c
	3c				16.6	0.96, d (6.6)	H-3b
MePhe	4		7.53, d (9.3)			8.00, d (9.3)	
	5						
	6	56.6	5.74, dd (12.3, 5.2)	H-7a	56.7	5.81, dd (12.0, 5.2)	H-7a
	6a	33.4	3.56, dd (15.1, 5.2) 2.87, dd (15.1, 12.3)	H-6c/6c'	33.4	3.64, dd (15.2, 5.2) 2.85, dd (15.2, 12.0)	H-6c/6c'
MeAla	6b	137.5			137.5		
	6c/6c'	128.5	7.16, m	H-6a (δ 3.56, 2.87)	128.5	7.17, m	H-6a (δ 2.85)
	6d/6d'	128.3	7.24, m		128.3	7.24, m	
	6e	126.3	7.23, m	H-6c/6c'	126.3	7.23, m	H-6c/6c'
	7a	30.5	2.95, s		31.5	2.96, s	
	8	174.8		H-7a, H-9a	173.9		H-7a, H-9a
Ala-2/	9	51.3	4.63, q (7.2)	H-9a, H-10a	51.6	4.38, q (6.8)	H-9a, H-10a
	9a	13.0	0.97, d (7.2)		13.0	0.88, d (6.8)	
	10a	31.1	3.07, s		31.6	3.03, s	
Val-2	11	173.2		H-10a, H-12, H-12a	172.6		H-10a
	12	46.3	4.82, dq (7.5, 6.7)	H-12a	54.9	5.02, dd (9.0, 2.1)	H-12b, H-12c
	12a	16.2	1.39, d (6.7)	H-12	30.9	2.29, m	H-12b, H-12c
	12b				20.8	0.99, d (6.8)	H-12c
	12c				16.3	0.87, d (6.8)	H-12b
MeVal/	13		6.86, d (7.5)			7.30, d (9.0)	
	14	167.9		H-13, H-15	170.4		H-15, H-15a
MeAla-2	15	61.4	4.68, d (11.0)	H-15b, H-15c, H-16a	53.5	5.20, q (6.8)	H-15a, H-16a
	15a	25.2	2.24, m	H-15b, H-15c	13.5	1.24, d (6.8)	
	15b	19.9	0.92, d (6.4)	H-15c			
	15c	17.8	0.70, d (6.8)	H-15b			
	16a	30.3	3.05, s	H-15	31.7	3.12, s	
MeThr	17	169.7		H-16a	169.9		H-16a
	18	53.5	5.72, d (3.2)	H-18b, H-19a	54.8	5.77, d (3.6)	H-18b, H-19a
	18a	67.7	5.57, qd (6.7, 3.2)	H-18b	68.8	5.72, qd (6.7, 3.6)	H-18b
	18b	17.6	1.30, d (6.7)		17.7	1.33, d (6.7)	
	19a	33.9	3.42, s		34.1	3.41, s	
DMBA	20	172.7		H-19a, H-21 (δ 2.68, 2.43)	172.7		H-19a
	21	35.8	2.43, dd (16.4, 6.7)	H-22a	35.8	2.46, dd (16.5, 5.6)	H-22a
			2.68, dd (16.4, 6.2)				2.71, dd (16.5, 7.6)
	22	40.5	2.76, m	H-21, H-22a	40.5	2.77, m	H-22a
	22a	15.1	1.18, d (6.6)		15.1	1.19, d (6.6)	
23	77.8	5.97, d (2.8)	H-22a	77.8	5.97, d (2.9)	H-22a	

^a ¹³C NMR chemical shifts were extracted from the 2D slices of the HSQC and HMBC spectra; MePhe C-1 signal was not observed in HMBC. ^b ¹³C NMR data for C-6b, C-6c/6c', C-6d/6d', and C-6e were nearly identical for the corresponding carbons in itralamides A and B.

