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Discovery and Synthesis of Caracolamide A, an Ion Channel Modulating Dichlorovinylidene Containing Phenethylamide from a Panamanian Marine Cyanobacterium cf. *Symploca* Species

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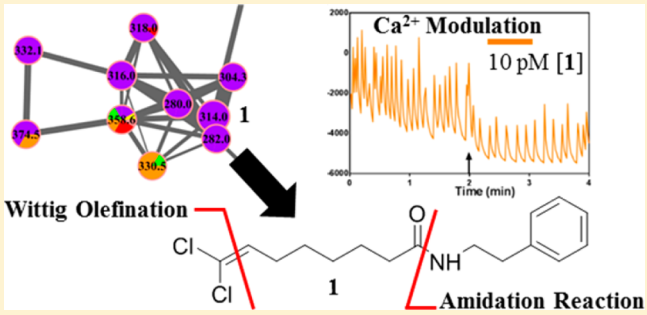
Supporting Information

ABSTRACT: A recent untargeted metabolomics investigation into the chemical profile of 10 organic extracts from cf. *Symploca* spp. revealed several interesting chemical leads for further natural product drug discovery. Subsequent target-directed isolation efforts with one of these, a Panamanian marine cyanobacterium cf. *Symploca* sp., yielded a phenethylamide metabolite that terminates in a relatively rare *gem*-dichlorovinylidene moiety, caracolamide A (**1**), along with a known isotactic polymethoxy-1-alkene (**2**). Detailed NMR and HRESIMS analyses were used to determine the structures of these molecules, and compound **1** was confirmed by a three-step synthesis. Pure compound **1** was shown to have in vitro calcium influx and calcium channel oscillation modulatory activity when tested as low as 10 pM using cultured murine cortical neurons, but was not cytotoxic to NCI-H460 human non-small-cell lung cancer cells in vitro (IC_{50} > 10 μM).



Halogenated secondary metabolites are known to exist quite widely in Nature, with more than 5000 of these having been described previously.[1] Perhaps unsurprisingly, marine natural products have a higher incidence of halogen atoms than those obtained from terrestrial or freshwater organisms.[2,3] Compared to the total number of halogenated natural products reported, however, polyketides and lipids that contain terminal halogenation are relatively rare.[4] Several halogenated polyketides and lipids have been isolated from marine cyanobacteria, such as the calcium channel oscillation modulators credneramides A and B, sodium channel blockers jamaicamide A, janthielamide A, and kimbeamides A–C, and in vitro cancer cell toxins veraguamides A, B, K, and L.[5−9] Pitiamides A and B are another family of fatty acid amides that terminate with a vinyl chloride moiety, and they were also shown to modulate intracellular calcium levels.[10,11] Even more rare in Nature are those that contain *gem*-dichlorovinylidene moieties, currently numbering only about 30 in the Dictionary of Natural Products (http://dnp.chemnetbase.com) database. The presence of such an atypical functional group, particularly among plant natural products, may in some cases be an experimental artifact. Chloroform can form a reactive dichlorocarbene species under certain conditions and lead to the formation of *gem*-dichlorovinylidene moieties.[12] However, marine natural products, such as the taveunamides, mutafuran, (*Z*)- and (*E*)-antazirine, and several analogous azirinecarboxylates that were isolated from the sponge *Dysidea fragilis*, have been shown to contain *gem*-dihalovinylidene moieties compris-

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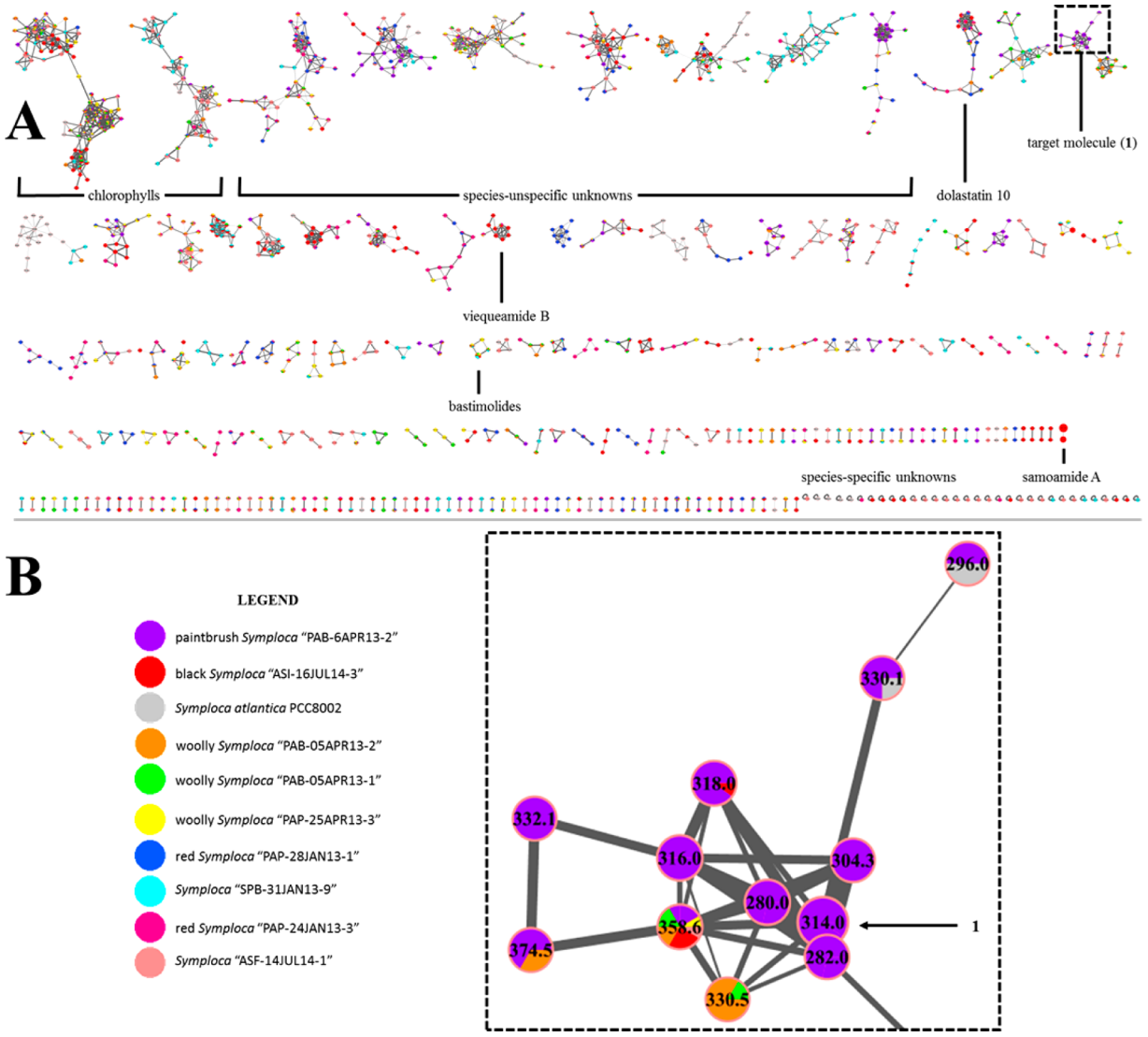

