A Cytotoxic Proteasome Inhibitor

Salinosporamide A: A Highly Cytotoxic Proteasome Inhibitor from a Novel Microbial Source, a Marine Bacterium of the New Genus *Salinospora***

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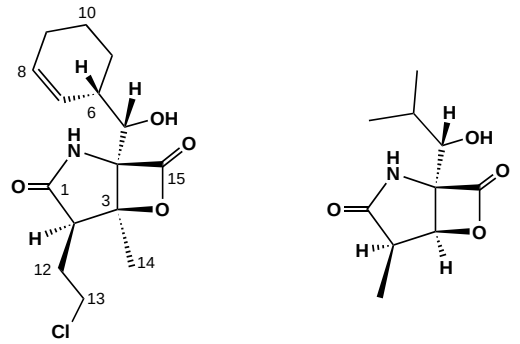
In memory of D. John Faulkner

We recently reported the cultivation and phylogenetic characterization of a new group of obligate marine actinomycete bacteria that is widely distributed in ocean sediments.[1] Analogous soil-derived actinomycetes have been the single most significant source of naturally occurring microbial antibiotics,[2] thus the discovery of a major new group of these bacteria in marine sediments suggests that the ocean represents an overlooked habitat from which to isolate these important microorganisms. Given that the rate of discovery of new biologically active compounds from common soil actinomycetes has been falling,[3] obligate marine actinomycetes represent a new resource for structurally diverse secondary metabolites.

To date, we have isolated in excess of 2500 strains belonging to this new taxon for which we have proposed the genus name "*Salinospora*" (a formal taxonomic description is in progress). "*Salinospora*" strains, with previously undescribed 16S rRNA gene sequences, have been recovered from five distinct tropical/subtropical ocean systems[1] and from depths as great as −1100 m, which indicates that they represent a widely distributed and taxonomically diverse group of sediment bacteria. All isolates display an obligate requirement of ionic sodium for growth, thus indicating a high level of marine adaptation.

In preliminary screening, a high percentage of the organic extracts of cultured "*Salinospora*" strains possessed antibiotic and anticancer activities, which suggests that these bacteria are an excellent resource for drug discovery. Herein we report the results of our first chemical investigation of a member of the "*Salinospora*" group and show that strain CNB-392 produces the chemically unique and highly bioactive metabolite salinosporamide A (**1**, Scheme 1). Salinosporamide A exhibits potent cancer cell cytotoxicity and appears to exert its cytotoxic effects through inhibition of the 20S proteasome.



1 **2**

Scheme 1. Structures of salinosporamide A (**1**) and *clasto*-lactacystin-β-lactone (omuralide, **2**).

"Salinospora" strain CNB-392 was isolated from a heat-treated marine sediment sample that was plated on a seawater-based agar nutrient medium. Liquid shake flask cultivation of this strain, followed by solid-phase extraction with Amberlite resin (XAD-16) and elution with acetone, resulted in a crude extract that was highly cytotoxic in vitro toward HCT-116 human colon carcinoma (IC_{50} ca. 80 ng mL^{-1}). Cytotoxicity-guided fractionation of the crude extract led to the isolation of salinosporamide A (**1**) as a colorless crystalline solid (yield: 7 mg L^{-1}). The complete structural assignment of **1** was accomplished by spectral analysis and by a single-crystal X-ray diffraction study.

Analysis of the low-resolution mass spectrum of salinosporamide A showed a characteristic $[M+2]^+$ peak indicative of the presence of a chlorine atom. High-resolution mass-spectral analysis provided the molecular formula $C_{15}H_{21}{}^{35}ClNO_4$ (calcd for $[M+H]^+$: m/z 314.1160, obsd: m/z 314.1144) for salinosporamide A. The infrared spectrum of **1** showed bands characteristic of amide and β-lactone carbonyl groups ($\tilde{\nu}$ = 1702 and 1819 cm^{-1}). Analysis of the ^{1}H NMR spectrum of **1** showed the presence of two protons on a *cis*-double bond (δ = 6.42 ppm, d and δ = 5.88 ppm, m, ^3J = 9.6 Hz), an amide proton (δ = 10.60 ppm), and one hydroxy proton (δ = 4.99 ppm, br s). Also evident was a single oxygen atom bearing a methine proton (δ = 4.24 ppm, t), an isolated methyl group (δ = 2.07 ppm, s), and a chloroethyl substituent.

