Molassamide, a Depsipeptide Serine Protease Inhibitor from the Marine Cyanobacterium *Dichothrix utahensis*†

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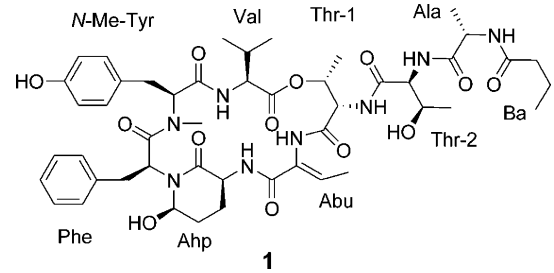
A new dolastatin 13 analogue, molassamide (**1**), was isolated from cyanobacterial assemblages of *Dichothrix utahensis* collected from the Molasses Reef, Key Largo, Florida, and from Brewer's Bay, St. Thomas, U.S. Virgin Islands. This is the first peptide reported from the cyanobacterial genus *Dichothrix* and the first natural product isolated from marine *Dichothrix* spp. Its planar structure was determined by NMR spectroscopic techniques, and the configurations of the asymmetric centers were assigned after chiral HPLC analysis of the hydrolysis products. The depsipeptide **1** exhibited protease-inhibitory activity, with IC_{50} values of 0.032 and 0.234 μM against elastase and chymotrypsin, respectively. There was no apparent inhibition of trypsin at 10 μM, the highest concentration tested.

Marine cyanobacteria have emerged as a rich source of biologically and ecologically active secondary metabolites, in particular peptides and depsipeptides.[1] While marine cyanobacteria are common inhabitants of tropical and subtropical coastal waters worldwide, under favorable environmental conditions they can grow rapidly and form harmful algal blooms.[2] Grounding of the M/V *Wellwood* on Molasses Reef in Key Largo, Florida Keys National Marine Sanctuary (FKNMS), on August 4, 1984, resulted in massive destruction to living corals and underlying reef framework. Further injury to this restoration site was caused during the 1998 storm season. Artificial reef structures were placed at the *Wellwood* grounding site in 2002 as a restoration action by FKNMS. National Oceanic and Atmospheric Administration scientists have attempted to settle larval corals in situ on these *Wellwood* restoration structures (WRS) for several years, and from 2003−2005, Southeast Fisheries Science Center and academic partners "seeded" tens of thousands of reef-building coral larvae and out-planted a few thousand newly settled coral polyps to the WRS for restoration purposes. They have identified only two survivors from this effort. The WRS has an obvious algal mat cover dominated by the cyanobacterium *Dichothrix utahensis*, which appears as "black puff balls". The cyanobacterial mat was 14 times more abundant on the WRS than on adjacent natural reef surfaces at similar depth (5−7 m). Preliminary experiments conducted using the cyanobacterial mat exudates with swimming coral larvae indicated significant reduction of larval settlement,[3] leading NOAA scientists to hypothesize that algal mat exudates inhibit coral success on the WRS and possibly other nearby restoration structures.[4] Therefore, we selectively collected a cyanobacterial assemblage of *D. utahensis* from the WRS and investigated the natural products chemistry of this bloom-forming cyanobacterium to better understand the chemical ecology of this species.

Here we report the isolation, structure elucidation, and protease-inhibitory studies of a new dolastatin 13 analogue, trivially named molassamide (**1**), as a component of the polar *n*-BuOH-soluble fraction. Molassamide contains the six common amino acid residues Val, *N*-Me-Tyr, Phe, Ahp, Abu, and Thr, which are common to dolastatin 13[5] and its analogues. Several dolastatin 13 analogues have been isolated from *Lyngbya* spp., namely, lyngbyastatin 4,[6] lyngbyastatins 5−7,[7] lyngbyastatins 8−10,[8] and symplostatin 2,[9] and from mixed assemblages of *L. majuscula* and *Schizothrix* species, somamides A and B,[10] all of which differ in the pendant side chain. The serine protease inhibitory activities of molassamide against elastase and chymotrypsin are comparable to other reported dolastatin analogues.[6,7,9]



