The Ammosamides: Structures of Cell Cycle Modulators from a Marine-Derived *Streptomyces* Species**

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Marine-derived actinomycete bacteria are emerging as a valuable resource for bioactive natural products encompassing a variety of unique structural classes.[1] In our hands, early detection of cell growth inhibitors using in vitro cytotoxicity assays against the colon carcinoma cancer cell line HCT-116, followed by extensive mechanism of action studies, has proven to be an effective approach. As such, the HCT-116 assay has been instrumental in the identification of potentially important anticancer agents.[2]

In the course of our continued studies, *Streptomyces* strain CNR-698[3] was isolated from bottom sediments collected at a depth of 1618 meters in the Bahamas Islands in 2003. Cytotoxicity-guided (HCT-116) fractionation by C_{18} flash chromatography and RP-HPLC of crude extract led to the isolation of ammosamides A (**1**) and B (**2**) as blue and red solids, respectively (3 and 4 mg L^{-1}). Structure assignments for **1** and **2** proved to be particularly difficult due to their inherent insolubility (soluble only in dimethyl sulfoxide (DMSO)) and a lack of descriptive NMR signals, ultimately requiring the integration of NMR spectral analysis, mass spectrometry data, and single crystal X-ray diffraction studies.

High-resolution (ESI) mass spectrometric analysis of ammosamide A (**1**) indicated a molecular formula $C_{12}H_{10}{}^{35}ClN_5OS$ (m/z [M+H]$^+$: 308.0303]. The molecular weight of ammosamide B (**2**) was found to be 16 amu lower (m/z [M+H]$^+$: 292.0604) consistent with the molecular formula $C_{12}H_{10}{}^{35}ClN_5O_2$. The UV/Vis spectrum of **1** was indicative of an unusually highly conjugated structure with absorptions at $\lambda_{max} = 580, 430, 350,$ and 290 nm.

Inspection of the ^1H NMR spectrum of **1** in [D$_6$]DMSO revealed six singlets between $\delta = 6.0$ and 9.0 ppm and one methyl singlet at $\delta = 4.03$ ppm, while the ^{13}C NMR spectra revealed the presence of eleven sp^2 hybridized carbon atoms

and a single sp^3 hybridized carbon atom at $\delta_c = 33.3$ ppm (Table 1). The addition of D$_2$O (20 μL) to the sample in [D$_6$]DMSO resulted in the immediate disappearance of

Table 1: NMR spectral data for **1** and **2** ([D$_6$]DMSO).

1   **2**

Position	Ammosamide A (1)			Ammosamide B (2)	
	δ_C[a,c]	δ_H[d]	HMBC	δ_C[b,c]	δ_H[d]
1a	33.3	4.03 (s)	C-2, C-8a	28.5	3.59 (s)
2	177.2			164.0	
3	116.5	8.47 (s)	C-2, C-4 C-5b, C-4a	115.3	8.34 (s)
4	144.6			144.7	
4a	166.0			166.2	
5b	119.7			119.0	
7	103.1			104.5	
8a	110.5			106.3	
CONH$_2$		7.68 (br s)	C-4		7.66 (br s)
		8.92 (br s)	C-4a		8.91 (br s)
NH$_2$ (C-6)		7.16 (br s)[e]	C-7		6.73 (br s)[f]
		6.63 (s)[e]			
NH$_2$ (C-8)		6.89 (s)	C-7, C-8a		6.18 (br s)[f]

[a] 75 MHz. [b] 125 MHz. [c] C-2a, C-5a, C-6, and C-8 (**1**: $\delta_C = 132.6, 134.7, 136.8, 142.7$ ppm; **2**: $\delta_C = 130.6, 130.8, 132.4, 140.5$ ppm) could not be unambiguously assigned. [d] 600 MHz. [e] At certain concentrations, these two one-proton signals coalesce to a two-proton signal at $\delta = 7.09$ ppm. [f] Assigned by analogy to **1**.