Figure 1. (A) LC-MS/MS-derived molecular network of extracts and systematically produced VLC fractions from 10 cf. *Symploca* spp. For more details about the generation of this network, see ref 14. (B) Expanded region from the top-right corner of panel A, made to highlight the cluster that contains caracolamide A (**1**) along with an organism extract legend.

ing only bromine or chlorine or atoms of each element, thus supporting their natural biosynthetic origins.[4,13]

Chemical profiling of extracts produced from 10 morphologically identified *Symploca* spp., comprising two field-identified collections from American Samoa, one from Saipan, six from Panama, and one grown in the laboratory from the Pasteur Culture Collection (PCC8002), was achieved using LC-MS/MS and molecular networking untargeted metabolomics (Figure 1, A).[14] This revealed several candidates for MS-based targeted natural product discovery.[14] For example, after utilization of the Global Natural Products Social Molecular Network (GNPS at http://gnps.ucsd.edu) Web site to correlate these data, it was found that several clusters of secondary metabolites were present with unique or very limited distribution among these taxa. It was hypothesized that the biosynthesis of these molecules might have a role in mediating specific biological interactions, and thus they were prioritized for natural product drug discovery efforts. Accordingly, the secondary metabolites of the cyanobacterium cf. *Symploca* sp. PAB-6APR13-2, collected near Punta Caracol, Panama, were selected for targeted isolation and structure elucidation (Figure 1, B). Because this discovery process was based on LC-MS and MS/MS data, it was clear from the outset that one of the targeted molecules, compound **1**, had an isotopic signature indicative of the presence of two chlorine atoms. This fact along with its candidate molecular formula of $C_{16}H_{21}Cl_2NO$ suggested its novelty compared to known compounds in the Dictionary of Natural Products and MarinLit (http://pubs.rsc.org/marinlit/) databases. In addition, a second metabolite was isolated in the course of these investigations and was identified by comparison of HRESIMS, specific rotation, and ^1H and ^{13}C NMR data with literature values as the previously reported isotactic polymethoxy-1-alkene 4(*S*),6(*S*),8(*S*),10(*S*),12(*S*),14(*R*),16(*R*),18(*R*),20(*R*),22(*R*)-decamethoxyheptacos-1-ene (**2**).[15−17]

RESULTS AND DISCUSSION

Structure Elucidation. Compound **1** was determined to have the molecular formula $C_{16}H_{21}Cl_2NO$ based on the sodium adduct ion peak in the HRESIMS at *m/z* 336.08983 $[M + Na]^+$. This molecular formula indicated six inherent degrees of unsaturation and, because of an isotopic pattern of *m/z* 336/

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338/340 in a ratio of 9:6:1, supported the occurrence of two chlorine atoms in the molecule. Analysis of the ^{1}H NMR spectrum of **1** in CD_3OD revealed several methylene groups, one of which, CH_2-2 [δ_H 2.14, t (J = 7.5 Hz); δ_C 36.9], formed a terminus for an acyl chain spin system. Following this, four more methylene units were determined through sequential COSY correlations from CH_2-2 to CH_2-3 [δ_H 1.57, pent (J = 7.5 Hz); δ_C 26.7], then to CH_2-4 (δ_H 1.29, m; δ_C 29.6) and CH_2-5 [δ_H 1.43, pent (J = 7.5 Hz); δ_C 28.8], and finally to CH_2-6 [δ_H 2.17, q (J = 7.4 Hz); δ_C 30.4]. The protons of C-6 had COSY correlations to an olefinic methine proton at δ_H 5.96 [t (J = 7.4 Hz); δ_C 131.5 (CH-7)]. The CH_2-6 protons also showed HMBC correlations to CH-7 as well as to a relatively shielded nonprotonated olefinic carbon at δ_C 120.7 (C-8). Considering the molecular formula of **1**, the chemical shifts of CH-7 and C-8 were indicative of a *gem*-dichlorovinylidene moiety.[4,18] Finally, the CH_2-2 and CH_2-3 protons showed cross-peaks by HMBC with an amide-type carbonyl carbon at δ_C 176.1 (C-1), establishing substructure **1a** (Figure 2).