Comprehensive analysis of 2D NMR data, including the results of COSY, HMQC, and HMBC experiments, enabled the complete planar structure of salinosporamide A to be assigned, as in **1**, to a 2-aza-6-oxabicyclo[3.2.0]heptane-3,7-dione skeleton substituted with cyclohexenylcarbinol and chloroethyl side chains.

Portions of the relative stereochemistry of salinosporamide A were readily assigned by NOE NMR spectral methods. However, several attempts to solve the absolute configuration by derivatization of the secondary hydroxy group with Mosher's reagent and esterification with *p*-bromobenzoic acid for CD analysis were unsuccessful, presumably because of steric hindrance at the C-5 hydroxy

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group. Subsequently, the absolute stereochemistry of **1**, and confirmation of the overall structure of salinosporamide A, was achieved by single-crystal X-ray diffraction analysis.

Crystallization of **1** from ethyl acetate/*iso*-octane resulted in single, cubic crystals, which diffracted as a monoclinic system P2(1). The unusual high unit-cell volume of 3009 Å[4] hosted four independent molecules in which different conformational positions were observed for the flexible chloroethyl substituent. The assignment of the absolute structure from the diffraction anisotropy of the chlorine substituent resolved the absolute stereochemistry of Salinosporamide A (**1**) as 2*R*,3*S*,4*R*,5*S*,6*S* (Figure 1) with a Flack parameter of 0.01 and an esd of 0.03.

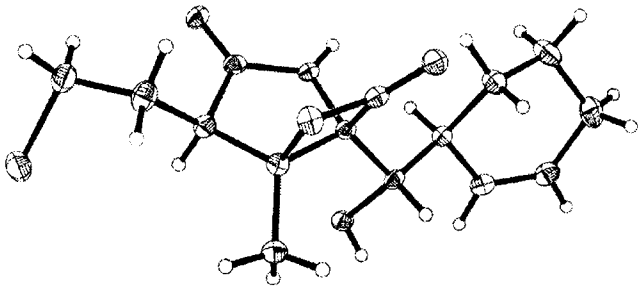

Figure 1. ORTEP plot of the final X-ray structure of salinosporamide A (**1**) depicting the absolute stereochemistry.

Salinosporamide A shares its fused γ-lactam-β-lactone bicyclic ring structure with *clasto*-lactacystin-β-lactone (**2**), a transformation product of the microbial metabolite lactacystin[5] originally isolated by Ōmura and co-workers from cultures of a terrestrial *Streptomyces* sp.[6a,b] *Clasto*-lactacystin-β-lactone, also called omuralide,[7] is remarkable since it was the first molecule discovered to be a truly specific inhibitor of the proteasome, a multicatalytic proteinase complex responsible for most nonlysosomal protein degradation in the cell. Omuralide is unique in that it specifically inhibits the proteolytic activity of the 20S subunit of the proteasome without inhibiting any other protease activities of the cell.[8] For this reason **2** has proven to be a valuable tool in the elucidation of the role of the proteasome in the degradation of regulatory proteins that control fundamental cellular processes, and selective proteasome inhibition has become an important target for drug discovery.

Although salinosporamide A (**1**) shares an identical bicyclic ring structure with omuralide (**2**, Scheme 1), it is uniquely functionalized. More specifically, **1** is methylated at the C-3 ring juncture, possesses a chloroethyl group instead of a methyl group at C-2, and, significantly, is functionalized with a cyclohexene instead of an isopropyl group at the C-5 position. The structural features of omuralide that are responsible for its activity have been studied in detail by Corey and co-workers who demonstrated that the C-5 isopropyl group (see Scheme 1) is required for activity and that substitution with a phenyl ring at this position completely abolished activity.[9] The fact that salinosporamide A (**1**) incorporates a cyclohexene substituent at this position, yet retains activity, suggests that it may interact with the proteasome in a manner that is different from omuralide. Future studies on the structure–activity relationships of salinosporamide A derivatives, or co-crystallization of **1** with purified 20S proteasome, may help elucidate these differences.

Salinosporamide A seems to be a direct product of the fermentation rather than a subsequent transformation product of a precursor similar in structure to that of lactacystin. Further, while some ring opening does occur in methanol during chromatography on silica gel, the β-lactone functionality seems to be somewhat stabilized in this molecule.