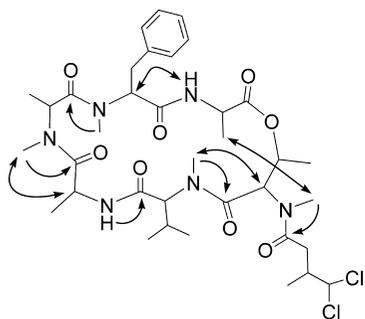


Figure 2. Key HMBC and ROESY correlations for itralamide A (**3**). HMBC correlations between ¹H and ¹³C are indicated by single-headed arrows, while ¹H,¹H-ROESY correlations are shown as double-headed arrows.

was subjected to reversed-phase C₁₈ column chromatography with a MeOH/H₂O step gradient. HPLC of the 80% aqueous MeOH fraction yielded hectochlorin and deacetylhectochlorin as determined by ¹H NMR.^{5,6} Repeated HPLC of the 100% MeOH eluate residue led to the isolation of fatty acid amides **1** (9.2 mg) and **2** (28.5 mg), the mixture of **3** and **4** (0.22 mg), and carriebowmide sulfone **6** (1.4 mg) [Luna C18(2), Phenomenex, 10 × 250 mm, MeCN/H₂O gradient: 20:80 to 50:50 (0–2 min), 50:50 to 100:0 (2–45 min), 100:0 (45–60 min); 3.3 mL/min]. Prior to advanced Marfey's analysis the mixture of **3** and **4**

Table 3. Cytotoxicity of Isolates against HEK-293 Cells^a

compound	IC ₅₀ (μM)
grenadamide B (1)	≥ 100
grenadamide C (2)	≥ 100
itralamide A (3)	54 ± 6
itralamide B (4)	6 ± 1
carriebowmide sulfone (6)	>50
deacetylhectochlorin	6 ± 1

^a Values ± 95% confidence intervals are averaged IC₅₀ determinations (*n* = 2) conducted on separate days from duplicate serial dilutions. Positive control was 400 nM daunorubicin (*n* = 12), which showed 82 ± 2% inhibition of cell viability.

was separated by reversed-phase HPLC using an ether-linked phenyl stationary phase [Synergi Polar RP, Phenomenex, 10 × 250 mm; MeOH/H₂O gradient with 0.10% formic acid: 60:40 to 100:0 (0–50 min), 100:0 (50–75 min); 3.0 mL/min; detection by PDA (200–400 nm)] to yield **3** (0.16 mg) and **4** (0.06 mg). The identical chromophores of **3** and **4** allowed for the calculation of yields as a proportion of the 220 μg mixture from the molar ratio of **3** and **4** (2.7:1.0) as determined by PDA detection.

Grenadamide B (1): colorless oil; [α]_D²³ −8.7 (*c* 0.40, CH₂Cl₂); UV (MeOH) λ_{max} (log ϵ) 208 nm (3.75); IR ν_{max} (film on NaCl) 2965, 2933, 2872, 1709, 1642, 1541, 1457, 668 cm^{−1}; ¹H (CDCl₃, 500 MHz) and ¹³C (CDCl₃, 125 MHz) NMR data (Table 1); ESIMS/MS (*m/z* 370.2 fragmented using 50% collision energy) *m/z* 334, 198, 180; ESI-TOF-MS *m/z* 370.2498, [M + H]⁺ (calcd for C₂₁H₃₇³⁵ClNO₂, 370.2507).

Grenadamide C (2): light yellow oil; $[\alpha]_D^{23} -17$ (*c* 0.40, CH₂Cl₂); UV (MeOH) λ_{\max} (log ϵ) 207 nm (3.84); IR ν_{\max} (film on NaCl) 2962, 2932, 2872, 1709, 1641, 1546, 1460, 1378, 1265, 738 cm⁻¹; ¹H (CDCl₃, 500 MHz) and ¹³C (CDCl₃, 125 MHz) NMR data (Table 1); ESIMS/MS (*m/z* 404.2 fragmented using 50% collision energy) *m/z* 368, 232, 215; ESI-TOF-MS *m/z* 404.2112, [M + H]⁺ (calcd for C₂₁H₃₆³⁵Cl₂NO₂, 404.2118).