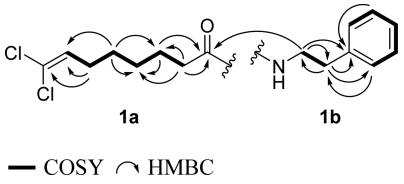
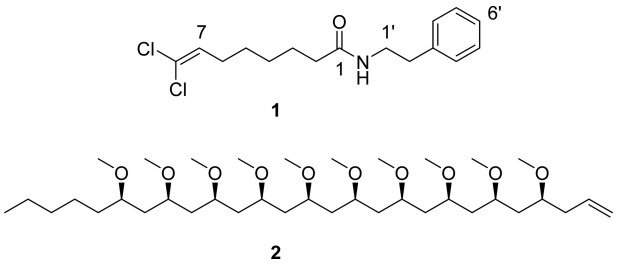


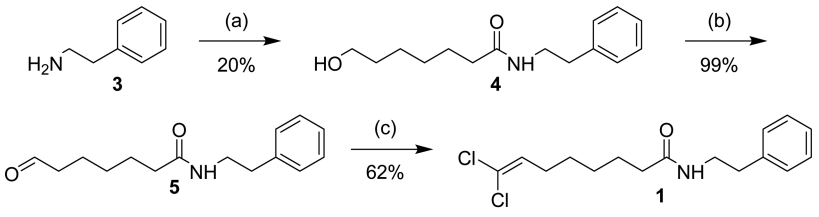
Figure 2. Selected correlations used to determine the two partial structures of caracolamide A (**1**). Bolded bonds represent correlated protons in the COSY spectrum. Arrows represent cross-peaks from the ^{1}H–^{13}C HMBC spectrum.



The ^{1}H NMR spectrum of **1** also exhibited five aromatic protons at δ_H 7.18–7.26, consistent with a monosubstituted phenyl ring. The phenyl protons *ortho* to the substitution, CH-4′ and CH-8′ [δ_H 7.21, d (J = 7.5 Hz), 2H; δ_C 129.8], possessed clear HMBC correlations to a benzylic methylene group [CH_2-2′, δ_H 2.79, t (J = 7.3 Hz); δ_C 36.5]. The latter protons were adjacent to another deshielded methylene group by COSY correlations [CH_2-1′, δ_H 3.41, t (J = 7.3 Hz); δ_C 41.9]. Furthermore, the protons of C-1′ showed HMBC correlations to the fully substituted carbon of the phenyl ring (C-3′, δ_C 140.5). The chemical shift of C-1′ at δ_C 41.9 was indicative of its direct attachment to a nitrogen atom, and this was confirmed by an HMBC correlation between CH_2-1′ and C-1, thus defining a phenethylamide moiety (**1b**) and connecting the two partial structures (Figure 2). Furthermore, the ^{1}H NMR spectrum of **1** recorded in $CDCl_3$ included one additional proton signal at δ_H 5.34 for the amide proton and thus completed the structural characterization of compound **1**, named here as caracolamide A.

Total Synthesis. To confirm the structure of compound **1** and provide material for further biological investigation, a three-step total synthesis was designed (Figure 3). The scheme initiated with the formation of an amide bond between two commercially available starting materials: 2-phenylethylamine (**3**) and 7-hydroxyheptanoic acid. This is analogous to the initiation of the synthesis developed for the structurally related credneramides A and B.[19] The resulting intermediate alcohol (**4**) was oxidized with Dess-Martin periodinane (DMP) to form the corresponding aldehyde (**5**), and this was converted into the final product (**1**) by Wittig reaction with dichloromethylenetriphenylphosphorane.[20,21] The purified reaction yields obtained for each of the three steps to form **4**, **5**, and **1** were 20%, 99%, and 62%, respectively, providing an overall yield of 12% (excluding recoverable starting materials). Synthetic compound **1** had ^{1}H and ^{13}C NMR spectra, along with HRESIMS data, that matched those of the isolated natural product.

Biological Evaluation. Pure compounds **1** and **2** were evaluated for their cytotoxic activity toward NCI-H460 non-small-cell lung cancer cells in vitro; however, neither of these molecules was found to be cytotoxic (IC_{50} > 10 μM). Structurally, **1** is related to a few other cyanobacterial natural products, including credneramide A and santacruzamate A.[5,22] These molecules each feature a 2-phenethylamide moiety with a modified fatty acid group. In the case of credneramide A, there is also a terminal vinyl chloride moiety installed during the biosynthesis of its presumed precursor molecule, credneric acid.[5] Because credneramide A and its analogues have been shown to inhibit spontaneous calcium oscillations in murine cerebrocortical neurons, caracolamide A (**1**) was evaluated in



Reagents and conditions:
(a): 7-hydroxyheptanoic acid (1 eq), HATU (1.1 eq), DIPEA (1 eq), rt in 4:1 DMF/CH_2Cl_2
(b): Dess-Martin periodinane (1.5 eq), $NaHCO_3$ (4 eq), rt in CH_2Cl_2
(c): triphenylphosphine (1 eq), $CHCl_3$ (1 eq), n-BuLi (1.1 eq), in THF at -78 C then rt

