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Ammosamide D, An Oxidatively Ring Opened Ammosamide Analog from a Marine-derived *Streptomyces variabilis*

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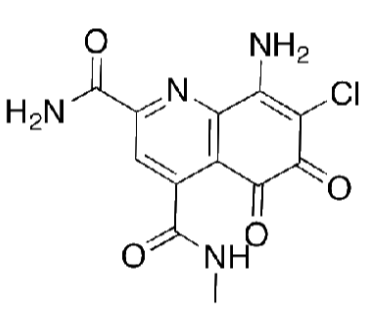
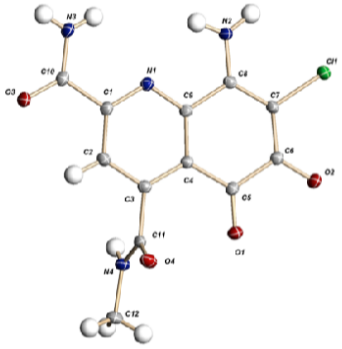
Abstract



ammosamide D



Ammosamide D (1), an oxidized analog of the ammosamide family, was isolated from a marine-derived *Streptomyces variabilis*. Pyrroloquinoline containing alkaloids are a growing class of natural products, with 1 being the first example of an oxidized analog resulting in a 5,6-dioxo-5,6-dihydroquinoline ring system. Attempts at chemical conversion of ammosamide B to ammosamide D revealed that a strong chemical oxidant is required. Ammosamide D has modest cytotoxicity to the MIA PaCa-2 pancreatic cancer cell line.

Cytotoxicity based screening for natural products has been the most succssful strategy for the discovery of bioactive natural products with new modes of action.[1] In 2008 the Fenical laboratory reported the isolation, characterization and molecular target of the cytotoxic agents ammosamides A and B (**2** – **3**).[2] These heteroaromatic alkaloids contain an unusual pyrroloquinoline moiety, which most likely derives from modification of tryptophan. Biological studies demonstrated that these compounds exert their cytotoxicity via covalent modification of myosin.[3] The interesting biology and structural features of the ammosamides have led to considerable interest from the synthetic community.[4] Recently, biosynthetic studies on the pyrrolquinoline containing natural product lymphostin (**4**) indicate that the pyrroloquinoline carbon skeleton is derived from tryptophan and further modified in an assembly line fashion.[5] This study also showed that **4** and related analogs are potent inhibitors of mTOR. There are now a growing number of natural products with the

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Supporting Information Available General procedures, bioassay protocols, chemical derivatization, data tables, NMR spectra and X-ray crystal data.

pyrrolo[4,3,2-de]quinoline core from multiple organisms, including mycenarubin A (**5**) from a mushroom.[6]

In our continuing efforts to search for natural products from marine bacteria with selective cytotoxicity against cancer cell lines, we screened a library of 1500 natural products fractions against a panel of cancer cell lines from multiple tissue types.[7] From this screen we obtained a series of fractions from a marine-derived *Streptomyces variabilis* (strain SNA-020)[8] that exhibited modest selectivity and potency for the MIA PaCa-2 pancreatic cancer cell line. Analysis of the active fractions by LC–UV-MS showed the presence of chlorinated compounds with a UV-Vis profile with absorptions at λ_{max} = 550, 420, 340, 280 and 235 nm. Based on this UV profile and MS, we could discern the presence of **2** and **3** in the active fractions. **2** and **3** exhibit activity against the colon tumor cell line HCT-116[2], but when tested against MIA PaCa-2 cells, there was no cytotoxicity < 20 μM, suggesting the presence of additional active metabolites in the fraction. Further analysis revealed additional chlorine bearing molecules with complex UV profiles similar to **2** and **3**, leading to the isolation of ammosamide D (**1**), which has modest cytotoxicity against MIA PaCa-2 (IC_{50} = 3.2 μM).

1 Was isolated as an orange solid. The positive ion HRESIMS at m/z 309.0224 $[M+H]^+$, corresponds to a molecular formula of $C_{12}H_9ClN_4O_3$. ^{1}H NMR in DMSO-d_6 exhibited five singlets: δ_H 9.35, 9.17, 8.55, 8.02, 8.00, one quartet proton at δ_H 8.18 (J = 4.7 Hz) and one methyl doublet at δ_H 2.77 (J = 4.7 Hz) (Table 1). Addition of a 30 μL of D_2O to the NMR sample lead to the disappearance of all signals in the ^{1}H NMR with the exception of the singlet at δ_H 8.00 ppm and the methyl doublet, suggesting there are five exchangeable protons. The ^{13}C NMR revealed the presence of 11 sp^2 carbons and a sp^3 carbon. The above data for **1**, along with the UV spectrum, suggested an ammosamide like structure.