Itralamide A (3): colorless oil; UV [PDA, MeOH/formic acid (1000:1)] λ_{\max} 219 nm; ¹H (CDCl₃, 500 MHz) NMR data and ¹³C chemical shifts derived from HMBC and HSQC (CDCl₃) NMR data (Table 2); ESIMS/MS (*m/z* 769.4 fragmented using 33% collision energy) *m/z* 737, 733, 680, 519, 434, 250; ESI-TOF-MS *m/z* 769.3457, [M + H]⁺ (calcd for C₃₆H₅₅Cl₂N₆O₈, 769.3458).

Itralamide B (4): colorless oil; UV [PDA, MeOH/formic acid (1000:1)] λ_{\max} 219 nm; ¹H (CDCl₃, 500 MHz) NMR data and ¹³C chemical shifts derived from HMBC and HSQC (CDCl₃) NMR data (Table 2); ESIMS/MS (*m/z* 797.4 fragmented using 33% collision energy) *m/z* 765, 761, 720, 680, 519, 434; ESI-TOF-MS *m/z* 797.3772, [M + H]⁺ (calcd for C₃₈H₅₉Cl₂N₆O₈, 797.3771).

Preparation of *N*-Methylthreonine Standards. A mixture of *N*-methylthreonine and *N,N*-dimethylthreonine was prepared using the method from Bowman and Stroud.²⁸ Separately *L*-threonine (0.84 mmol) and *L-allo*-threonine (0.43 mmol) were dissolved in distilled water. To each solution were added 10% Pd/C (0.10 and 0.05 g, respectively) and 38% (w/w) formaldehyde (0.14 and 0.07 mL, respectively). The reaction solutions were bubbled with H₂ (g) for 1.5–2.5 h, while the reaction progress was monitored using direct injection ESIMS. Once the concentration of the monomethyl derivative plateaued, the solutions were filtered and dried under vacuum. The desired monomethylated product was separated from starting material and dimethylated product by reversed-phase HPLC (Atlantis dC18, Waters, 3.0 × 250 mm) using 0.10% aqueous formic acid as the mobile phase. The resulting *N*-methyl-*L*-threonine (98% purity by ELSD) demonstrated ¹H NMR and ¹³C NMR signals consistent with literature values.²⁹ MS analysis confirmed the identification: ESI-TOF-MS *m/z* 134.0805 [M + H]⁺ (calcd for C₅H₁₂NO₃, 134.0812); ESIMS/MS (*m/z* 134.1 fragmented using 50% collision energy) *m/z* 98, 88, and 70. The specific rotation was $[\alpha]_D^{27} -12$ (*c* 0.093 6 N HCl). The resulting *N*-methyl-*L-allo*-threonine (91% purity by ELSD) gave the following NMR data: ¹H NMR (D₂O, 300 MHz) δ 4.34 (1H, m, H-3); 3.63 (1H, d, *J* = 3.7 Hz, H-2); 2.75 (3H, s, *NMe*); 1.21 (3H, d, *J* = 6.7 Hz, H-4); ¹³C NMR (D₂O, 300 MHz) δ 171.5 (C-1); 69.3 (C-2); 65.8 (C-3); 33.5 (*NMe*); 17.5 (C-4). MS analysis confirmed the identification: ESI-TOF-MS *m/z* 134.0801 [M + H]⁺ (calcd for C₅H₁₂NO₃, 134.0812); ESIMS/MS (*m/z* 134.1 fragmented using 50% collision energy) *m/z* 98, 88, 70. The specific rotation was $[\alpha]_D^{28} +13$ (*c* 0.10 6 N HCl).