Figure 3. Synthetic scheme for preparation of caracolamide A (**1**). HATU: 1*H*-1,2,3-triazolo[4,5-*b*]pyridinium, 1-[bis(dimethylamino)methylene]-, hexafluoro-phosphate(1-), 3-oxide (9CI). DIPEA: *N*,*N*-diisopropylethylamine. DMF: dimethylformamide. Reagents and conditions: (a) 7-hydroxyheptanoic acid (1 equiv), HATU (1.1 equiv), DIPEA (1 equiv), rt in 4:1 DMF/CH_2Cl_2; (b) Dess-Martin periodinane (1.5 equiv), $NaHCO_3$ (4 equiv), rt in CH_2Cl_2; (c) triphenylphosphine (1 equiv), $CHCl_3$ (1 equiv), *n*-BuLi (1.1 equiv), in THF at −78 C then rt.

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this assay system. Remarkably, at subnanomolar concentrations, caracolamide A displayed statistically significant effects on calcium oscillation amplitude in neocortical neurons (Table 2 and Figure 4). The frequency of calcium oscillations observed during the same biological assay, however, was not statistically different at any of the concentrations tested (Supporting Information).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Caracolamide A (1) in CD_3OD[a]

position	δ_C, type	δ_H, mult (J in Hz)	HMBC[b]
1	176.1, C		
2	36.9, CH_2	2.14, t (7.5)	1, 3, 4
3	26.7, CH_2	1.57, t (7.5)	1, 2, 4, 5
4	29.6, CH_2	1.29, m	2, 3, 6
5	28.8, CH_2	1.43, pent (7.5)	3, 6, 7
6	30.4, CH_2	2.17, q (7.4)	4, 5, 7, 8
7	131.5, CH	5.96, t (7.4)	5
8	120.7, C		
1′	41.9, CH_2	3.41, t (7.3)	1, 2′, 3′
2′	36.5, CH_2	2.79, t (7.3)	1′, 3′, 4′, 8′
3′	140.5, C		
4′, 8′	129.8, CH	7.21, d (7.5)	4′, 6′, 8′
5′, 7′	129.5, CH	7.27, t (7.5)	3′, 5′, 7′
6′	127.3, CH	7.18, t (7.5)	4′, 8′

[a]Data recorded at 298 K, 600 MHz (^{1}H) and 150 MHz (^{13}C). Assignments supported by 2D NMR. [b]HMBC correlations, optimized for 8 Hz, are from proton(s) to the indicated carbon.

Because metabolite **1** is a phenethylamide, the commercially available and structurally simpler molecule 2-phenylethylamine (**3**) was also tested in this bioassay and elicited similar effects at the same concentrations as **1** (Supporting Information). The modulatory effects observed on calcium oscillation amplitude suggest a profound regulation of neuronal function and are further predictive of sodium channel blocking and neurotoxic activities. Additional investigations will be necessary to confirm these hypotheses for metabolite **1**; however, compound **3** is a known neuroactive agent and "trace amine", indicating that it is present at much lower concentrations than the more common biogenic amines such as serotonin or dopamine.[23,24] However, the ion channel modulating properties observed for the amide (**1**) and the primary amine (**3**) may be unrelated, as these molecules will have different charge states and attendant physical properties under the conditions of the biological assay. Alternatively, it may be that the biological activity of **1** is related to that of pitiamides A and B, two fatty acid amides with terminal alkyl halide groups.[10,11] Pitiamides A and B were previously shown to modulate plasma membrane potentials, increase intracellular calcium levels, and have low μM IC_{50} values against HCT116 cells.[11] Further pharmacological studies using the pitiamides, **1**, and other structurally related molecules will be necessary to make more meaningful comparisons between these agents.

CONCLUSIONS

The utilization of MS/MS-based molecular networking facilitated the targeted isolation and structural characterization of a new natural product, caracolamide A (**1**), from the Panamanian cyanobacterium cf. *Symploca* sp. (Collection code PAB-6APR13-2). The relatively simple molecular architecture of compound **1** allowed for its three-step total synthesis, and this both confirmed the structure of **1** and provided material for biological testing. This new metabolite has some structural similarity to the credneramides and pitiamides and was further demonstrated to share the biological properties of these molecules by modulating calcium channel function in murine cerebrocortical neurons in vitro. Future studies using synthetically produced caracolamide A (**1**), as well as analogues created via similar synthetic procedures, will enable the development of a broader knowledge of its biological and ecological properties.