Analysis of the 2D data provided only a few correlations for assignment of the structure. A COSY correlation between the exchangeable ^{1}H at δ 8.18 and the methyl doublet at δ_H 2.77 was suggestive of an *N*-methyl amide, a deviation from the previously reported ammosamides. Additionally, we observed four carbons shifted downfield of 160 ppm, whereas **2** and **3** only have two carbons downfield of 160 ppm (Table S1). Although the data suggested that **1** had the basic carbon framework as **3**, there were clearly some differences. Further examination of the HMBC revealed correlations from the aromatic proton H3 at δ_H 8.00 to C2 at δ_C 166.3 and C4a at δ_C δ_c 163.7, which established the locations of two amide carbons, C4a and C2.

Due to the lack of NMR correlations, we turn our attention towards obtaining an X-ray crystal structure. Following a similar procedure as that of ammosamide A[9] we were able to obtain small crystals. The X-ray assignment of **1** revealed that the pyrrole ring had been cleaved at C8a to give an *N*-methylamide and a ketone at C8a (Figure 2). The presence of carbonyls at C8 and C8a are consistent with the two additional downfield signals in the ^{13}C NMR. Additionally, the crystal structure explained the presence of only five exchangeable protons. Thus, **1** contains a 5,6-dioxo-5,6-dihydroquinoline ring system.

There is a significant change in the bond lengths of the *ortho*- quinone portion of **1** upon lose of aromaticity. The bond length from C-5b to C-8a has increased by 0.12 Å to 1.48 Å while C-5b to C-8a has increased by 0.14 Å to 1.53 Å. The 5,6-dioxo-5,6-dihydroquinoline ring system explains the dramatic hypsochromic UV shift from 520 nm in **3** to 475 nm in **1**. Although we would anticipate an even much shorter λ for the non-aromatic ring system of **1**. DFT calculations of the UV spectra of **1** and **3** reasonably predicted the λ_{max} for both molecules (**3** $\lambda_{calculated}$ 580 nm; **1** $\lambda_{calculated}$ 500 nm).[10] This suggests that the pyridine-2,4-

dicarboxyamide moiety, which exists in both structures plays a key determinant in the long wavelength UV transition.

It is clear from the previous isolation studies on **2** and **3** that these compounds are susceptible to oxidation. In particular, it was shown that the thiolactam moiety of **2** could be converted to the lactam upon storage or rapidly via treatment with H_2O_2 in MeOH. We envision that **1** results from oxidation of **3** to give the peroxy intermediate. This is mechanistically similar to the oxidation of flavin to flavin hydroperoxide.[11] There are a number of pathways that by which the peroxy species could lead to the oxidatively ring opened product. One possiblity is elimination to form the acyliminium ion, which could then react with H_2O to form the aminal and open to iminoquinone **6** (Figure 3). With this proposed mechanism in mind, there is a probability that **1** is an artifact of the isolation, via reaction with O_2. To test this possibility we subjected **3** to a series of oxidation conditions to look for conversion to **1** or other ring open compounds (Figure 4).

As a control, **3** was dissolved in 1:1 CH_3OH/H_2O and allowed to stand at 25 °C for 7 days exposed to air. These conditions were meant to mimic the solvent/air exposure that compounds receive during isolation conditions. Other than a small amount of decomposition, we observed only starting material. Under more forcing conditions, we placed **3** under an atmosphere of O_2 in aq. methanol and heated to 50 °C. Under these conditions we still failed to see conversion of **3** to **1**. In fact we found **3** to be incredibly stable under these conditions and only saw a small amount of decomposition. A third option we looked into was the possibility that **3** could be converted to **1** in the fermentation media. As the slightly basic (pH 8.0) fermentation media contains trace metals and μM concentrations of Fe, we thought these might play a role in mediating the oxidative ring opening. After addition of 50 μL of a 10 mM DMSO stock solution of **3** to the fermentation media and shaking for 7 days (the same time as for fermentation) we saw no conversion to **1**.

We decided to undertake more forcing conditions and looked at using chemical oxidants such as $PhI(OAc)_2$ and AgOAc that are frequently used to generate quinones.[12,13] Treatment of **3** with $PhI(OAc)_2$ in DMF at room temperature gave a 3:1 mixture of products, with the predominant product observed being **1** (LC-MS trace Figure S1) and a compound with MS data consistent with iminoquinone **6** or the C8 imine tautomer. Upon purification by reversed phase C18 HPLC, the only product observed is **1** in an overall 46% yield. It is not surprising that under the purification conditions potential intermediate **6** be converted to **1** by addition of H_2O. As it required chemical intervention to convert **3** to **1**, we propose that the opening of the pyrrole ring is not likely a simple artifact of the isolation, but is an enzyme catalyzed, biosynthetic product.