Acid Hydrolysis of 3–6. Itralamides A (3, 50 μ g) and B (4, 21 μ g), authentic caribbowmide (5, 75 μ g), and caribbowmide sulfone (6, 200 μ g) were hydrolyzed in 0.4 mL of 6 N HCl (Pierce, 24308) at 110 °C for 18.5 h. The samples were dried under a N₂ stream at 60 °C, then twice reconstituted with 100 μ L of H₂O and redried. The hydrolysate was eluted through a C₁₈ SPE column (Phenomenex, Strata C₁₈-E) using MeOH/H₂O (10:90, v/v) to yield free amino and hydroxy acids.

Advanced Marfey's Method for the Analysis of the Acid Hydrolysates of 3–6. The acid hydrolysates of 3–6 were reconstituted with 90, 40, 80, and 100 μ L of H₂O, respectively. One-half volume of each resulting solution was reacted with 30/20/40/50 μ L of aqueous 1.0 M NaHCO₃ and 30/20/40/50 μ L of a 0.20% (w/v) solution of *L*-FDLA in acetone, respectively. The reaction mixtures were heated at 80 °C for 3 min and allowed to cool. The reaction solutions for 3–6 were then acidified with 60/40/80/100 μ L of 2.0 N HCl and diluted with 30/10/30/250 μ L of MeCN, respectively. Standard FDLA derivatives were prepared for each constituent α -amino acid by reacting 50 μ L aliquots of a 5 mM aqueous solution of the standard *L*-isomer using separately both *L*- and *DL*-FDLA reagents. Standard FDLA derivatives of AMHA were prepared similarly by separately reacting 50 μ L of 3.5 mM solutions of the (2*R*,3*R*)- and (2*S*,3*R*)-diastereomers with both *L*- and *DL*-FDLA reagents. (The AMHA standards were reported in a previous study³⁰ and provided by Dr. Philip Williams, University of Hawaii.) Each reaction product mixture was chromatographed by reversed-phase HPLC (Eclipse XDB-18, Agilent, 4.6 × 150 mm) using a gradient of MeCN/H₂O with 0.10% formic acid [20:80 to 80:20 (0–30 min), 80:20 (30–35 min); 0.8 mL/min] and detection by ESIMS. In

order to resolve the FDLA-Met(O₂) products that co-eluted under the above conditions, the derivatized hydrolysate and standards were similarly chromatographed on a second reversed-phase column [Luna C₁₈, Phenomenex, 5 μ m, 4.6 × 250 mm; MeCN/H₂O gradient with 0.10% formic acid: 20:80 to 90:10 (0–40 min), 90:10 (40–45 min); 0.8 mL/min]. For authentic caribbowmide (5), only the configuration of the AMHA residue was analyzed. As determined by reconstructed SIC of [M + H]⁺ ions, the retention times of the *L*-FDLA-amino acids in each hydrolysate reaction product were compared with retention times of standard FDLA-amino acids (see Supporting Information).

Advanced Marfey's Analysis of MePhe-Ala1 from the Hydrolysate of 3 and MePhe-Val1 and MeAla1-Val2 from the Hydrolysate of 4. The dipeptide MePhe-Ala1 was isolated from the hydrolysate of 3, and the dipeptides MePhe-Val1 and MeAla1-Val2 were isolated from that of 4, both by reversed-phase HPLC-PDA [Waters Atlantis dC₁₈ 5 μ m, 3.0 × 250 mm; MeCN/H₂O gradient with 0.1% formic acid: 0:100 (0–10 min), 0:100 to 50:50 (10–23 min); 0.3 mL/min]. Each dipeptide was hydrolyzed for 72 h in 0.4 mL of 6 N HCl at 110 °C. Workup of the hydrolysate product, preparation of *L*-FDLA derivatives, and LC-ESIMS analyses of the Marfey's products were conducted as described above (see Supporting Information).