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The wet samples collected in 2007 and 2008 were stored frozen. After freeze-drying, the dry materials were extracted with organic solvents. The *n*-BuOH-soluble fractions of the MeOH extracts of the two samples were fractionated separately by C_{18} column chromatography. The subfractions, which eluted with H_2O−MeOH (1:3) on repeated reversed-phase HPLC, yielded purified molassamide (**1**) as a white, amorphous solid. The NMR data combined with a $[M + Na]^+$ peak at *m/z* 985.4645 in the HRESI/APCIMS suggested a molecular formula of $C_{48}H_{66}N_8O_{13}$. The IR spectrum displayed strong absorption bands at 1735 and 1642 cm^{-1}, indicating the presence of ester and amide functionalities. The ^1H and ^{13}C NMR spectra were indicative of a depsipeptide (Table 1). Following the interpretation of DQF COSY, edited HSQC, and HMBC experiments, the ^1H and ^{13}C NMR signals were assignable to nine partial structures, which accounted for all atoms except one H atom in an amide group. These partial structures consisted of the amino acids alanine, valine, phenylalanine, *N*-methyltyrosine, and two threonines. In Thr-1, there was no visible coupling between the H-2 and H-3, similar to other compounds with the same cyclic core. NOESY and HMBC (Table 1) correlations established the partial structure of Thr-1. Additionally, partial structures corresponding to 2-amino-2-butenoic acid (Abu) and 3-amino-6-hydroxy-2-piperidone (Ahp) units were deduced, both of which are present in other dolastatin 13 analogues. A butanoic acid unit (Ba) was

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Table 1. NMR Spectroscopic Data for Molassamide (**1**) in CD_3CN (600 MHz)

unit	position	δ_C mult.	δ_H (*J* in Hz)	COSY[a]	HMBC[b]	NOESY[c]
Val	1	173.2, qC				
	2	61.6, CH	3.87, m	3, NH	1, 4, 5	3, 4, 5, NH
	3	30.2, CH	1.97, m	2, 4, 5	1, 2, 4, 5	2, NH
	4	20.0, CH_3	0.94, d (6.2)	3	2, 3, 5	2
	5	19.3, CH_3	0.92, d (6.2)	3	2, 3, 4	2, NH, 3 (Thr-1)
	NH		8.28, br	2		2, 3, 5, 2 (*N*-Me-Tyr)
N-Me-Tyr	1	173.2, qC				
	2	62.7, CH	4.86, dd (12.3, 2.8)	3a, 3b	1, 3, 4, N-Me	3, N-Me, NH (Val)
	3a	33.7, CH_2	3.54, dd (−13.7,2.8)	2, 3b	2, 4, 5/9	2, 3b
	3b		2.75, dd (−13.7, 12.3)	2, 3a	2, 4, 5/9	2, 3a
	4	129.7, qC				
	5/9	131.5, CH	7.10, d (9.0)	6/8	3, 7	6/8
	6/8	116.4, CH	6.83, d (9.0)	5/9	4, 5/9, 7	5/9
	7	156.8, qC				
	N-Me	31.5, CH_3	2.80, s		2, 1 (Phe)	2, NH (Val)
Phe	1	171.9, qC				
	2	52.5, CH	4.88, dd (11.6, 4.1)	3a, 3b	1, 3, 4, 6 (Ahp)	3a, 3b, 6 (Ahp)
	3a	35.5, CH_2	2.91, dd (−14.4, 4.1)	2, 3b	2, 4, 5/9	2, 3b, 6 (Ahp)
	3b		2.11, dd (−14.4, 11.6)	2, 3a	2, 4, 5/9	2, 3a, 5/9
	4	137.9, qC				
	5/9	130.4, CH	6.87, d (7.2)	6/8	3, 4, 7	6/8, 3b
	6/8	128.9, CH	7.21, t (7.2)	5/9, 7	4, 5/9, 7	5/9, 7
	7	127.3, CH	7.17, t (7.2)	6/8	5/9	6/8
Ahp	2	170.6, qC				
	3	49.70[d], CH	4.04, m	NH, 4a, 4b	2, 4	4b, 5b
	4a	22.5, CH_2	2.23, m	3, 4b, 5a, 5b	3, 5, 6	4b
	4b		1.70, m	3, 4a, 5a, 5b	2, 5, 6	4a, 3
	5a	30.4, CH_2	1.84, m	4a, 4b, 5b, 6	3, 4, 6	5b, 6
	5b		1.78, m	4a, 4b, 5a, 6	3, 4, 6	3, 5a, 6
	6	75.6, CH	5.26, br s	5a, 5b	4, 2 (Phe)	5a, 5b, 2 (Phe), 3a (Phe)
	NH		7.09, d (4.8)	3	3, 1 (Abu)	3
Abu	1	164.6, qC				
	2	129.8, qC				
	3	133.8, CH	6.68, q (6.9)	4	1, 2, 4	4
	4	13.8, CH_3	1.56, d (6.9)	3	1, 2, 3	3, 2 (Thr-1)
	NH		8.58			NH (Ahp), NH (Thr-1)
Thr-1	1	169.9, qC				
	2	57.1, CH	4.60, d (9.6)	NH	1, 3, 4	3, 4, NH, 4 (Abu)
	3	73.4, CH	5.27, d (6.2)	4	1	2, 4, 5 (Val)
	4	20.8, CH_3	1.43, d (6.2)	3	2, 3	2, 3, NH
	NH		7.19, d (9.6)	2	1, 2, 3, 1 (Thr 2)	2, 4, 4 (Thr-2)
Thr-2	1	170.7, qC				
	2	56.2, CH	4.98, m	3, NH	2	3, 4
	3	68.0, CH	4.35, m	2, 4	1, 2, 4	2, 4, NH
	4	18.0, CH_3	1.04, d (6.2)	3	2, 3	2, NH, NH (Thr-1)
	4-OH		4.10, s	3		
	NH		7.11, d (6.3)	2	1, 2, 1 (Ala)	3, 4, 3 (Ala)
Ala	1	173.2, qC				
	2	49.68[d], CH	4.32, m	3, NH	1, 3	3
	3	17.4, CH_3	1.24, d (7.6)	2	1	2, NH, NH (Thr-2)
	NH		6.66, d (7.5)	2	2, 3, 1 (Ba)	3, 2 (Ba)
Ba	1	174.0, qC				
	2	38.3, CH_2	2.11, m (2H)	3	1, 3, 4	3, 4, NH
	3	19.8, CH_2	1.56, m (2H)	2, 4	1, 2	2, 4
	4	13.9, CH_3	0.87, t (6.8)	3	2, 3	2, 3