Based on previous reports of biological activity for ammosamides A and B (cytotoxicity) and lymphostin (mTOR inhibitor), we probed the activity of **1** in a variety of biological assays. Cytotoxicity measurements against the pancreatic cancer cell line MIA PaCa-2 revealed only modest cytotoxicity for **1**, with an IC_{50} of 3.2 μM. **1** Was not found to be an mTOR inhibitor (tested up to 20 μM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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8. Bacterial strain SNA-020 was isolated from a sediment sample collected at Sweetings Cay, Bahamas (N 26° 33′27″, W 77° 51′15″) using a starch based isolation media. The phylogeny was established using 16S rRNA analysis with the universal bacterial primers F27 and R1492. The strain showed 99.9% identity to *Streptomyces variabilis*. The 16S gene sequence is deposited in the NCBI as JQ815387).
9. **1** was dissolved in methanol and water in a 1 dram vial and allowed to stand for 30 days, after which small crystals were obtained.
10. The theoretical UV λ_{max} was calculated using DFT calculations at the B3LYP/6-31+G level in the GAUSSIAN 09 software package.
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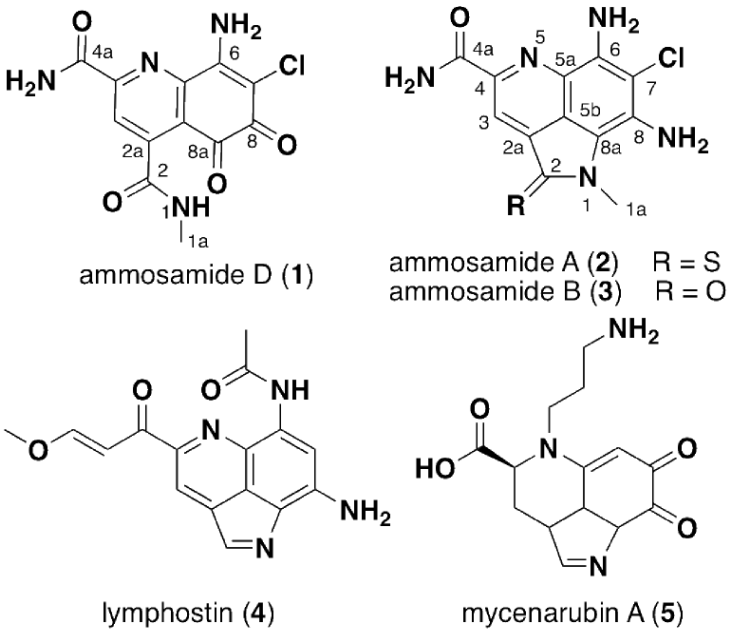

Figure 1.
Structures of new compound **1** and other pyrrolo[4,3,2-de]quinoline natural products

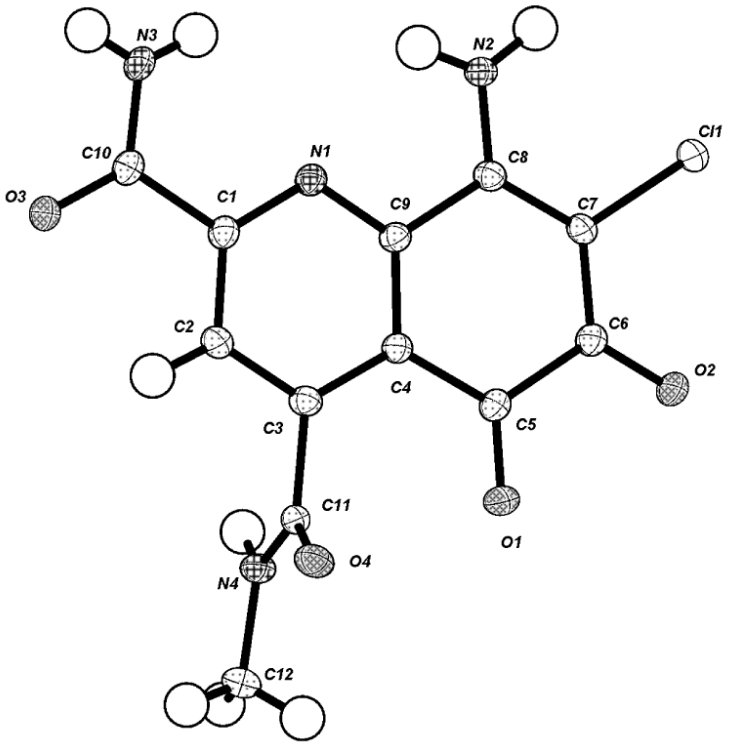

Figure 2.
X-ray crystal structure of **1**.