Table 2. Statistical Analysis for Caracolamide A (1)-Induced Changes in Spontaneous Ca^{2+} Oscillations in Neocortical Neurons

dose[a]	total no. of oscillations[b]	mean amplitude of oscillations	95% CI of amplitude	P-value of amplitude[c]
baseline	359	1660	1567 to 1753	
0.01 nM [**1**]	282	1053	942 to 1164	0.0279
baseline	330	1883	1759 to 2007	
0.1 nM [**1**]	272	1103	991 to 1213	0.0085
baseline	323	1676	1560 to 1792	
1 nM [**1**]	266	894	803 to 984	0.0060
baseline	361	1781	1684 to 1878	
10 nM [**1**]	324	1388	1286 to 1489	0.0191
baseline	363	1464	1387 to 1541	
100 nM [**1**]	334	887	827 to 947	0.0111
baseline	353	1332	1250 to 1413	
1000 nM [**1**]	330	727	678 to 776	0.0069
baseline	214	2307	2159 to 2456	
control[d]	179	2430	2238 to 2623	0.1803

[a]Baseline amplitude was calculated as peak minus trough for each oscillation during 120 s baseline reading. Compound dose amplitude was calculated as peak minus trough for each oscillation during 120 s reading following compound addition. Amplitudes from 10 replicates were pooled. [b]The total number of oscillations observed during the 2 min before and after application of caracolamide A (**1**) was determined to not be significantly different (Supporting Information). [c]A two-tailed paired t test was performed to assess differences in oscillation amplitude between the means from before and after application of caracolamide A (**1**). [d]Solvent control (DMSO, 0.04% final concentration) was performed in cortical culture on a different date from the other assays.

EXPERIMENTAL SECTION

General Experimental Procedures. A JASCO P-2000 polarimeter was used to measure optical rotations at 25 °C. Ultraviolet and visible (UV−vis) spectra were recorded using a Beckman Coulter DU 800 spectrophotometer. Infrared (IR) spectra were obtained on a Thermo Nicolet IT 100 FT-IR (Thermo Fisher Scientific). A Bruker Avance III 600 NMR spectrometer equipped with a Bruker cryoplatform and a 5 mm inverse detection triple resonance (H−C/N/D) cryoprobe with z-gradients was used to record NMR data at 298 K using standard Bruker pulse sequences. Additional NMR data were obtained on a JEOL ECZ 500 NMR spectrometer equipped with a 3 mm inverse detection probe with z-gradients and a Varian vx 500 NMR equipped with a Varian XSens 2 channel ($^{1}H/^{13}C$) NMR cold

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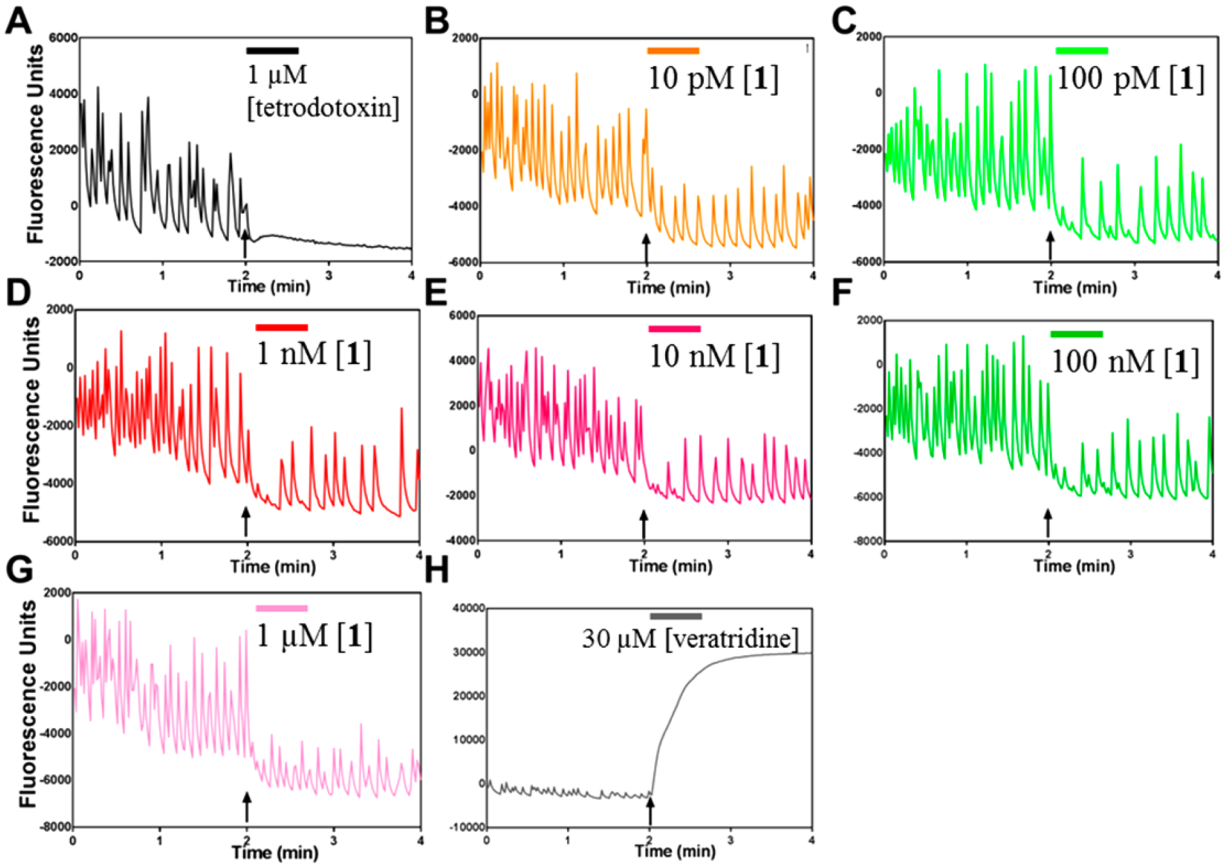

Figure 4. Representative time and concentration response relationships for caracolamide A (**1**)-induced changes in spontaneous Ca^{2+} oscillations in neocortical neurons. (A) Tetrodotoxin (1 μM) was used as a negative control. (B−G) Results from testing of 10 pM through 1 μM of compound **1**, as individually indicated. (H) Veratridine (30 μM) was used as a positive control. Representative data shown from two experiments with five replicates per dose per experiment. Statistical analysis is presented in Table 2.