[a] $^1H-^1H$ COSY correlations are from proton(s) stated to the indicated proton(s). [b] HMBC correlations, optimized for $^{2/3}J_{CH} = 8$ Hz, are from proton(s) stated to the indicated carbon. [c] NOESY correlations are from proton(s) stated to the indicated proton(s). [d] These carbon signals are interchangeable.

identified as the only non amino acid unit in the molecular structure. The residue sequence for **1** was determined from NOESY data and supported by HMBC data (Table 1). Two weak NOESY correlations between the amide NH of Val (δ 8.28) and H-2 (δ 4.86) and *N*-Me (δ 2.80) of *N*-Me-Tyr connected these two units. The chemical shift values of H-2 of *N*-Me-Tyr (δ 4.86) and H-2 of Phe (δ 4.88) are too close for unambiguous assignment of NOE correlations involving these protons, but the presence of a strong HMBC correlation between the *N*-Me (δ 2.80) of *N*-Me-Tyr and amide carbonyl of Phe (δ 171.9) connected these two residues. A NOESY correlation between H-2 of Phe and H-6 of Ahp (δ 5.26) and HMBC correlation between H-6 of Ahp and C-2 of Phe (δ 52.5) extended the sequence to the Ahp unit. Similarly, a NOESY correlation between 3-NH of Ahp (δ 7.09) and 2-NH of Abu (δ 8.58), an HMBC correlation between 3-NH of Ahp and the amide carbonyl of Abu (δ 164.6), and a NOESY correlation between H_3-4 of Abu (δ 1.56) and H-2 of Thr-1 (δ 4.60) expanded the sequence to Val-*N*-Me-Tyr-Phe-Ahp-Abu-Thr-1. Further, NOE correlations between NH of Thr-1 (δ 7.19) and H_3-4 of Thr-2 (δ 1.04), NH of Thr-2 (δ 7.11) and H_3-3 of Ala (δ 1.24), and NH of Ala (δ 6.66) and H_2-2 of Ba (δ 2.11) allowed us to determine the linear sequence for all eight amino acids including the terminal butanoic acid group. The low-field chemical shift of H-3 of Thr-1 (δ 5.27) indicated the presence of an ester bond at this position. The NOESY correlation between H-3 of Thr-1 and H_3-5 of Val (δ 0.92) supported the ester bond between these amino acids and thereby established the cyclic planar structure of **1**, which is

