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Thiol-Based Probe for Electrophilic Natural Products Reveals That Most of the Ammosamides Are Artifacts

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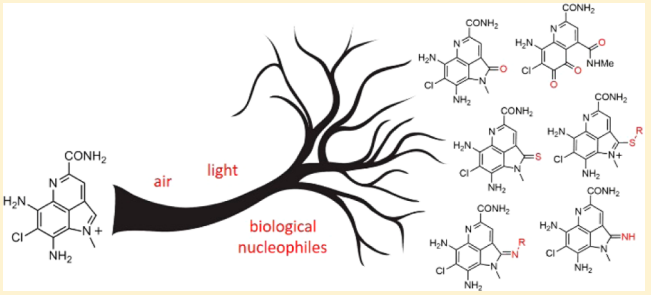
Supporting Information

ABSTRACT: To date, 16 members of the ammosamide family of natural products have been discovered, and except for ammosamide D each of these metabolites is characterized by an unusual chlorinated pyrrolo[4,3,2-*de*]quinoline skeleton. Several ammosamides have been shown to inhibit quinone reductase 2, a flavoenzyme responsible for quelling toxic oxidative species in cells or for killing cancer cells outright. Treatment of the extract from an ammosamide-producing culture (*Streptomyces* strain CNR-698) with a thiol-based reagent designed to label electrophilic natural products produced an ammosamide C-thiol adduct. This observation led us to hypothesize, and then demonstrate through experimentation, that all of the other ammosamides are derived from ammosamide C via nonenzymatic processes involving exposure to nucleophiles, air, and light. Like many established electrophilic natural products, reaction with the thiol probe suggests that ammosamide C is itself an electrophilic natural product. Although ammosamide C did not show substantial cytotoxicity against cancer cells, its activity against a marine *Bacillus* bacterial strain may reflect its ecological role.



One of the many difficulties associated with the study of natural products is the isolation of artifacts, compounds derived from natural products that are formed via non-enzymatic processes during the cultivation and extraction of the organism or during the process of purification. Here, we use the term "artifact" broadly to include metabolites modified by both biotic and abiotic reagents, provided the transformations proceed without the need for catalysis. In most cases, an artifact arises from reaction between an electrophilic site on a natural product and a nucleophilic metabolite or solvent (Figure 1). For example, the plant metabolite palmatine (**1**) has been shown to react with solvent during isolation to form pseudobase **2** and artifacts **3**−**5**.[1] Trichloromethyl artifact **5** stems from a common extraction procedure for plant alkaloids involving a mixture of ammonium hydroxide and $CHCl_3$. Extraction of a *Hyrtios* sponge with MeOH yielded puupehenone (**6**) and dimethoxypuupehenol (**7**),[2] and aqueous extraction of a *Zyzzya* sponge gave makaluvamine H (**8**) and damirone A (**9**).[3] In both cases, the formation of **7** and **9** is directly observed upon treatment of pure **6** and **8** with the appropriate conditions, which provides strong evidence that **7** and **9** are indeed artifacts. The oxidation of natural products to produce artifacts is also common. For instance, cryptolepine (**10**) oxidizes to cryptolepinone (**11**) during isolation and purification.[4] Artifacts arising from a nonenzymatic reaction between a nucleophile-containing natural product and an external electrophile are much less common. The formation of bohemamine dimer **13** from bohemamine (**12**), due to the presence of formaldehyde in the growth medium, is a salient example.[5]

The ammosamides are a family of biologically active natural products isolated from marine-derived *Streptomyces* sp. CNR-698.[6] Although many related pyrroloiminoquinone natural products have been reported from marine sponges, including the batzellines, damirones, isobatzellines, makaluvamines, discorhabdins, prianosins, epidardins, tsitsikammamines, velutamine, zyzzyanone, and wakayin,[7] the ammosamides and lymphostin are the only natural products known to possess a pyrrolo[4,3,2-*de*]quinoline skeleton.[8] In their initial disclosure, ammosamides A (**14**) and B (**15**) were shown to be highly cytotoxic against the HCT-116 colon cancer cell line. Ammosamide B was then determined to target the motor protein myosin using an immunofluorescent probe, and the activity of the metabolite was thus attributed to inhibition of crucial myosin-based cellular functions.[9] Ammosamide B has also been shown to inhibit quinone reductase 2.[10] In contrast to the initial reported cytotoxicity of **14** and **15**, subsequent publications have noted their lack of cytotoxicity against several cancer cell lines.[10,11] A later report detailing the first total synthesis of **15** included the serendipitous discovery of a minor metabolite, ammosamide C (**16**).[12] Since then, ammosamide D (**17**) has been discovered, and ammosamides E−P have been produced by supplementing the culture medium with a set of