probe optimized for direct observe ^{13}C NMR. All NMR data were calibrated to the solvent residual peak(s) observed in the ^{1}H and ^{13}C NMR spectra (C_6D_6 δ_H 7.16, δ_C 128.06; $CDCl_3$ δ_H 7.26, δ_C 77.16; CD_3OD δ_H 3.31, δ_C 49.00).[25] Nominal mass resolution LC-MS data were collected using an HPLC comprising a Thermo Finnigan Surveyor PDA Plus detector, Autosampler Plus, and LC Pump Plus coupled to an LCQ Advantage Plus mass spectrometer (all Thermo Fisher Scientific), with a Phenomenex Kinetex C_{18} 100 × 4.6 mm × 5 μm analytical column installed. Exact mass HRESIMS data were recorded using an Agilent 6530 Accurate-Mass QTOF mass spectrometer in positive ion mode. Semipreparative HPLC was performed using a Kinetex C_{18} 150 × 10.0 mm × 5 μm semipreparative column (Phenomenex) connected to an HPLC system comprising a Thermo Dionex UltiMate 3000 pump, RS autosampler, RS diode array detector, and automated fraction collector (all Thermo Fisher Scientific). HPLC-grade CH_3CN was purchased from Thermo Fisher Scientific, and HPLC-grade H_2O was obtained by filtration using a Milli-Q Direct water purification system (Millipore). Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories.

Organism Collection and Identification. Small colonies of a morphologically identified cf. *Symploca* species (e.g., reddish-purple color and a stiff "paintbrush" shape) were obtained in April 2013 by scuba diving to 3−6 m at Punta Caracol (9°22′24.0″ N 82°18′06.0″ W), Bocas del Toro, Panama. The organism was preserved in 0.5 L of 1:1 seawater−2-propanol solution, transported to the laboratory in San Diego, CA, USA, and frozen at −20 °C until extraction.

Extraction, Molecular Networking, and Isolation. The raw biomass of this sample (88.2 g) was extracted exhaustively with 2:1 CH_2Cl_2−MeOH to yield 1.5 g of organic extract (A2143) after solvent evaporation. This material was fractionated by vacuum liquid chromatography (VLC) over silica gel to produce nine subfractions of increasing polarity by elution with 300 mL each using a step gradient of hexanes (A2143A; 50.4 mg), 9:1 hexanes−EtOAc (A2143B; 54.0 mg), 4:1 hexanes−EtOAc (A2143C; 65.4 mg), 3:2 hexanes−EtOAc (A2143D; 76.8 mg), 2:3 hexanes−EtOAc (A2143E; 50.9 mg), 1:4 hexanes−EtOAc (A2143F; 58.0 mg), 100% EtOAc (A2143G; 29.1 mg), 3:1 EtOAc−MeOH (A2143H; 235.8 mg), and 100% MeOH (A2143I; 722.4 mg). These subfractions were analyzed by nominal mass LC-MS as described previously.[14] Briefly, samples were individually preprocessed using C_{18} SPE cartridges by application of 0.3 mg of sample and elution with 1 mL of CH_3CN. A 10 μL aliquot of each sample was injected into the LC-MS and eluted at 0.7 mL/min by a gradient program of CH_3CN−H_2O (0.1% formic acid modifier): 30% for 5 min to 99% in 17 min, held for 3 min, to 30% in 1 min, and held for 4 min. The mass spectrometer was set to observe m/z 190−2000 in positive ESI mode and with an automated data-dependent MS/MS scan enabled. The resulting data were utilized to create a molecular network of 10 extracts from morphologically identified samples of distinct cf. *Symploca* spp. for the purpose of dereplication and molecular targeting.[14] Accordingly, fractions A2143E−G were selected for further isolation studies because they had network nodes and clusters that were unique to this analysis.

Fractions A2143E and A2143F were each further purified by RP-HPLC using a Phenomenex Synergi Fusion C_{18} column (250 × 10.00 mm × 4 μm) and gradient solvent system (65% CH_3CN−H_2O for 5 min, linear gradient to 80% CH_3CN in 15 min, linear gradient to 100% CH_3CN in 5 min) at 4.0 mL/min. Subfractions from each were analyzed and combined to yield compound **1** (t_R = 7.6 min; 0.7 mg, 0.0008% w/w extraction yield).

An aliquot of fraction A2143G (21 mg) was further separated using a GracePure SPE C_{18}-Max cartridge with a step gradient of solvents:

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65% CH_3CN-H_2O (A2143G.1; 8.6 mg), 75% CH_3CN-H_2O (A2143G.2; 5.8 mg), 85% CH_3CN-H_2O (A2143G.3; 0.5 mg), 95% CH_3CN-H_2O (A2143G.4; 1.0 mg), CH_3CN (A2143G.5; 0.5 mg), and MeOH (A2143G.6; 2.8 mg). Fraction A2143G.1 was pooled with A2143G.2 and subjected to RP-HPLC purification using a Phenomenex Kinetex C_{18} column (150 × 10.00 mm, 5 μm) and gradient solvent system (65% CH_3CN-H_2O for 5 min, linear gradient to 80% CH_3CN in 15 min, linear gradient to 100% CH_3CN in 5 min) at 4.0 mL/min to yield compound **2** (t_R = 17.6 min; 1.8 mg, 0.02% w/w extract yield). Spectroscopic, spectrometric, and specific rotation data {$[\alpha]^{23}_D$ +7.30 (*c* 0.15, MeOH)]} obtained for compound **2** matched corresponding literature values {e.g., $[\alpha]^{25}_D$ +4.73 (*c* 0.43, $CHCl_3$)]} for the isotactic polymethoxy-1-alkene 4(*S*),6(*S*),8(*S*),10(*S*),12(*S*),14(*R*),16(*R*),18(*R*),20(*R*),22(*R*)-decamethoxyheptacos-1-ene.[15−17]