the same cyclic core present in dolastatin 13, symplostatin 2, somamides A and B, and lyngbyastatins 4−10.

The absolute configurations of the amino acids were determined by chiral HPLC analysis of the acid hydrolysates of **1** and its CrO_3 oxidation product. Oxidation followed by acid hydrolysis liberated glutamic acid and Phe, allowing the assignment of the C-3 position of the Ahp unit. The analysis revealed L-configurations for all amino acids. In the Ahp ring system, NOESY correlations were observed between the diaxial H-3 (δ 4.04) and H-5b (δ 1.78), and H-5b and the equatorial H-6 (δ 5.26, br s), with two small coupling constants.[6] These observations and NOESY correlations observed between H-2 of L-Phe (δ 4.88) and H-6 of Ahp (δ 5.26) supported a 2-*S*-Phe, 6-*R*-Ahp configuration similar to the stereochemical assignments in symplostatin 2,[9] lyngbyastatins 4−7,[6,7] and somamide A.[10] A four-bond HMBC correlation between H_3-4 and C-1 of Abu indicates a "w" configuration[11,12] for bonds between these atoms and, therefore, the *Z* geometry for the double bond. NOESY correlations from H_3-4 of Abu (δ 1.56) to H-2 of Thr-1 (δ 4.60) and H_3-4 of Thr-2 (δ 1.04) also support this configuration.

Because of its structural similarity with other serine protease inhibitors, **1** was evaluated for serine protease-inhibitory activity. Molassamide exhibited protease-inhibitory activity, with IC_{50} values of 0.032 ± 0.004 and 0.234 ± 0.003 μM against elastase and chymotrypsin, respectively. There was no apparent inhibition of trypsin at 10 μM, the highest concentration tested. This is a similar selectivity profile to that previously observed for lyngbyastatins 4−7.[6,7] Protease inhibitors are known from taxonomically diverse cyanobacteria; they have been reported to have allelochemical activity and are commonly regarded as digestion inhibitors.[13,14]

Experimental Section

General Experimental Procedures. The optical rotation was recorded on a Perkin-Elmer model 343 polarimeter. UV spectrophotometric data were acquired on a Hitachi U-3010 spectrophotometer. IR spectroscopic data were obtained on a Bruker Vector 22 FT-IR spectrometer. NMR data were collected on a JEOL ECA-600 spectrometer operating at 600.17 MHz for ^{1}H and 150.9 MHz for ^{13}C. The edited-HSQC experiment was optimized for J_{CH} = 140 Hz, and the HMBC spectrum was optimized for $^{2/3}J_{CH}$ = 8 Hz. ^{1}H NMR chemical shifts (referenced to residual CH_3CN observed at δ 1.93) were assigned using a combination of data from 2D DQF COSY and multiplicity-edited HSQC experiments. Similarly, ^{13}C NMR chemical shifts (referenced to CD_3CN observed at δ 118.2) were assigned on the basis of multiplicity-edited HSQC experiments. The HRMS data were obtained using an Agilent 6210 LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector at the Mass Spectrometer Facility at the University of California, Riverside, California. Silica gel 60 (EMD Chemicals, Inc., 230−400 mesh) and Varian BondElut octadecyl (C_{18}) were used for column chromatography. All solvents used were of HPLC grade (Fisher Scientific).

Collection, Extraction, and Isolation. The sample of *Dichothrix utahensis* was collected in June 2007 from Wellwood Restoration Site at Molasses Reef, Key Largo, Florida, at a depth of 6 m. Subsequently, another *Dichothrix* assemblage was collected in June 2008 from Brewer's Bay rocks in the U.S. Virgin Islands at a depth of 1 m. The samples were identified by two of us (M.W.M. and V.J.P.) on the basis of morphological characteristics of the genus (Littler and Littler 2000),[15] and voucher specimens (MWM-Wellwood mat-001 and VP-Brewers-06-08) are maintained at the Smithsonian Marine Station, Fort Pierce, FL.