Cytotoxicity Testing. Resazurin is a nonfluorescent dye that is reduced by viable cells to the fluorescent resorufin. The protocol used was adapted from O'Brien and co-workers.³¹ Human embryonic kidney (HEK-293) cells were grown in culture flasks with DMEM media (Dulbecco's modified Eagle's medium, Cellgro), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2.0 mM *L*-glutamine, 100 U/mL penicillin, and 0.10 mg/mL streptomycin, in a humidified incubator (5% CO₂ at 37 °C). The cells were plated (6000 cells/well) on poly-*L*-lysine-coated black/clear-bottom 96-well plates (Greiner). Following the addition of test substances or vehicle, plates were incubated in growth media for 48 h, at which time media was completely removed, and wells were washed with Krebs Ringer Hepes (KRH) buffer (consisting of 1.5 mM CaCl₂, 5.6 mM glucose, 20 mM Hepes, 5 mM KCl, 1.5 mM MgCl₂, and 135 mM NaCl) and incubated for 2.5 h (5% CO₂ at 37 °C) in 13 μ M resazurin (Sigma) in KRH. The production of resorufin was measured using a scanning fluorometer (FLEXstation 3, Molecular Devices; 9 points/well; emission read at 590 nm following excitation at 530 nm). Controls included wells receiving 0.1% saponin (MP Biomedicals), which serve as an "all dead" (background) and wells receiving 400 nM daunorubicin (Sigma) as a positive control. Duplicate serial dilutions for each compound were tested on two separate days. Data were normalized by expressing compound-treated well fluorescence as a percent of vehicle control (% vehicle) after subtracting average fluorescence of background (saponin-treated) wells from vehicle control and compound-treated wells. For each serial dilution, IC₅₀ values were determined from dose–response curves fitted with Prism statistical software (GraphPad Software, version 5.01; log of inhibitor concentration vs normalized response, variable slope). Independent IC₅₀ values were averaged and are reported in Table 3.

Beet Armyworm Activity (BAW: *Spodoptera exigua*). Six replicates of each of 1 and 2 were overlaid in 15% EtOH on top of a wheat germ/casein-based artificial diet in 96-well plates. The screening diet was augmented with antibiotics to control bacterial overgrowth. Template-inoculated filter papers containing temporally synchronized armyworm eggs were then matched to the well orifice of the diet/extract plate, and both were covered with a ventilated plate lid. The eggs hatched and the neonates dropped to the treated diet. After five days the percent mortality was determined in comparison with control (vehicle) wells. A positive control was included as serial dilutions of Javelin (Thermo Trilogy's *Bacillus thuringiensis*).

Acknowledgment. This publication was made possible by NIH Grant P20 RR-16467 from the National Center for Research Resources and by the HPU Trustees' Scholarly Endeavors Fund. Funds for the upgrade of the Varian Unity INOVA 500 were provided by the CRIF program of the National Science Foundation (CH E9974921) and the Elsa U. Pardee Foundation. The purchase of the Agilent LC-TOF-MS was funded by Grant W911NF-04-1-0344 from the Department of Defense. We thank Dr. G. M. L. Patterson for the taxonomic identification, Dr. P. Williams for providing FDLA reagent and reference AMHA samples,

Drs. S. Gunasekera and V. Paul for providing a sample of authentic carriebowmide, and Mr. D. Feher for assistance in collecting TOF-MS data.

Supporting Information Available: Amino acid analysis of **3**, **4**, and **6**; FDLA retention times for standard amino acids and hydrolysates of **3**, **4**, and **6** and AMHA residue in the hydrolysate of authentic carriebowmide (**5**); chiral HPLC of α -hydroxy isovaleric acid from **6**; preparation of methionine sulfone standard; ^1H and ^{13}C NMR spectra of **1**, **2**, and **6**; ^1H NMR spectrum of the mixture of **3** and **4**; and a proposed MS fragmentation of **1** and **2** are included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References and Notes