*Caracolamide A (**1**):* slightly yellow, oily residue; UV (MeOH) λ_{max} (log ε) 201 (4.31), 280 (2.85) nm; IR (film) ν_{max} 3301, 2927, 2857, 1643, 1546, 1457, 1369, 879, 740, 698 cm^{-1}; ^1H NMR and ^{13}C NMR, Table 1; HRESIMS *m*/*z* 336.08983 $[M + Na]^+$ (calcd for $C_{16}H_{21}Cl_2NONa$, 336.08924); LR MS/MS *m*/*z* 313.97 $[M + H]^+$, 121.81, 105.02.

Synthesis of 7-Oxo-*N*-phenethylheptanamide (4). To a solution of 2-phenylethylamine (**3**; 2.487 g, 20.52 mmol; Sigma-Aldrich), 7-hydroxyheptanoic acid (1.5 g, 10.26 mmol; OxChem), and HATU (4.29 g, 11.29 mmol; Sigma-Aldrich) in dimethylformamide (DMF; 6.84 mL)/CH_2Cl_2 (27.4 mL) was quickly added DIPEA (1.792 mL, 10.26 mmol). The reaction mixture was stirred overnight at room temperature (rt) to generate compound **4**. The mixture was worked up by addition of excess EtOAc, washed three times with 1 M HCl and then saturated sodium bicarbonate and brine. The organic layer was dried using anhydrous sodium sulfate, filtered, and evaporated under reduced pressure. The residue was purified on normal-phase silica flash chromatography using a gradient of 0−10% MeOH in EtOAc to obtain **4** (515.3 mg; 2.07 mmol; 20% yield).

*7-Hydroxy-N-phenethylheptanamide (**4**):* ^1H NMR (500 MHz) δ 7.30 (t, *J* = 7.4 Hz, 2H), 7.22 (m, 1H), 7.19 (m, 2H), 3.60 (t, *J* = 6.5 Hz, 2H), 3.50 (q, *J* = 6.9 Hz, 2H), 2.80 (t, *J* = 6.9 Hz, 2H), 2.12 (t, *J* = 7.4 Hz, 2H), 1.59 (pent, *J* = 7.4 Hz, 2H), 1.54 (pent, *J* = 7.4 Hz, 2H), 1.33 (m, 2H); ^{13}C NMR (125 MHz) δ 173.2, 139.0, 128.9, 128.7, 126.6, 62.8, 40.6, 36.7, 35.8, 32.6, 29.0, 25.7, 25.5; HRESIMS *m*/*z* 250.1800 $[M + H]^+$ (calcd for $C_{15}H_{24}NO_2$, 250.1802).

Synthesis of 7-Oxo-*N*-phenethylheptanamide (5). To a solution of **4** (68 mg, 0.273 mmol; Sigma-Aldrich) and sodium bicarbonate (92 mg, 1.091 mmol; Sigma-Aldrich) in CH_2Cl_2 (1.364 mL) was added DMP (173 mg, 0.409 mmol) in one portion at rt. The reaction mixture was stirred at this temperature for 1 h to generate **5**. The reaction was monitored by normal-phase silica TLC for the conversion of **4** (5:1 CH_2Cl_2−acetone; R_f = 0.33) to **5** (5:1 CH_2Cl_2−acetone; R_f = 0.50). The reaction mixture was worked up by dilution with 15 mL of EtOAc and the addition of 5 mL of a saturated solution of sodium thiosulfate in H_2O, then extracted twice with 10 mL of EtOAc, washed with brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. After workup, the crude material was determined to be of high enough purity by NMR and LC-MS to carry forward in the synthesis (67.5 mg; 0.273 mmol; 99% yield).

*7-Oxo-N-phenethylheptanamide (**5**):* ^1H NMR (500 MHz, $CDCl_3$) δ 9.73 (t, *J* = 1.6 Hz, 1H), 7.30 (m, 2H), 7.22 (m, 1H), 7.19 (m, 2H), 3.50 (q, *J* = 7.0 Hz, 2H), 2.80 (t, *J* = 7.0 Hz, 2H), 2.42 (td, *J* = 7.4, 1.6 Hz, 2H), 2.11 (t, *J* = 7.4 Hz, 2H), 1.61 (q, *J* = 7.4 Hz, 2H), 1.60 (q, *J* = 7.4 Hz, 2H), 1.29 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.7, 172.9, 139.0, 128.8, 128.7, 126.6, 43.7, 40.6, 36.5, 35.7, 28.7, 25.4, 21.7; HRESIMS *m*/*z* 248.1642 $[M + H]^+$ (calcd for $C_{15}H_{22}NO_2$, 248.1645).

