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Ammosamide D, an Oxidatively Ring Opened Ammosamide Analog from a Marine-Derived *Streptomyces variabilis*

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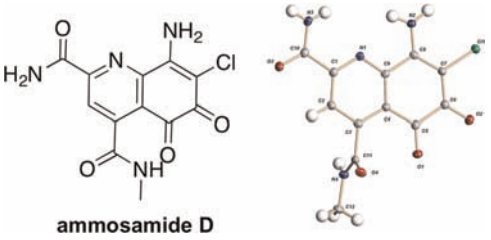
ABSTRACT



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 successful 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** and **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 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

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(1) Shoemaker, R. H. *Nat. Rev. Cancer* **2006**, *6*, 813.
(2) Hughes, C. C.; MacMillan, J. B.; Gaudencio, S. P.; Jensen, P. R.; Fenical, W. *Angew. Chem., Int. Ed.* **2009**, *48*, 725.
(3) Hughes, C. C.; MacMillan, J. B.; Gaudencio, S. P.; Fenical, W.; LaClair, J. J. *Angew. Chem., Int. Ed.* **2009**, *48*, 728.
(4) (a) Hughes, C. C.; Fenical, W. *J. Am. Chem. Soc.* **2010**, *132*, 2528. (b) Reddy, P. V. N.; Banerjee, B.; Cushman, M. *Org. Lett.* **2010**, *12*, 3112.
(5) For isolation, see: (a) Nagata, H.; Ochiai, K.; Aotani, Y.; Ando, K.; Yoshida, M.; Takahashi, I.; Tamaoki, T. *J. Antibiot.* **1997**, *50*, 537. (b) Aotani, Y.; Nagata, H.; Yoshida, M. *J. Antibiot.* **1997**, *50*, 543. For biosynthesis, see: Miyanaga, A.; Janso, J. E.; McDonald, L.; He, M.; Liu, H.; Barbieri, L.; Eustáquio, A. S.; Fielding, E. N.; Carter, G. T.; Jensen, P. R.; Feng, X.; Leighton, M.; Koehn, F. E.; Moore, B. S. *J. Am. Chem. Soc.* **2011**, *133*, 13311.
(6) Peters, S.; Spiteller, P. *Eur. J. Org. Chem.* **2007**, 1571.
(7) Hu, Y.; Espindola, A. P.; Stewart, N. *Biorg. Med. Chem.* **2011**, *19*, 5183.
(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.

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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**, Figure 1), which has modest cytotoxicity against MIA PaCa-2 (IC_{50} = 3.2 μM).

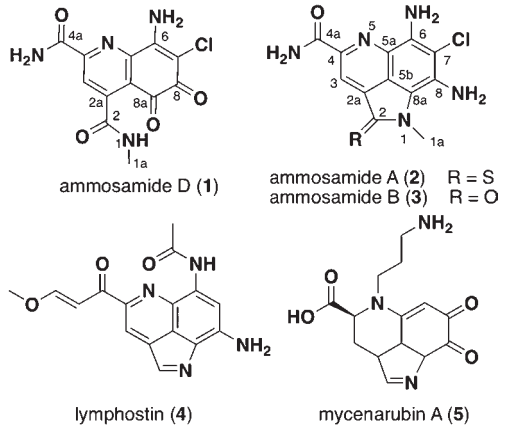

Figure 1. Structures of new compound **1** and other pyrrolo-[4,3,2-*de*]quinoline natural products.

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 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 an 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 same basic carbon framework as that of **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 163.7, which established the locations of two amide carbons, C4a and C2.

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

Due to the lack of NMR correlations, we turn our attention toward 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 loss of aromaticity. The bond length from C-5b to C-8a increased by 0.12 to 1.48 Å, while that from C-5b to C-8a increased by 0.14 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**. Yet, we would anticipate an even much shorter λ for the nonaromatic 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 role in the long wavelength UV transition.

(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.