- Burja, A. M.; Banaigs, B.; Abou-Mansour, E.; Burgess, J. G.; Wright, P. C. *Tetrahedron* **2001**, *57*, 9341–9377. Welker, M.; von Dohren, H. *FEMS Microbiol. Rev.* **2006**, *30*, 530–563. Tan, L. T. *Phytochemistry* **2007**, *68*, 954–979.
- Dictionary of Natural Products*; CRC Press, v16.2, and *MarinLit*; University of Canterbury, vpc14.6.
- Sitachitta, N.; Gerwick, W. H. *J. Nat. Prod.* **1998**, *61*, 681–684.
- We herein recommend the use of “grenadamide A” for the original grenadamide reported in ref 3. The compound name “itralamide” is taken from the root “itral”, which means “natural” in Grenadian colloquial speech and reflects the natural occurrence of these amides.
- Marquez, B. L.; Watts, K. S.; Yokochi, A.; Roberts, M. A.; Verdier-Pinard, P.; Jimenez, J. I.; Hamel, E.; Scheuer, P. J.; Gerwick, W. H. *J. Nat. Prod.* **2002**, *65*, 866–871.
- Suntornchashweij, S.; Chaichit, N.; Isobe, M.; Suwanborirux, K. *J. Nat. Prod.* **2005**, *68*, 951–955.
- Gunasekera, S. P.; Ritson-Williams, R.; Paul, V. J. *J. Nat. Prod.* **2008**, *71*, 2060–2063.
- Whitfield, R. E. *Science* **1963**, *142*, 577–579.
- Nagle, D. G.; Park, P. U.; Paul, V. J. *Tetrahedron Lett.* **1997**, *38*, 6969–6972.
- Cardellina, J. H.; Daliotos, D.; Marner, F.-J.; Mynderse, J. S.; Moore, R. E. *Phytochemistry* **1978**, *17*, 2091–2095. Cardellina, J. H.; Marner, F.-J.; Moore, R. E. *J. Am. Chem. Soc.* **1979**, *101*, 240–242. Ainslie, R. D.; Barchi, J. J.; Kuniyoshi, M.; Moore, R. E.; Mynderse, J. S. *J. Org. Chem.* **1985**, *50*, 2859–2862. Praud, A.; Valls, R.; Piovetti, L.; Banaigs, B. *Tetrahedron Lett.* **1993**, *34*, 5437–5440. Milligan, K. E.; Marquez, B.; Williamson, R. T.; Davies-Coleman, M.; Gerwick, W. H. *J. Nat. Prod.* **2000**, *63*, 965–968. Nogle, L. M.; Gerwick, W. H. *J. Nat. Prod.* **2003**, *66*, 217–220.
- Gerwick, W. H.; Reyes, S.; Alvarado, B. *Phytochemistry* **1987**, *26*, 1701–1704.
- Wu, M.; Milligan, K. E.; Gerwick, W. H. *Tetrahedron* **1997**, *53*, 15983–15990.
- Kan, Y.; Sakamoto, B.; Fujita, T.; Nagai, H. *J. Nat. Prod.* **2000**, *63*, 1599–1602.
- Edwards, D. J.; Marquez, B. L.; Nogle, L. M.; McPhail, K.; Goeger, D. E.; Roberts, M. A.; Gerwick, W. H. *Chem. Biol.* **2004**, *11*, 817–833.
- Williamson, R. T.; Singh, I. P.; Gerwick, W. H. *Tetrahedron* **2004**, *60*, 7025–7033.
- The *Dictionary of Natural Products* (CRC Press, v16.2) shows 183 natural products (72 of cyanobacterial origin) that possess the 1-oxa-4,7,10,13,16-pentaazacyclononadecane-2,5,8,11,14,17-hexaone depsipeptide ring system. Of the seven chlorinated cyanobacterial compounds, each possessed an aromatic chloride.