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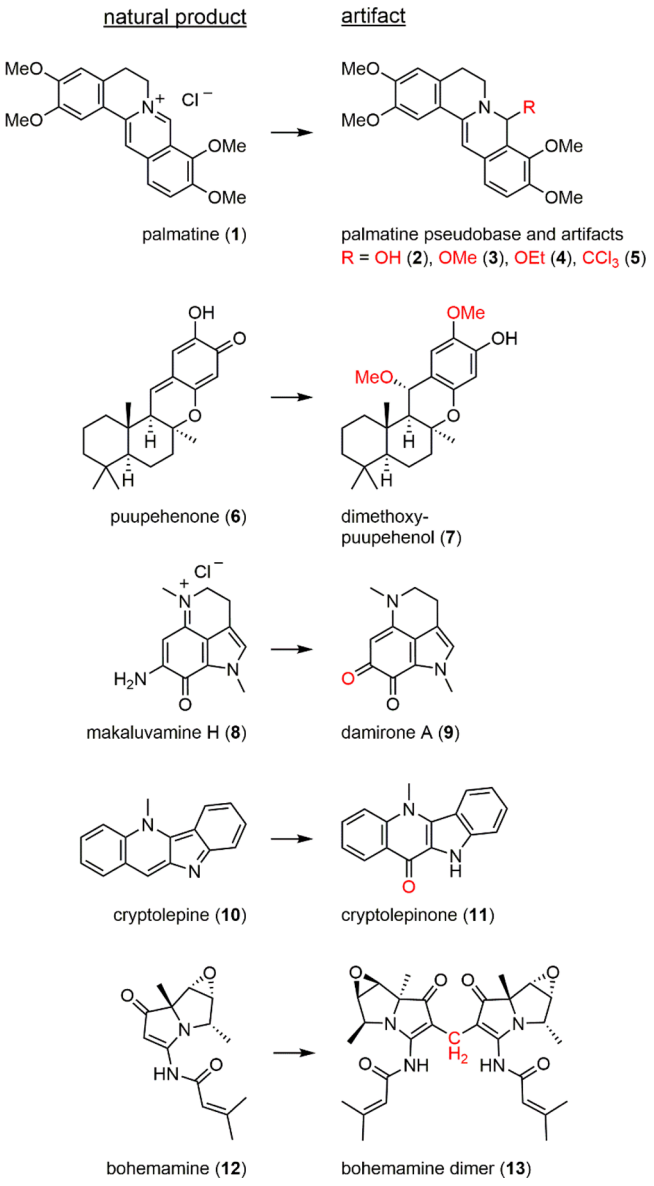
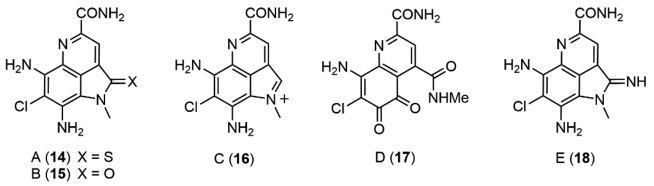


Figure 1. Isolation artifacts of natural products.

precursors.[11,13] Three additional total syntheses of ammosamide B (**15**) have since been completed.[14−16]



RESULTS AND DISCUSSION

In our ongoing efforts to detect electrophilic natural products using thiol-based probe **19** and triethylamine,[17] we observed that the addition of **19** to the extract of *Streptomyces* sp. strain CNR-698 produced a single adduct (**20**) (Scheme 1).[18] In accordance with the method, the thiol adduct bore a conspicuous isotopic pattern by virtue of the probe's bromine atom. The structure of **20** was determined using HRMS and NMR spectrosopy, and retrosynthetic analysis then revealed that the adduct originated from ammosamide C (**16**). Curiously, the red-purple labeling reaction mixture of **19**, **20**, and triethylamine gradually turned blue due to the formation of ammosamide A (**14**) and bromide **21**. On the basis of previous applications of **19** to bacterial extracts, an effective bond-forming reaction with **16** suggests that the metabolite binds irreversibly to its cellular target(s) and that the electrophilic iminium ion is therefore essential for the metabolite's biological activity.[17] However, to date, both the cellular target and mechanism of action of ammosamide C have not been determined.

This observation led us to hypothesize that ammosamide C (**16**) is the precursor to all other ammosamides, which are themselves artifacts formed from uncatalyzed chemical transformations owing to the electrophilicity of the reactive C-2 iminium functionality. In a key experiment, a small quantity of purified **16** was treated with A1 medium buffered to pH 10 to mimic the alkalinity associated with the producing culture and after 5 days yielded ammosamides B (**15**) and E (**18**). A1 medium at pH 6.8 effected very little conversion (Figures 2 and S1). Furthermore, C (**16**) cleanly converted to B (**15**) using an aqueous sodium bicarbonate solution. When treated with cysteine at pH 10, C (**16**) converted to B (**15**), E (**18**), and A (**14**). Although the notion that **14** and **15** are derived from **16** has been previously suggested,[12] this transformation has not been heretofore demonstrated experimentally. To be fair, we do acknowledge that ammosamides A (**14**), B (**15**), and E (**18**), and others, may be formed via enzyme-catalyzed processes in addition to the nonenzymatic conditions described above.

To further investigate the reactivity of ammosamide C (**16**), we required larger quantities of material. Originally, this metabolite was purified as a minor metabolite from cultivations of strain CNR-698 because its hydrophilicity meant that extractions with organic solvent and hydrophobic resins were ineffective. When a 3-day-old culture was directly treated with MeOH containing 0.1% trifluoroacetic acid, filtered through Celite, concentrated to remove the organic solvent, fractionated with C18 silica gel, and purified by HPLC, 100 mg of **16** per liter of culture was obtained. Acidification of the culture before concentration preserved the natural product and greatly reduced the formation of artifacts, while the addition of organic solvent precipitated cellular material that would otherwise clog the C18 silica gel column.

With adequate amounts of ammosamide C (**16**) in hand, we treated the compound with various nucleophiles in order to assess the reactivity of the iminium functionality and to obtain a sufficient quantity of each adduct for full structural characterization (Scheme 2). In contrast to a previously reported study by Pan et al., in which new ammosamide derivatives were generated by supplementing the culture with amines,[11] here similar derivatives were prepared in vitro using amines, thiols, and water. Treatment of **16** with amine nucleophiles (*n*-propylamine and 4-chloroaniline) furnished **23** and ammosamide G (**24**), while treatment with thiol nucleophiles (*N*-acetylcysteamine, ethanethiol, and 4-bromobenzyl mercaptan) provided **26**−**28**. Conversion of **16** to ammosamide B (**15**) was performed using basic aqueous conditions, as before. Presumably, these transformations proceed via acetals **22**, **25**, and **29**, which oxidize when exposed to air. When conducted under an inert atmosphere, clear color changes accompany both formation of the acetals and subsequent oxidation. Given the wide range of possible ammosamide C-nucleophile adducts, the reactive iminium functionality may represent a strategy in