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^1H NMR signals at $\delta = 7.16$ (1H), 6.63 (1H), 6.89 ppm (2H) and the slower disappearance of singlets at $\delta = 8.92$ (1H), and 7.68 ppm (1H) (less than 10 min). The exchangeable protons at $\delta = 7.16$, 6.63 and 6.89 ppm were assigned as aromatic amines at C-6 and C-8 (based on HMBC correlations), while the slowly exchanging protons at $\delta = 8.92$ and 7.68 ppm were assigned to a primary amide on the basis of COSY and HMBC correlations. The only non-exchangeable hydrogen atoms were the methyl singlet resonance at $\delta = 4.03$ ppm and a one-proton singlet at $\delta = 8.47$ ppm. The ^{13}C NMR spectrum of **1** indicated the presence of two carbonyl groups ($\delta_c = 177.2$ and 166.0 ppm), as well as two upfield sp^2 carbon atoms ($\delta_c = 103.1$ and 110.5 ppm). HMBC correlations between the downfield carbonyl ($\delta_c = 177.2$ ppm) and the proton methyl

singlet at $\delta = 4.03$ ppm, we thought, defined an *N*-methyl amide, although a carbon chemical shift so far downfield would not be expected. In addition to correlations from the aromatic $\delta = 8.47$ ppm singlet, the only other HMBC correlations were from the exchangeable protons at $\delta = 7.16/6.63$ ppm to C-7 ($\delta_c = 103.1$ ppm) and from $\delta = 6.89$ ppm to C-7 and C-8a ($\delta_c = 110.5$ ppm).

The spectral data for **1** suggested a highly unsaturated aza-aromatic metabolite possessing three rings. However, the lack of definitive NMR assignments that could be used to link these features forced us to concentrate efforts toward obtaining an X-ray crystal structure. We were fortunate to obtain small crystals of **1** by the slow diffusion of H_2O into a saturated solution in DMSO.[4] The X-ray assignment of ammosamide A (**1**) is shown in Figure 1. Once X-ray data became clear, the spectral data for **1** could be assigned.
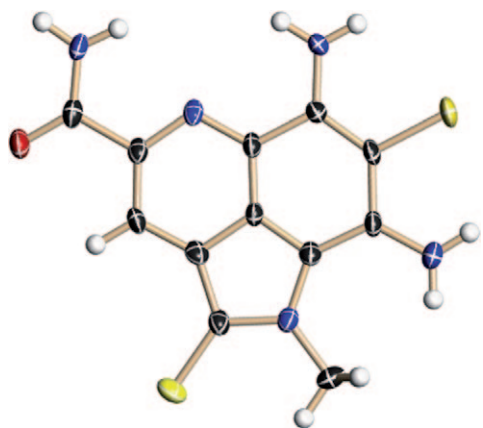

Figure 1. X-ray crystal structure of ammosamide A (**1**). Red O, blue N, yellow Cl, black C, white H.

The structure assignment of ammosamide B (**2**) followed from analysis of spectral data and chemical interconversion. Comparison of the C-2 carbonyl chemical shifts in **1** ($\delta_C = 177.2$ ppm) and **2** ($\delta_C = 164.0$ ppm) revealed a difference of 13 ppm, consistent with the typical ^{13}C chemical shift difference between a carbonyl and a thiocarbonyl (ca. 20 ppm).[5] In order to chemically confirm the presence of the thiolactam functionality, we used Lawesson's reagent [2,4-bis(*p*-methoxyphenyl)-1,3-dithiadiphosphetane-2,4-disulfide] to convert lactam **2** into thiolactam **1**.[6] The low yield of this reaction is likely attributable the nucleophilic amines in **2**.[7] Exposed to air during storage, **1** was gradually converted to ammosamide B (**2**). Notably, the transformation could also be accomplished in 10 min, upon treatment of **1** with hydrogen peroxide in aqueous methanol.[8] This reactivity has been previously observed in other thioamide-containing compounds.[9]

The structural similarities between the ammosamides and the microbial product lymphostin (**3**) are clear,[10] as is the relationship of the ammosamides to several sponge-derived pyrroloiminoquinone natural products, including batzelline A (**4**),[11a] isobatzelline D (**5**),[11b] and makaluvamine A (**6**)[11c] (Scheme 1). The sponge metabolites **4–6** possess different
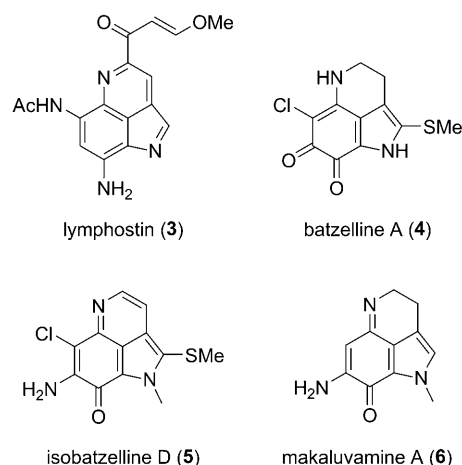
Scheme 1. Related metabolites from bacteria and sponges.