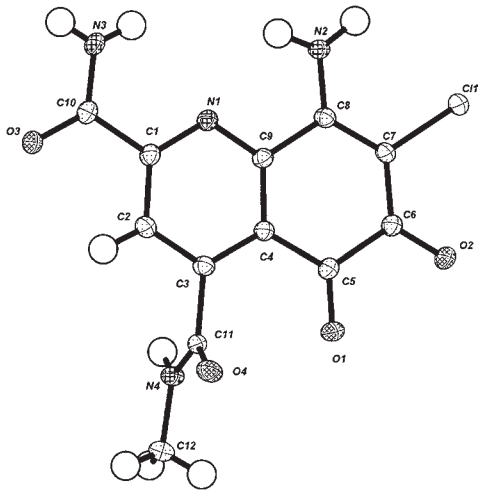

Figure 2. X-ray crystal structure of **1**.

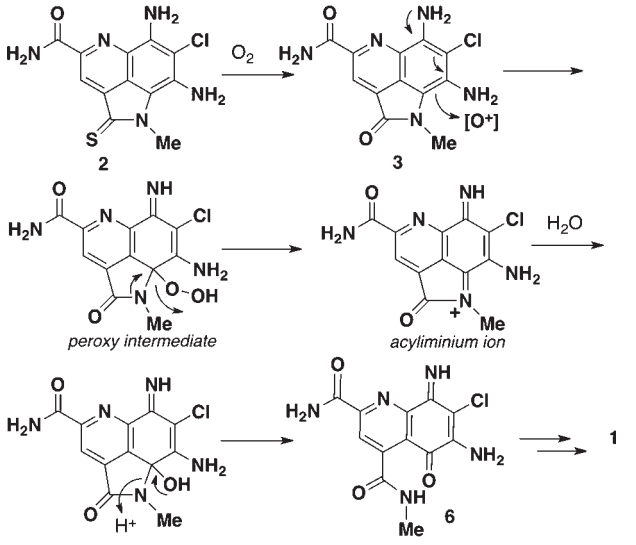

Figure 3. Proposed biosynthetic pathway from **3** to **1**.

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 by which the peroxy species could lead to the oxidatively ring opened product. One possibility is elimination to form the acyliminium ion, which could then react with H_2O to form the aminal and open to become iminoquinone **6** (Figure 3). With this proposed mechanism in mind, it likely 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 it 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 observed 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 micromolar concentrations of Fe, we thought these might play a role in mediating the oxidative ring opening. After the 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 that for fermentation) we observed no conversion to **1**.

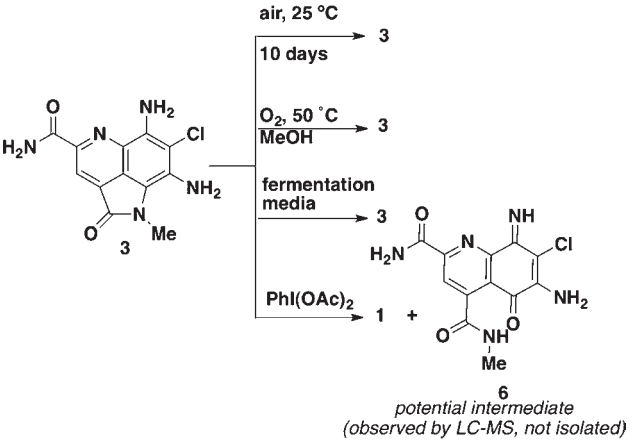

Figure 4. Attempts at conversion of **3** to **1**.

We decided to undertake more forcing conditions and examined 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** could be

(11) (a) Kemal, C.; Bruce, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 995. (b) Mager, H. I. X.; Berends, W. *Tetrahedron* **1976**, *32*, 2303.

(12) Nicolaou, K. C.; Sugita, K.; Baran, P. S.; Zhong, Y.-L. *Angew. Chem., Int. Ed.* **2001**, *40*, 207.

(13) Adams, R.; Nagarkatti, A. S. *J. Am. Chem. Soc.* **1950**, *72*, 4601.

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).

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Supporting Information Available. General procedures, bioassay protocols, chemical derivatization, data tables, NMR spectra, and X-ray crystal data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.