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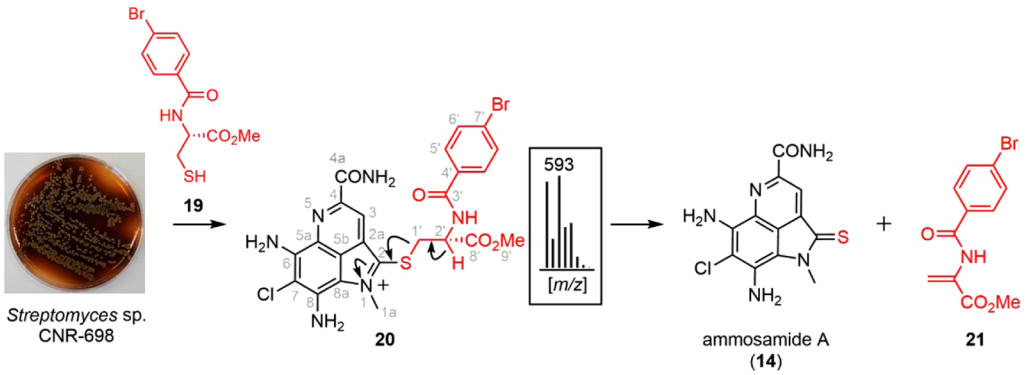
Scheme 1. Treatment of the Extract of Strain CNR-698 with Cysteine-Based Probe 19 Gave 20 and Adduct 20 Fragmented to Ammosamide A (14) and 21



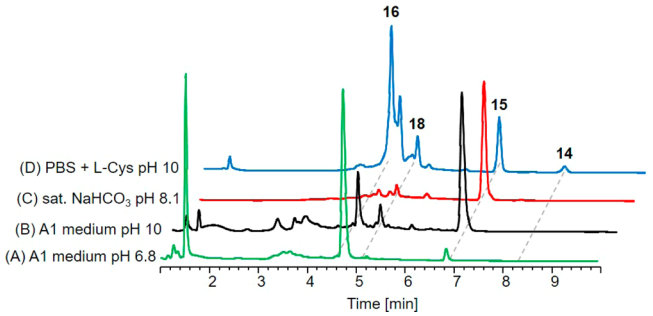


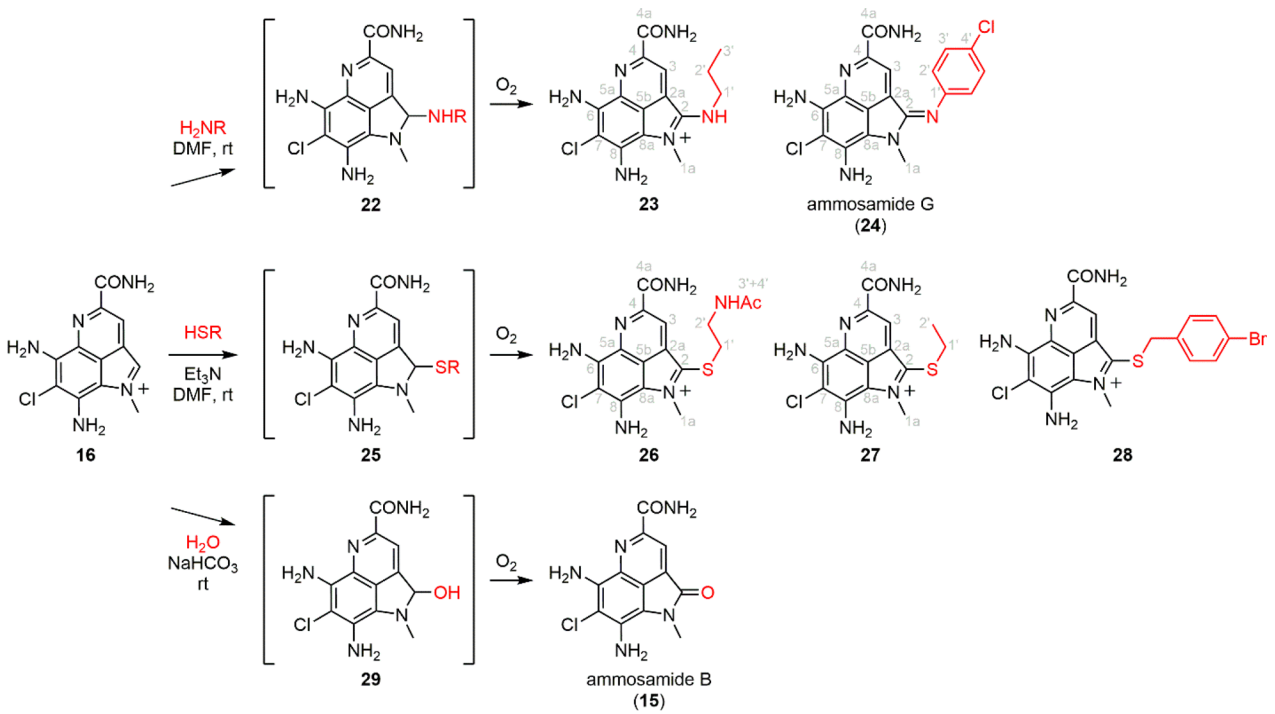
Figure 2. UV/vis-HPLC chromatograms (254 nm) showing the conversion of ammosamide C (**16**). (A) **16** in A1 medium at pH 6.8 after 5 days (green). The conversion of **16** after 5 days into (B) ammosamides B (**15**) and E (**18**) in A1 medium at pH 10 (black), (C) ammosamide B (**15**) in an aqueous sodium bicarbonate solution at pH 8.1 (red), and (D) ammosamides B (**15**), E (**18**), and A (**14**) in a PBS solution containing excess cysteine at pH 10 (blue).