The freeze-dried Molasses Reef material (153 g) was successively extracted with hexanes, EtOAc, and then MeOH. Concentration of the extracts by rotary evaporation at 45 °C under reduced pressure furnished 0.180 g (0.1% yield, dry wt) of the hexanes extract, 0.360 g (0.2% yield) of the EtOAc extract, and 16.1 g (10.5% yield) of the MeOH extract. The MeOH-soluble fraction was partitioned between *n*-BuOH and H_2O. Concentration of these extracts furnished 0.510 g (3.3%) of an *n*-BuOH-soluble fraction and 15.5 g (10.1%) of H_2O-soluble material. The *n*-BuOH-soluble fraction (0.480 g) was chromatographed on a column of C_{18} (10 g) using a MeOH−H_2O step gradient system to give six subfractions. The subfraction 3 (150 mg), eluted with MeOH−H_2O (3:1), was further separated by reversed-phase HPLC (semipreparative, 5 μm, RP-18) using CH_3CN−H_2O (4:6) to give six subfractions. Subfraction 6 (4.0 mg) was further purified by reversed-phase HPLC using CH_3CN−H_2O (35:65) to give 2.0 mg of molassamide (**1**, yield, 0.001% dry wt). The freeze-dried U.S. Virgin Island material (252 g) was extracted first with EtOAc−MeOH (1:1) followed by MeOH. Concentration of the extracts gave 10.5 g (7.7% yield, dry wt) of the organic extract and 14.5 g (5.7% yield) of a polar extract. The organic extract was partitioned between EtOAc and H_2O. The H_2O-soluble fraction (9.7 g) was then partitioned between *n*-BuOH and H_2O. Concentration of these extracts furnished 0.650 g (0.25%) of an *n*-BuOH-soluble fraction and 9.1 g (3.6%) of H_2O-soluble material. Similarly, the polar extract (14.5 g) was partitioned between *n*-BuOH and H_2O. Concentration of these extracts furnished 0.800 g (0.32%) of an *n*-BuOH-soluble fraction and 13.5 g (5.3%) of H_2O-soluble material. The combined *n*-BuOH-soluble fractions (1.45 g) were chromatographed on a column of C_{18} (20 g) using a MeOH−H_2O step gradient system to give eight subfractions. Subfractions 4 and 5 (136 mg), which eluted with MeOH−H_2O (3:1), were further separated by reversed-phase HPLC (semipreparative, 5 μm, RP-18, flow 3.0 mL/min) using CH_3CN−H_2O (35:65) to give molassamide (**1**) (1.5 mg) at 13.5 min and another compound (6.0 mg) at 24 min, in addition to several mixed fractions. The latter compound appeared to be decomposing with time. This fraction was further purified by reversed-phase HPLC using CH_3CN−H_2O (35:65) to give an additional 2.0 mg of molassamide (**1**, total yield, 0.001% dry wt).

Molassamide (1): colorless, amorphous powder; $[\alpha]^{25}_D$ −2.7 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 212 (3.84), 230 (3.69), 280 (2.72) nm; IR (film) ν_{max} 3374, 2957, 1735, 1642, 1538, 1518, 1240, cm^{-1}; ^{1}H NMR, ^{13}C NMR, DQF COSY, HMBC, and NOESY data, see Table 1; HRESI/APCIMS *m/z* 985.4645 [M + Na]$^+$ (calcd for $C_{48}H_{66}N_8O_{13}Na$, 985.4642).