Synthesis of 8,8-Dichloro-*N*-phenethyloct-7-enamide (Caracolamide A; 1). To a solution of PPh_3 (90 mg, 0.342 mmol; Sigma-Aldrich) and $CHCl_3$ (0.0274 mL, 0.342 mmol) in tetrahydrofuran (THF; 1.315 mL) at −78 °C was added dropwise *n*-BuLi (0.147 mL, 0.368 mmol) to generate dichloromethylenetriphenylphosphorane. The reaction turned a dark brown-orange color and was stirred for 15 min at this temperature before a solution of **5** (65.1 mg, 0.263 mmol) in THF (0.3 mL) was added dropwise. The reaction was stirred at −78 °C for 30 min, at which time the dry ice bath was removed and the reaction was stirred for an additional 1 h. The mixture was diluted in EtOAc (5 mL) and then quenched with saturated aqueous NH_4Cl (5 mL) and H_2O (5 mL). The layers were partitioned, and the aqueous phase was extracted with EtOAc (3 × 5 mL). The combined organic layers were washed with brine (10 mL), dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude reaction product was purified via normal-phase silica flash chromatography (0−25% EtOAc in hexanes) to afford pure **1** (51 mg, 0.162 mmol, 62% yield). Characterization data of the synthesized compound **1** exactly matched that of the natural product.

In Vitro Cytotoxicity Test Protocols. Samples were evaluated for cytotoxicity to NCI-H460 cells according to previously published protocols with the exception of the concentrations studied.[26] Briefly, 180 μL of suspended cells were added to each well of several 96-well plates at 3.33 × 10^4 cells/mL in RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin. The plates were incubated overnight at 37 °C in a 5% CO_2 chamber before application of test samples. Samples were dissolved in DMSO and diluted in RPMI 1640 medium without FBS to final concentrations of 1 or 10 μg/mL for crude samples, 10 final concentrations with 3-fold serial dilutions starting from 100 μg/mL, screened in duplicate, for pure compounds. Doxorubicin (1 μg/mL) and DMSO in RPMI 1640 without FBS were used as positive and negative controls, respectively. Plates were incubated for 48 h and then stained with MTT (thiazolyl blue tetrazolium bromide 98%; Sigma-Aldrich) prior to being measured on a ThermoElectron Multiskan Ascent plate reader (Thermo) at 630 and 570 nm. Dose−response curves were generated using nonlinear regression in GraphPad Prism computer software.

Cerebrocortical Neuron Culture. Cerebrocortical neurons were harvested and cultured according to previously described protocols.[27,28] Briefly, Swiss-Webster gravid dams were euthanized by CO_2 asphyxiation and embryonic day 16 mice were removed under sterile conditions. Animal care and handling complied with protocols approved by the Creighton University Institutional Animal Care and Use Committee and employed measures to minimize pain and discomfort. Cerebrocortices were dissected, stripped of meninges, minced, and incubated with trypsin for 25 min at 37 °C. Cells were dissociated by trituration in isolation buffer containing soybean trypsin inhibitor and DNase, centrifuged, and resuspended in Eagle's minimal essential medium with Earle's salt (MEM) supplemented with 1 mM L-glutamine, 10% FBS, 10% horse serum, 100 IU/mL penicillin, and 0.10 mg/mL streptomycin (pH 7.4). Cells were plated onto poly-L-lysine-coated 96-well (9 mm), clear-bottomed, black-well culture plates (MidSci) at a density of 1.5 × 10^5 cells/well. Cells were then incubated at 37 °C in a 5% CO_2 and 95% humidity atmosphere. Cytosine arabinoside (ARA-C; 10 μM) was added to the culture medium 24 h after plating, to prevent proliferation of non-neuronal cells. From day 4 in vitro, culture media was changed every other day using a serum-free growth medium containing neurobasal medium supplemented with B-27, 100 IU/mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM L-glutamine.

In Vitro Intracellular Ca^{2+} Channel Oscillation Monitoring Protocols. Cerebrocortical neuron cultures were used for $[Ca^{2+}]$ measurements at day 12 in vitro (DIV12) as previously described.[28] Briefly, the growth medium was removed and replaced with dye loading medium (100 μL per well) containing 4 μM fluo-3 AM and 0.04% pluronic acid in Locke's buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM $MgCl_2$, 2.3 mM $CaCl_2$, 0.0001 mM glycine, pH 7.4). After 1 h of incubation in dye loading medium, the neurons were washed four times in fresh Locke's buffer (180 μL per well) using an automated microplate washer (Bio-Tek Instruments Inc.) and transferred to a FLIPR II (Molecular Devices). Cells were excited at 488 nm, and Ca^{2+}-bound fluo-3 emission was recorded at 515−575 nm at 0.5 s intervals. After recording baseline fluorescence for 2 min, 20 μL of 10× concentrations of compounds was added to wells at a rate of 20 μL/s, and the fluorescence was monitored for an additional 2 min. GraphPad Prism 7 software was used to analyze time and concentration−response relationships. Fluo3

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fluorescence was expressed as $F_{max} - F_0$, where F_{max} is the maximum and F_0 is the baseline fluorescence measured in each well.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00367.

^{1}H NMR, ^{13}C NMR, COSY, HSQC, HMBC, LRMS, and LRMS/MS spectra for compound **1**, along with ^{1}H NMR and ^{13}C NMR spectra for compounds **2**, **4**, and **5**, LC-MS/MS molecular networking output data, as well as ion channel modulation activity data for phenylethylamine (**3**) and statistical analysis of ion channel modulation activity for caracolamide A (**1**) (PDF)

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Notes

The authors declare no competing financial interest.

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