Nature for the deliberate diversification of the ammosamide scaffold, yielding a whole class of molecules that are capable of exhibiting a variety of biological functions.

We then noticed that ammosamide B (**15**) after prolonged exposure to sunlight and air in MeOH converted to ammosamide D (**17**) (Figure 3). This conversion did not appear to occur in other solvents, such as THF or DMSO, suggesting that a protic solvent is required (Figure S5d). This observation is in stark contrast to a previous study describing the discovery of the natural product from cultures of *Streptomyces* sp. strain SNA-020, wherein an enzyme-catalyzed process yielding **17** was postulated.[13] Under standard cultivation conditions in the laboratory with artificial light, ammosamide D is formed only in small amounts as a minor compound.

The cytotoxicity of selected compounds against the HCT-116 colon cancer cell line was assessed in-house using the MTS

Scheme 2. Reaction of Ammosamide C (16) with Various Nucleophiles Followed by Oxidation in Air[a]



[a]Pyrroloquinolinium compounds **23** and **26**–**28** were isolated as TFA salts.

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Table 1. NMR Spectroscopic Data (^{1}H 500 MHz, ^{13}C 125 MHz, DMSO-d_6) for Compounds 20, 23, 24, 26, and 27

	20		23		24		26		27	
position	δ_C, type[a]	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1a	36.4, CH_3	4.31, s	33.5, CH_3	3.92, s	30.1, CH_3	3.69, s	36.7, CH_3	4.39, s	36.4, CH_3	4.39, s
2	137.4, C		152.5, C		153.0, C		138.4, C		138.6, C	
3	118.3, CH	8.66, s	119.6, CH	8.69, s	115.7, CH	7.51, s	118.4, CH	8.73, s	118.2, CH	8.71, s
4	144.4, C		143.7, C		144.5, C		144.5, C		144.3, C	
4a	165.5, C		165.6, C		ND		165.4, C		165.3, C	
4a-NH_2		8.93, br s		8.98, br s		7.51, s		9.00, s		9.00, s
		7.84, br s		7.80, br s		8.78, s		7.88, s		7.89, s
5b	118.8, C		118.8, C		120.4, C		119.0, C		118.9, C	
6-NH_2		9.19, br s/8.26, br s[b]		7.37, br s[c]		6.45, br s		8.94, br s[d]		8.91, br s[e]
8-NH_2	–	6.58, br s[b]		7.20, br s[c]		5.92, br s		9.27, br s/8.32, br s[d]		9.26, br s/8.30, br s[e]
8a	114.0, C		106.4, C		110.5, C		114.2, C		114.0, C	
1′	35.7, CH_2	3.67, dd (14.2, 4.0)	46.3, CH_2	3.82, t (7.2)	149.4, C		36.0, CH_2	3.28, m	30.4, CH_2	3.24, q (7.4)
		3.75, dd (14.2, 10.0)								
2′	53.6, CH	4.75, m	21.8, CH_2	1.82, m	122.6, CH	7.01, d (8.6)	38.8, CH_2	3.22, q (5.6)	14.8, CH_3	1.24, t (7.4)
3′	164.4, C		11.3, CH_3	1.06, t (7.4)	129.2, CH	7.40, d (8.6)	169.4, C			
4′	ND[d]				126.3, C		22.3, CH_3	1.63, s		
5′	128.2, CH	7.26, d (8.6)								
6′	130.6, CH	7.39, d (8.6)								
7′	125.5, C									
8′	169.7, C									
9′	52.2, CH_3	3.60 (s)								
1′-NH				9.33, br s						
2′-NH		8.77, d (8.2)						8.04, t (5.6)		

[a]Carbon chemical shifts were based on HSQC and HMBC data. [b,c,d,e]These assignments may be switched. [f]Could not be determined.

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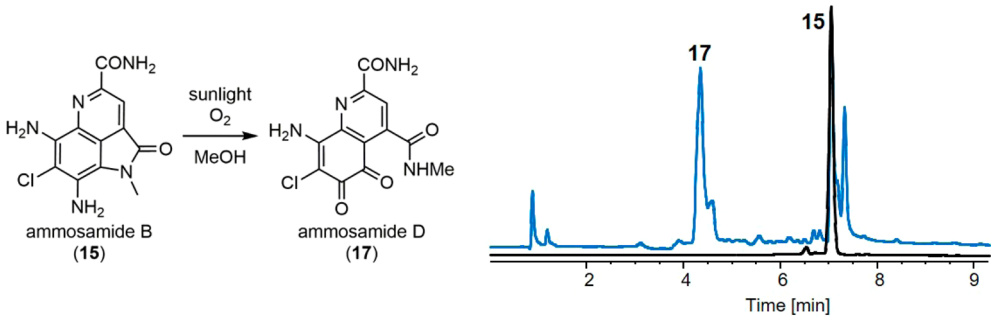

Figure 3. Ammosamide B (**15**) converts to ammosamide D (**17**) after prolonged exposure to sunlight and air in MeOH. UV/vis-HPLC chromatogram (254 nm) at 0 days (black) and after 6 days (blue).

assay and against the 60-cell-line panel at the National Cancer Institute (Figure S2).[19] Ammosamide C (**16**) displayed modest cytotoxicity, exhibiting the highest potency against the human colorectal cancer cell line HCT-116 of all tested cancer cell lines. Importantly, **16** persisted for weeks in the bioassay media and only slowly converted to ammosamide B (**15**) with a $t_{1/2} \approx$ 10 days, so the bioactivity can be attributed exclusively to **16**. Ammosamides A (**14**), B (**15**), D (**17**), and G (**24**) showed very little cytotoxicity, in agreement with other reports,[10,11,13] while propylamine adduct **23** presented activity similar to **16**. Interestingly, ammosamides L and M, carrying the aliphatic amines hexylamine and isopropylamine, also showed significant cytotoxicity against the HCC44 cancer cell line (0.50 and 1.1 μM).[11] The fact that **23** showed activity was unexpected and defies our hypothesis that the unsubstituted electrophilic iminium functionality is tied to its biological activity, assuming cytotoxicity toward eukaryotic cells is related to the metabolite's ecological function.