patterns of Cl and NH_2 substitution and assume *p*-iminoquinone and *o*-quinone structures. The presence of an amino group at C-8 in the ammosamides results in a fundamentally different structure type in which the quinoline tautomer predominates. The pyrrole moiety in **3–6** is uniquely oxidized to the pyrrolidinone in ammosamide B (**2**). Finally, though methyl sulfides are present in **4** and **5**, ammosamide A (**1**) is the first natural product to contain a thio-γ-lactam functionality.[12]

The fact that the ammosamides are highly colored (**1**: $\lambda_{max} = 580$ nm; **2**: $\lambda_{max} = 530$ nm), yet lack quinone or iminoquinone functionalities, leads to speculation about the electronic character and reactivity of these metabolites. The intense colors of these compounds could reflect a strong charge separation between the two six-membered aromatic rings due to the effects of electron-donating groups on the chlorine-containing ring and electron-withdrawing substituents on the pyridine ring. It is, conceptually, also explained by the potential for ammosamide A to exist in an equilibrium with its bis-iminoquinone tautomer (Scheme 2). Furthermore,
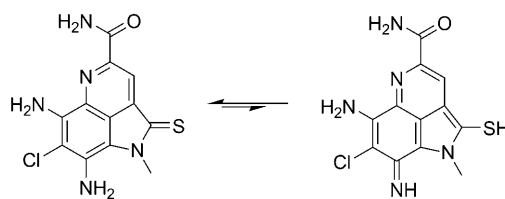
Scheme 2. Possible tautomeric form of ammosamide A (**1**).

in **1** and **2** the chlorine atom at C-7 is poised to engage in nucleophilic aromatic substitution with a suitable nucleophile, particularly when the molecule exists as its bis-iminoquinone tautomer.[13] This reactivity may be relevant to the molecule's interaction with its protein target.[14]

Ammosamides A (**1**) and B (**2**) exhibited significant in vitro cytotoxicity against HCT-116 colon carcinoma, each with $IC_{50} = 320$ nM. These compounds also demonstrated

pronounced selectivity in a diversity of cancer cell lines with values ranging from 20 nm to 1 μm, indicating a specific target mechanism of action. To explore the intracellular target of the ammosamides, ammosamide B (**2**) was converted to a highly fluorescent molecule by conjugation.[14] Treatment of HCT-116 colon carcinoma or HeLa cells with this fluorescent molecule produced immediate and irreversible labeling of a specific protein in the cellular cytosol. Using a cell and molecular biology approach, the target of the ammosamides was identified as a member of the myosin family, important cellular proteins that are involved in numerous cell processes, including cell cycle regulation, cytokinesis, and cell migration.

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[3] Strain CNR-698 was isolated from a sediment sample collected at Little San Salvador, Bahamas. The strain does not require seawater for growth. Its partial 16S rRNA sequence (Genbank #EU863183) is 100% identical to another bacterium of the family Streptomycetaceae, also isolated from marine sediments (20 m) (#EU099411).

[4] X-ray measurements were made on a SMART CCD area detector with graphite monochromated Mo$_{K\alpha}$ radiation ($\lambda = 1.54178$ Å). **1** (C$_{12}$H$_{10}$ClN$_5$OS): crystal dimensions $0.30 \times 0.20 \times 0.10$ mm, monoclinic, space group $P2_1/c$, $a = 9.3766(8)$, $b = 18.2930(12)$, $c = 7.0462(6)$ Å, $V = 1208.55(17)$ Å^3, $Z = 4$, $\rho_{calcd} = 1.691$ mg m^{-3}, $\mu = 4.456$ mm^{-1}, $T = 100(2)$ K, $2\theta_{max} = 136.3°$, 7060 measured reflections, 1872 independent reflections ($R_{int} = 0.0399$), 183 parameters refined, $R = 0.0541$ (for 4205 reflections with $I > 2.00\sigma(I)$), R$_w$ = 0.1523, max/min residual peaks in the final difference map 0.495/−0.817 e Å^{-3}. CCDC 694293 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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