- Sauleau, P.; Retailleau, P.; Vacelet, J.; Bourguet-Kondracki, M.-L. *Tetrahedron* **2005**, *61*, 955–963.
- Erickson, K.; Wells, R. *Aust. J. Chem.* **1982**, *35*, 31–38. Unson, M. D.; Rose, C. B.; Faulkner, D. J.; Brinen, L. S.; Steiner, J. R.; Clardy, J. *J. Org. Chem.* **1993**, *58*, 6336–6343. Isaacs, S.; Berman, R.; Kashman, Y.; Gebreyesus, T.; Yosief, T. *J. Nat. Prod.* **1991**, *54*, 83–91. Dumdei, E. J.; Simpson, J. S.; Garson, M. J.; Byriell, K. A.; Kennard, C. H. L. *Aust. J. Chem.* **1997**, *50*, 139–144. Stapleton, B. L.; Cameron, G. M.; Garson, M. J. *Tetrahedron* **2001**, *57*, 4603–4607.
- Harrigan, G. G.; Goetz, G. H.; Luesch, H.; Yang, S.; Likos, J. *J. Nat. Prod.* **2001**, *64*, 1133–1138.
- Harrigan, G. G.; Goetz, G. *J. Appl. Phycol.* **2002**, *14*, 103–108.
- Ridley, C. P.; Bergquist, P. R.; Harper, M. K.; Faulkner, D. J.; Hooper, J. N.; Haygood, M. G. *Chem. Biol.* **2005**, *12*, 397–406.
- Orjala, J.; Gerwick, W. H. *J. Nat. Prod.* **1996**, *59*, 427–430. Jimenez, J. I.; Scheuer, P. J. *J. Nat. Prod.* **2001**, *64*, 200–203.
- Medina, R. A.; Goeger, D. E.; Hills, P.; Mooberry, S. L.; Huang, N.; Romero, L. L.; Ortega-Barria, E.; Gerwick, W. H.; McPhail, K. L. *J. Am. Chem. Soc.* **2008**, *130*, 6324–6325.
- Ford, P. W.; Gustafson, K. R.; McKee, T. C.; Shigematsu, N.; Maurizi, L. K.; Pannell, L. K.; Williams, D. E.; Dilip de Silva, E.; Lassota, P.; Allen, T. M.; Van Soest, R.; Andersen, R. J.; Boyd, M. R. *J. Am. Chem. Soc.* **1999**, 5899–5909. Plaza, A.; Gustchina, E.; Baker, H. L.; Kelly, M.; Bewley, C. A. *J. Nat. Prod.* **2007**, *70*, 1753–1760.
- Lee, K. K.; Gloer, J. B.; Scott, J. A.; Malloch, D. *J. Org. Chem.* **1995**, *60*, 5384–5385. Komatsu, K.; Shigemori, H.; Kobayashi, J. *J. Org. Chem.* **2001**, *66*, 6189–6192. Chen, C. H.; Lang, G.; Mitova, M. I.; Murphy, A. C.; Cole, A. L.; Din, L. B.; Blunt, J. W.; Munro, M. H. *J. Org. Chem.* **2006**, *71*, 7947–7951.
- Bodanzky, M.; Izdebski, J.; Muramatsu, I. *J. Am. Chem. Soc.* **1969**, *91*, 2351–2358.
- Nociari, M. M.; Shalev, A.; Benias, P.; Russo, C. *J. Immunol. Methods* **1998**, *213*, 157–167.
- Bowman, R.; Stroud, H. *J. Chem. Soc.* **1950**, 1342–1345.
- Aurelio, L.; Brownlee, R. T. C.; Hughes, A. B.; Sleebs, B. E. *Aust. J. Chem.* **2000**, *53*, 425–433.
- Williams, P. G.; Moore, R. E.; Paul, V. J. *J. Nat. Prod.* **2003**, *66*, 1356–1363.
- O’Brien, J.; Wilson, I.; Orton, T.; Pognan, F. *Eur. J. Biochem.* **2000**, *267*, 5421–5426.

NP900173D