Table 2. Cytotoxicity of Selected Compounds against Human Colorectal Cancer Cell Line HCT-116[a]

compound	HCT-116 IC_{50} (μM)
14 (A)	NSA
15 (B)	29
16 (C)	5.5
17 (D)	NSA
23	6.8
24 (G)	39
26	NSA

[a]NSA = no significant activity. Growth inhibitions (%) at the highest test concentration (78.125 μg/mL) were measured as 19% (**14**), 97% (**17**), and 26% (**26**). Values are averages of three measurements.

Lastly, the antimicrobial activities of ammosamides A–C (**14**–**16**) were determined against *Bacillus oceanisediminis* CNY-977 and *Escherichia coli* MG1655 using a disk diffusion assay.[20] Although little activity was observed against the *E. coli* strain, a significant zone of inhibition for **16** alone was apparent against the *Bacillus* strain (Figure S3). The antimicrobial activity of **16** against a Gram-positive marine bacterial strain may reflect the true ecological role of the natural product.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H NMR and 2D NMR spectra were recorded at 500 MHz in DMSO-d_6 (residual solvent referenced to 2.50 ppm for ^{1}H, 39.52 ppm for ^{13}C) on a Varian Inova 500 MHz NMR spectrometer or a Jeol 500 MHz NMR spectrometer. Reactions were analyzed with an analytical 1100 Series Agilent Technologies HPLC system coupled to an ELSD and UV/vis detector (210, 254, and 360 nm) using a Phenomenex Luna reversed-phase (RP) C18(2) column (5 μm, 100 Å, 100 × 4.6 mm) with a 10 min solvent gradient from 10% to 100% or 1% to 60% CH_3CN in H_2O containing 0.1% formic acid (FA) and a flow rate of 1.0 mL min^{-1}. Using the same column and solvent gradients, liquid chromatography–high-resolution mass spectrometry (LC-HRMS) was performed on an analytical Agilent 1260 Infinity Series LC system coupled to a 6530 Series Q-TOF mass spectrometer. Metabolites and reaction products were purified using RP UV/vis-HPLC (Millipore Waters 600E) with a semipreparative Phenomenex Luna RP C8(2) column (5 μm, 100 Å, 250 × 10 mm), semipreparative Phenomenex Luna RP CN column (5 μm, 100 Å, 250 × 10 mm) or a preparative Phenomenex Luna RP C18(2) column (5 μm, 100 Å, 250 × 21.2 mm), and a flow rate of 3 and 13 mL min^{-1}, respectively. Thiol **19** was synthesized as described previously.[17] All reagents and solvents were purchased commercially and were used without further purification.

Extraction and Isolation of Ammosamides A–C (14–16). The marine *Streptomyces* sp. strain CNR-698 was cultivated in a 2.8 L Fernbach flask containing 1 L of a seawater-based A1 medium (10 g L^{-1} starch, 4 g L^{-1} yeast extract, 2 g L^{-1} peptone, 75% seawater (La Jolla, CA, Pacific Ocean), 25% deionized (DI) water) at 230 rpm and 27 °C under artificial light. For the isolation of **14** and **15**, a starter culture of 25 mL was grown for 3 days and inoculated into fresh A1 medium (1 L). After 7 days of cultivation, XAD7HP resin was added to the culture and left to stir for 1 h. The cells and resin were filtered through cheesecloth, extracted with acetone (200 mL), and concentrated under reduced pressure to remove the organic solvent. The aqueous layer was then extracted with EtOAc (500 mL). The organic layer was dried over sodium sulfate, filtered, and concentrated to afford 57.7 mg. The crude material was dry-loaded onto C18 silica gel and fractionated using a solid-phase extraction RP C18 cartridge (20%, 40%, 60%, 80%, and 100% CH_3CN in H_2O + 0.1% TFA). The 40% and 60% fractions were combined and evaporated to dryness to yield 17.1 mg. Amides **14** and **15** were purified by preparative C18 HPLC at 254 nm (25% CH_3CN in H_2O, 0.1% TFA, for 13 min, then 30% CH_3CN in H_2O, 0.1% TFA, t_R(**15**) = 12 min, t_R(**14**) = 23 min) to give 4.0 mg of **14** as blue and 4.0 mg of **15** as purple solids. For the isolation of **16**, a starter culture of 25 mL was grown for 3 days and inoculated into fresh A1 medium (1 L). After 3 days of cultivation, MeOH (500 mL) containing 0.1% TFA was added to the broth, and the resulting heterogeneous solution was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure to remove the organic solvent, and the aqueous solution was then loaded onto a C18 column. The column was flushed thoroughly with H_2O + 0.1% TFA, and ammosamide C (**16**) was then eluted with 20% CH_3CN in H_2O + 0.1% TFA. The fraction was concentrated to give 432 mg. A portion of the fraction (43.2 mg, 10%) was dissolved in DMSO and purified by preparative C18 HPLC at 254 nm (35% MeOH in H_2O, 0.1% TFA, t_R = 11 min) to yield 10.0 mg of ammosamide C (**16**) as a bright red solid. All HRMS, UV/vis, and NMR data agreed with reported data. HRMS (ESI-Q-TOF) for **14**: m/z [M + H]$^+$ 308.0372 (calcd for $C_{12}H_{11}ClN_5OS$, 308.0367, Δ0.72 ppm); for **15**: m/z [M + H]$^+$ 292.0596 (calcd for $C_{12}H_{11}ClN_5O_2$,

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292.0596, Δ1.79 ppm); for **16**: *m/z* $[M]^+$ 276.0646 (calcd for $C_{12}H_{11}ClN_5O^+$, 276.0647, Δ0.27 ppm).