Acid Hydrolysis and Chiral HPLC Analysis. Compound **1** (0.3 mg) was suspended in 6 N HCl (0.3 mL) and heated at 115 °C for 18 h in a sealed tube. The hydrolysate was concentrated to dryness. The residue was reconstituted in 0.3 mL of H_2O and analyzed by chiral HPLC, comparing the retention times with those of authentic standards [Phenomenex Chirex (D) Penicillamine, 4.6 × 250 mm, 5 μm]; solvent 2.0 mM $CuSO_4$ and mixtures of 2.0 mM $CuSO_4$−CH_3CN (95:5 or 90:10); detection at 254 nm. Using 2.0 mM $CuSO_4$ with a flow rate of 0.8 mL/min, the retention times (t_R min) for authentic standards were L-Thr (12.4), D-Thr (14.6), L-allo-Thr (17.2), and D-allo-Thr (20.5). Using 2.0 mM $CuSO_4$−CH_3CN (90:10) with a flow rate of 1.0 mL/min, the retention times (t_R min) for authentic standards were *N*-Me-L-Tyr (23.9), *N*-Me-D-Tyr (18.8), L-Phe (73.0), and D-Phe (75.6). Similarly, using 2.0 mM $CuSO_4$−CH_3CN (95:5) with a flow rate of 1.0 mL/min, the retention times (t_R min) for authentic standards were L-Ala (5.9), D-Ala (7.2), L-Val (14.5), and D-Val (19.8). The retention times in min (and respective HPLC conditions) of the amino acids in the hydrolysate were 12.4 (100:0), 23.9 (90:10), 73.0 (90:10), 5.9 (95:5), and 14.5 (95:5), indicating the presence of L-Thr, *N*-Me-L-Tyr, L-Phe, L-Ala, and L-Val in the hydrolysate.

Oxidation of **1** was performed as described in the literature.[16] Compound **1** (0.3 mg) was dissolved in glacial acetic acid (0.3 mL) and mixed with CrO_3 (2.0 mg). After stirring at room temperature for 4 h, the reaction mixture was separated using a C_{18} SPE cartridge eluting with H_2O followed by MeOH. The resulting oxidized material from the MeOH fraction was hydrolyzed with 6 N HCl (0.3 mL) at 115 °C for 18 h in a sealed tube. The hydrolysate was subjected to chiral HPLC as described above, using 2 mM $CuSO_4$−IPA (95:5) as the mobile phase with a flow rate of 1.0 mL/min. L-Glu liberated from the reaction mixture was detected at t_R 37.4 min. The retention times (t_R, min) for standards L-Glu and D-Glu were 37.4 and 40.4, respectively.

Protease Inhibitory Assays. Elastase activity was assessed using high-purity porcine pancreatic elastase (Elastin Products Company, EC134). The assay buffer used was 1 M Tris-HCl (pH 8.0). Assay buffer (79 μL), elastase solution (75 μg/mL in assay buffer, 5 μL), and various concentrations of **1** (1 μL, dissolved in DMSO) were preincubated for 15 min at room temperature in a microtiter plate. After this time, 15 μL of substrate solution was added (2 mM *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide in assay buffer) to each well, and the reaction was followed by measuring the absorbance at 405 nm every 30 s. Inhibition of chymotrypsin and trypsin was determined using α-chymotrypsin from bovine pancreas (Sigma, C4129) and trypsin from porcine pancreas (Sigma, T0303). The assay buffer in both cases was

50 mM Tris-HCl, 100 mM NaCl, and 1 mM $CaCl_2$ (pH 7.8). Assay buffer (39 μL), enzyme solution (100 μg/mL in assay buffer, 10 μL), and various concentrations of **1** (1 μL, dissolved in DMSO) were preincubated for 10 min at 37 °C, before 50 μL of substrate solution was added (1.5 mM *N*α-benzoyl-DL-arginine-4-nitroanilide dissolved in DMF and diluted to 7% v/v with assay buffer for trypsin, 1.5 mM *N*-succinyl-Gly-Gly-Phe-*p*-nitroanilide dissolved in assay buffer for chymotrypsin). The reaction was followed by measuring the absorbance at 405 nm every 30 s. For each assay, enzyme activity of each well was calculated using the initial slope of each progress curve, expressed as a percentage of the slope of the uninhibited reaction. Lyngbyastatin 7[7] was used as a positive control for inhibition of elastase and chymotrypsin, while phenylmethylsulfonyl fluoride was used as a positive control for inhibition of trypsin.[7]

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Supporting Information Available: ^{1}H, ^{13}C, COSY, NOESY, HSQC, and HMBC NMR spectra in CD_3CN for molassamide (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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