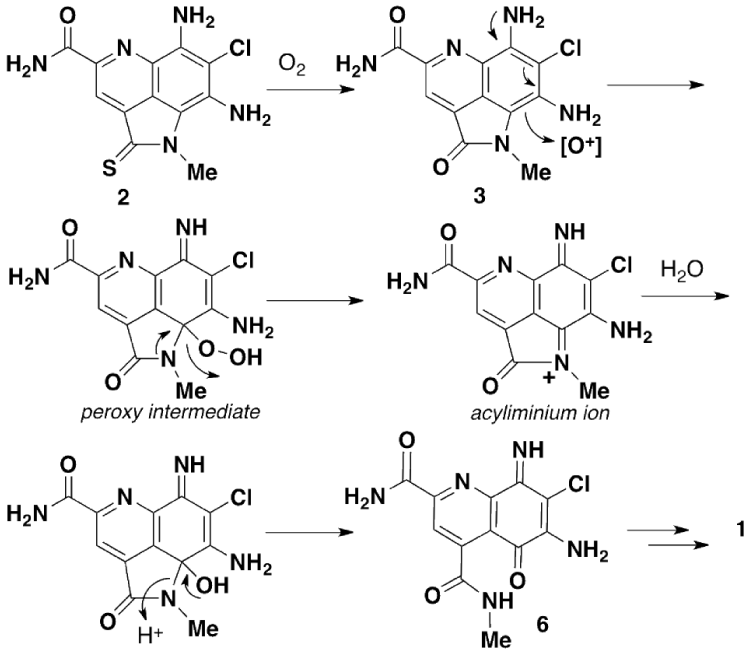

Figure 3.
Proposed biosynthetic pathway from **3** to **1**.

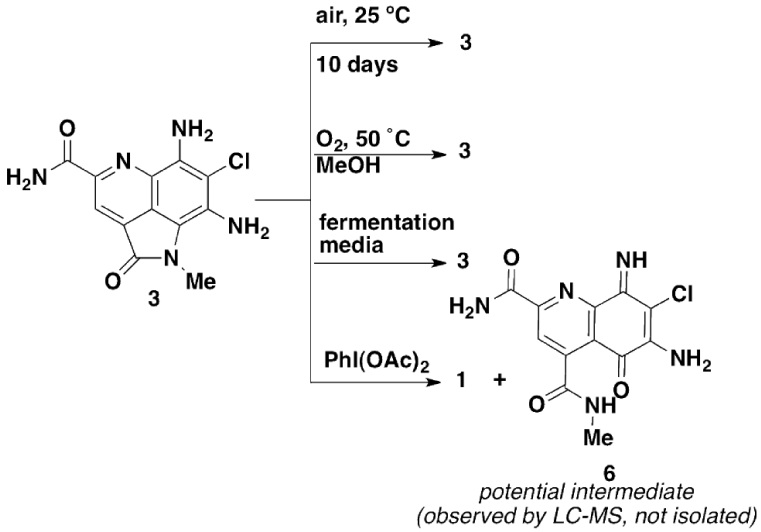

Figure 4.
Attempts at conversion of **3** to **1** Table 1.

Table 1

^{1}H and ^{13}C NMR of **1** in DMSO-d_6

no.	δ_H, m (*J* Hz)	δ_C	COSY	HMBC
1	8.18 q (4.7)		H1a	C1a,C2
1a	2.77 d (4.7)	25.9	H1	C2
2	-	166.3		
2a	-	146.8		
3	8.00 s	122.7		C2,C2a,C4a,C5b
4	-	152.0		
4a	-	163.7		
5a	-	146.7		
5b	-	125.4		
6	-	151.7		
7	-	106.9		
8	-	168.9		
8a	-	178.3		
CONH$_2$-a	9.17 s;	-	CONH$_2$-b	C4a
CONH$_2$-b	8.02 s		CONH$_2$-a	C4
NH$_2$(6)-a	9.35 s;		NH$_2$(6)-b	C5a,C7,C8
NH$_2$(6)-b	8.55 s		NH$_2$(6)-a	C5a,C5b,C6,C7