Conversion of Ammosamide C in Cultivation Medium, pH 10. Ammosamide C TFA (**16**) (1.0 mg, 0.0026 mmol) was dissolved in 500 μL of seawater-based A1 medium at pH 10.0. As a control reaction, the same amount of **16** was dissolved in seawater-based A1 medium at pH 6.8. The solutions were kept at room temperature (rt). HPLC samples were prepared by diluting the reaction mixture (10 μL) with CH_3CN (20 μL), and 5 μL was analyzed for the consumption of **16** (t_R = 4.8 min) and the formation of **15** (t_R = 6.9 min) and **18** (t_R = 5.3 min) by analytical C18 ELSD-UV/vis-HPLC (1% to 60% CH_3CN in H_2O, 0.1% formic acid) for 8 days and after 14, 18, 20, 24, and 28 days (Figure 2a, Figures S1a and S1b).

Conversion of Ammosamide C in PBS Buffer, pH 10. Ammosamide C TFA (**16**) (2.0 mg, 0.0051 mmol) was dissolved in 1.0 mL of phosphate-buffered saline (PBS) buffer at pH 10 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). The solution was kept at rt, and the conversion of **16** (t_R = 4.8 min) to **15** (t_R = 6.9 min) and **18** (t_R = 5.3 min) was monitored by analytical C18 ELSD-UV/vis-HPLC and LC-HRMS after 1, 2, and 5 days, as described for the cultivation medium above (Figure S1d).

Conversion of Ammosamide C in Aqueous Sodium Bicarbonate Solution, pH 8.1. Ammosamide C TFA (**16**) (2.0 mg, 0.0051 mmol) was dissolved in 1.0 mL of saturated $NaHCO_3$ solution at pH 8.1. The solution was kept at rt, and the conversion of **16** (t_R = 4.8 min) to **15** (t_R = 6.9 min) was monitored by analytical C18 ELSD-UV/vis-HPLC and LC-HRMS after 1, 2, and 5 days, as described for the cultivation medium above (Figure 2b, Figure S1c).

Conversion of Ammosamide C in PBS Buffer, pH 10, Supplemented with L-Cysteine. Ammosamide C TFA (**16**) (0.40 mg, 0.0010 mmol, 1 equiv) and L-cysteine (1.3 mg, 0.0072 mmol, 7 equiv) were dissolved in 400 μL of PBS buffer at pH 10. The solution was kept at rt, and the conversion of **16** (t_R = 4.8 min) to **14** (t_R = 8.3 min), **15** (t_R = 6.9 min), **18** (t_R = 5.3 min), and **20** (t_R = 4.9 min) was monitored by analytical C18 ELSD-UV/vis-HPLC and LC-HRMS after 1, 3, 4, 5, 6, 7, and 10 days as described for the cultivation medium above (Figure 2c, Figure S1e).

Conversion of Ammosamide B into Ammosamide D (17) with Light. For three different small-scale reactions ammosamide B (**15**) (1.0 mg, 0.0034 mmol) was dissolved in MeOH (500 μL), THF (500 μL), or DMSO (500 μL) and exposed to direct sunlight, while allowing for the exchange of air using a loosened cap for up to 21 days. The reaction was monitored by analytical C18 ELSD-UV/vis-HPLC (1% to 60% CH_3CN in H_2O, 0.1% FA) after 0, 2, 6, 9, 13, and 21 days. The conversion of **15** (t_R = 6.9 min) to **17** (t_R = 4.4 min) in MeOH was indicated by a color change from purple to bright orange (Figure S5). For the isolation and purification of **17**, ammosamide B (**15**) (6.3 mg, 0.022 mmol) was dissolved in MeOH (1.0 mL) and exposed to the same conditions. As the reaction is light dependent, cloudy weather conditions required a longer experiment length (5 weeks) for good conversion (~50%). The reaction was concentrated under N_2, and the product was purified by semipreparative C8 HPLC at 260 nm (18% CH_3CN in H_2O, 0.1% TFA, t_R = 5.5 min) to furnish 3.1 mg (47%) as a bright orange solid. All HRMS, UV/vis, and NMR data agreed with reported data. ^1H NMR δ_H 9.36 (1H, s), 9.17 (1H, s), 8.55 (1H, s), 8.18 (1H, q, *J* = 4.7 Hz), 8.02 (1H, s), 8.00 (1H, s), 2.77 (3H, d, *J* = 4.7 Hz) ppm; HRMS (ESI-Q-TOF) *m/z* $[M + H]^+$ 309.0389 (calcd for $C_{12}H_{10}ClN_4O_4$, 309.0385, Δ1.82 ppm).

Labeling Experiment of *Streptomyces* sp. CNR-698 with the Cysteine Thiol Probe. An extract from 9 × 1 L cultures of the marine *Streptomyces* sp. CNR-698 was prepared as described previously.[17] The extract was dry-loaded onto C18 silica gel and fractionated using a solid-phase extraction RP C18 cartridge (20% and 50% CH_3CN in H_2O, 0.1% TFA). Both fractions were combined and evaporated to dryness to provide 2.19 g. The crude fraction and cysteine thiol probe (**19**) (200 mg, 0.629 mmol) were dissolved in dry and sparged DMF (20 mL) in a vial purged with N_2. Dry Et_3N (175 μL, 1.26 mmol) was added, and the reaction mixture was kept overnight under N_2 at rt. Analysis of the reaction mixture by analytical C18 ELSD-UV/vis-HPLC (10% to 100% CH_3CN in H_2O, 0.1% FA) showed labeling of **16** (t_R = 3.0 min) with probe **19** (t_R = 7.1 min) to give adduct **20** (t_R = 3.8 min) (Figure S7). In this instance, a significant amount of ammosamide A (**14**) formed via cleavage of **20** during workup and purification.

Synthesis and Purification of Ammosamide C Cysteine Thiol Probe Adduct (20). Ammosamide C TFA (**16**) (10.0 mg, 0.0257 mmol, 1 equiv) and cysteine thiol probe (**19**) (17 mg, 0.053 mmol, 2 equiv) were dissolved in dry and sparged DMF (1.0 mL) in a vial purged with N_2. For cleaner conversion to **20** Et_3N was not added. The reaction mixture was kept under N_2 for 22 h at rt. The reaction mixture was concentrated under N_2 and high vacuum overnight, and the product was purified by semipreparative C8 HPLC at 254 nm (35% CH_3CN in H_2O, 0.1% TFA, t_R = 6.0 min) to afford 6.4 mg (35%) as a red-purple TFA salt. UV (MeOH) λ_{max} 435 (3.17), 560 (2.67) nm; ^1H and ^{13}C NMR, see Table 1; HRMS (ESI-Q-TOF) *m/z* $[M]^+$ 591.0230 (calcd for $C_{23}H_{21}BrClN_6O_4S^+$, 591.0211, Δ3.29 ppm).

Synthesis and Purification of Ammosamide C Propylamine Adduct (23). Ammosamide C TFA (**16**) (6.0 mg, 0.015 mmol, 1 equiv) and propylamine (2.7 μL, 0.033 mmol, 2.1 equiv) were each dissolved in dry and sparged DMF (500 μL) in vials purged with N_2. The propylamine solution was added to the ammosamide C solution, and the reaction mixture was kept under N_2 for 22 h at rt. Analysis of the mixture by analytical C18 ELSD-UV/vis-HPLC (10% to 100% CH_3CN in H_2O, 0.1% FA) showed complete conversion of the starting material **16** (t_R = 3.0 min) to product **23** (t_R = 4.0 min) (Figure S8). The reaction mixture was concentrated under N_2 and high vacuum overnight, and the product was purified by semipreparative CN HPLC at 260 nm (19% CH_3CN in H_2O, 0.1% TFA, t_R = 10.0 min) followed by semipreparative C8 HPLC at 260 nm (25% CH_3CN in H_2O, 0.1% TFA, t_R = 7.0 min) to give 5.9 mg (82%) as a blue TFA salt. UV (MeOH) λ_{max} 345 (3.76), 430 (3.34), 575 (3.45) nm; ^1H and ^{13}C NMR, see Table 1; HRMS (ESI-Q-TOF) *m/z* $[M]^+$ 333.1230 (calcd for $C_{15}H_{18}ClN_6O^+$, 333.1225, Δ0.27 ppm).

Synthesis and Purification of Ammosamide G (24). Ammosamide C TFA (**16**) (10.8 mg, 0.0277 mmol, 1 equiv) and 4-chloroaniline (7.5 mg, 0.059 mmol, 2.1 equiv) were each dissolved in dry and sparged DMF (500 μL) in vials purged with N_2. The 4-chloroaniline solution was added to the ammosamide C solution, and the reaction was kept under N_2 for 18 h at rt. Analysis of the mixture by analytical C18 ELSD-UV/vis-HPLC (10% to 100% CH_3CN in H_2O, 0.1% FA) showed a 25−30% conversion of the starting material **16** (t_R = 3.0 min) to product **24** (t_R = 4.7 min) (Figure S9). The reaction mixture was concentrated under N_2 and high vacuum overnight, and the product was purified by semipreparative C8 HPLC at 260 nm (30% CH_3CN in H_2O, 0.1% TFA, t_R = 10.0 min) to give 3.5 mg (32%) as a blue solid. UV (MeOH) λ_{max} 335 (3.38), 575 (2.95) nm; ^1H and ^{13}C NMR, see Table 1; HRMS (ESI-Q-TOF) *m/z* $[M + H]^+$ 401.0691 (calcd for $C_{18}H_{15}Cl_2N_6O$, 401.0679, Δ1.29 ppm).

Synthesis and Purification of Ammosamide C *N*-Acetylcysteamine Adduct (26). Ammosamide C TFA (**16**) (6.0 mg, 0.015 mmol, 1 equiv) and *N*-acetylcysteamine (3.5 μL, 0.033 mmol, 2.1 equiv) were each dissolved in dry and sparged DMF (500 μL) in vials purged with N_2. The *N*-acetylcysteamine solution was added to the ammosamide C solution, followed by dry Et_3N (6.1 μL, 0.044 mmol, 2.8 equiv), and the reaction mixture was kept under N_2 for 22 h at rt. Analysis of the mixture by analytical C18 ELSD-UV/vis-HPLC (10% to 100% CH_3CN in H_2O, 0.1% FA) showed nearly complete conversion of the starting material (**16**, t_R = 3.0 min) to product **26** (t_R = 3.3 min) (Figure S10). The reaction mixture was concentrated under N_2 and high vacuum overnight, and the product was purified by semipreparative C8 HPLC at 270 nm (10% CH_3CN in H_2O, 0.1% TFA, t_R = 12.5 min) followed, again, by semipreparative C8 HPLC at 270 nm (20% CH_3CN in H_2O, 0.1% TFA, t_R = 5.5 min) to give 5.6 mg (72%) as a red TFA salt. UV (MeOH) λ_{max} 450 (3.37), 545 (3.00) nm; ^1H and ^{13}C NMR, see Table 1; HRMS (ESI-Q-TOF) *m/z* $[M]^+$ 393.0888 (calcd for $C_{16}H_{18}ClN_6O_2S^+$, 393.0895, Δ1.67 ppm).

Synthesis and Purification of Ammosamide C Ethanethiol Adduct (27). Ammosamide C TFA (**16**) (3.0 mg, 0.008 mmol, 1 equiv) and ethanethiol (1.2 μL, 0.017 mmol, 2.2 equiv) were each dissolved in dry and sparged DMF (250 μL) in vials purged with N_2.

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The ethanethiol solution was added to the ammosamide C solution, followed by dry Et_3N (3.0 μL, 0.022 mmol, 2.8 equiv), and the reaction mixture was kept under an atmosphere of air (conversion was <5% after 48 h under oxygen-free conditions) for 22 h at rt. Analysis of the mixture by analytical C18 ELSD-UV/vis-HPLC (10% to 100% CH_3CN in H_2O, 0.1% FA) showed nearly complete conversion of the starting material **16** (t_R = 3.0 min) to a mixture of products **27** (t_R = 3.9 min), **14** (t_R = 5.5 min), and **15** (t_R = 4.6 min) (Figure S11). The reaction mixture was concentrated under N_2 and high vacuum overnight, and the product was purified by semipreparative C8 HPLC at 270 nm (20% CH_3CN in H_2O, 0.1% TFA, t_R = 14.5 min) to give 1.4 mg (40%) as a light red TFA salt. ^1H and ^{13}C NMR, see Table 1; HRMS (ESI-Q-TOF) m/z $[M]^+$ 336.0691 (calcd for $C_{14}H_{15}ClN_5OS^+$, 336.0680, Δ0.12 ppm).

Synthesis and Purification of Ammosamide C 4-Bromobenzyl Mercaptan Adduct (28). Ammosamide C TFA (**16**) (6.0 mg, 0.015 mmol, 1 equiv) and 4-bromobenzyl mercaptan (6.6 mg, 0.033 mmol, 2.1 equiv) were each dissolved in dry and sparged DMF (500 μL) in vials purged with N_2. The bromobenzyl mercaptan solution was added to the ammosamide C solution, followed by dry Et_3N (6.1 μL, 0.044 mmol, 2.8 equiv), and the reaction was kept under an atmosphere of air (conversion was <5% after 48 h under oxygen-free conditions) for 22 h at rt. Analysis of the mixture by ELSD-UV/vis-HPLC (10% to 100% CH_3CN in H_2O, 0.1% FA) showed a mixture of starting material and products **28** (t_R = 5.1 min), **14** (t_R = 5.5 min), and **15** (t_R = 4.6 min) (Figure S12). The reaction mixture was concentrated under N_2 and high vacuum overnight, and the product was purified by semipreparative C8 HPLC at 260 nm (30% CH_3CN in H_2O, 0.1% TFA, t_R = 17.0 min) to give 1.3 mg (14%) as a red TFA salt. HRMS (ESI-Q-TOF) m/z $[M]^+$ 475.9941 (calcd for $C_{19}H_{16}BrClN_5OS^+$, 475.9942, Δ0.17 ppm). The adduct could not be characterized by UV and NMR since it readily decomposed to ammosamide A.

Cytotoxicity Assays. The human colorectal cancer cell line HCT-116 (ATCC CCL-247) was cultured and passaged as described previously.[17] Cytotoxicity in half-maximal inhibitory concentrations (IC_{50}) of ammosamides A–D (**14**–**17**) and the synthetic derivatives (**23**, **24**, **26**) was tested using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) bioassay.[21] Compounds in DMSO were added at a concentration of 10 mg mL^{-1}, and the bioassay was conducted as reported previously.[17] IC_{50} determination was performed using a four-parameter sigmoidal fit. DMSO (ATCC) and the standard anticancer drug etoposide VP-16 (Sigma-Aldrich), with an IC_{50} of 0.49–4.9 μM at a concentration of 4 mg mL^{-1} in DMSO, were tested as negative and positive controls, respectively.

NCI60 Cancer Cell Line Screening. Ammosamide C (**16**) was submitted to the U.S. National Cancer Institute 60 human tumor cell line anticancer drug screen including blood cancer (CML, ALL, CLL), NSCLC, colon cancer, central nervous system cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer cell lines.[19] **16** was tested at a single high dose (10 μM) in the full panel comprising all 60 cell lines. Values were reported for growth inhibition (GI_{50}) corresponding to percentage of growth of treated cells relative to nontreated cells.

Disk Diffusion Assay. Disk diffusion tests for antimicrobial activity were carried out according to the Kirby–Bauer method.[20] Inoculums of the Gram-negative *Escherichia coli* MG1655 and the Gram-positive marine *Bacillus oceanisediminis* CNY-977 were prepared from 24 h precultures in LB (Luria–Bertani) medium, pH 7.0, cultivated at rt and 200 rpm on a rotary shaker. LB agar plates were overlaid with inoculums of the strains, which were streaked over the agar surface using a sterile swab. Compounds **14**–**16** (10 μg) were spotted on sterile paper discs with a 6 mm diameter (Becton Dickinson, BBL blank paper discs) at a concentration of 10 mg mL^{-1} in MeOH (10 μL) and dried prior to their application on the inoculated agar surface. MeOH (10 μL) and ciprofloxacin (5 μg) (Becton Dickinson, BBL Sensi-Disc) were tested as negative and positive controls, respectively. All agar plates were incubated for 48 h at rt or 37 °C for *B. oceanisediminis* and *E. coli*, respectively, and inhibition zone diameters were measured thereafter.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00773.

One-dimensional (^1H) and two-dimensional (COSY, HSQC, HMBC) NMR spectra of **17**, **20**, **23**, **24**, **26**, and **27** as well as UV/vis and HRMS data of all characterized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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