Salinosporamide A (**1**) displayed potent in vitro cytotoxicity against HCT-116 human colon carcinoma with an IC_{50} value of 11 ng mL^{-1}. This compound also displayed potent and highly selective activity in the NCI's 60-cell-line panel with a mean GI_{50} value (the concentration required to achieve 50% growth inhibition) of less than 10 nM and a greater than 4 log LC_{50} differential between resistant and susceptible cell lines. The greatest potency was observed against NCI-H226 non-small cell lung cancer, SF-539 CNS cancer, SK-MEL-28 melanoma, and MDA-MB-435 breast cancer (all with LC_{50} values less than 10 nM). Salinosporamide A was tested for its effects on proteasome function because of its structural relationship to omuralide (**2**). When tested against purified 20S proteasome (Calbiochem, cat. no. 539158), salinosporamide A inhibited proteasomal chymotrypsin-like proteolytic activity with an IC_{50} value of 1.3 nM.[10] This compound is approximately 35 times more potent than omuralide (IC_{50} = 49 nM in our assay) which was tested as a positive control in the same assay. Thus, the unique functionalization of the core bicyclic ring structure of salinosporamide A appears to have resulted in a molecule that is a significantly more potent proteasome inhibitor than omuralide.

Experimental Section

Cultivation of strain CNB-392: Strain CNB-392 was isolated from a sediment sample collected at a depth of about 1 m in June 1989 from a mangrove environment in Chub Cay, Bahamas. The strain was cultured in 2.8-L Fernbach flasks (20 × 1 L) in a seawater-based medium (0.2% chitosan, 0.2% kelp powder, 0.2% menhaden meal, 0.2% fish solubles, 0.5% starch) and shaken at 230 rpm at 27 °C). After nine days of cultivation, sterilized Amberlite XAD-16 resin (20 g L^{-1}) was added and the resin was collected 24 h later by filtration, washed with deionized water, and eluted with acetone to afford 5 g of a cytotoxic, crude organic extract after removal of solvent under reduced pressure.

1: The crude extract was partitioned between ethyl acetate and water, and the organic fraction was purified by flash column chromatography on silica gel, using increasing concentrations of acetone in CH_2Cl_2, to yield 250 mg of a fraction enriched in salinosporamide A (**1**). Recrystallization of the semi-purified fraction from ethyl acetate/*iso*-octane (3/1) provided 150 mg of **1** as colorless needles: m.p. 169–171 °C. $[\alpha]_D^{25} = -72.9°$ (c = 0.55, MeOH); NMR data ([D$_5$]pyridine): see Table 1; IR (NaCl): $\tilde{\nu}$ = 2920, 1819 m, 1702 s, 1255 m, 1085 s, 1020 s, 797 s cm^{-1}; UV (MeOH): λ_{max} nm (logε) = 225 (3.3), 205 (4.03); LC/(+)ESI-MS (70 eV, 3500 eV): R_t = 10.1 min (acetonitrile/water, 15 min gradient): m/z 314.1 $[M+H]^+$, 336 $[M+Na]^+$; HR-MALDI: m/z 314.1144 $[M+H]^+$, calcd for $C_{15}H_{21}{}^{35}ClNO_4$, m/z 314.1159.

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Table 1: NMR spectral data for salinosporamide A (**1**).

C no.	δ_C	δ_H, mult., int., J [Hz]	COSY correlations	HMBC correlations
1	176.4 C			
2	46.2 CH	3.17, t, 1H, 7.0	H-12	C-1, 3, 12, 13, 14
3	86.1 C			
4	80.2 C			
5	70.9 CH	4.24, d, 1H, 9.0	H-6	C-3, 6, 7
6	39.2 CH	2.85, m, 1H	H-5, H-11a/b	
7	128.4 CH	6.42, d, 1H, 9.6	H-6, 8, 9	C-9
8	128.8 CH	5.88, m, 1H	H-7, 9	
9	25.3 CH_2	1.91, m, 2H	H-8, 10b	C-11
10	21.7 CH_2	a 1.66, m, 1H	H-9, 10b	C-6, 8, 9
		b 1.38, m, 1H	H-9, 10a	
11	26.5 CH_2	a 2.37, m, 1H	H-6, 11b	C-6, 7, 9, 10
		b 1.66, m, 1H	H-6, 10b	C-5, 6
12	29.0 CH_2	a 2.48, m, 1H	H-2, 12b, 13	C-1, 2, 3, 13
		b 2.32, m, 1H	H-2, 12a, 13	C-1, 2, 3, 13, 14
13	43.2 CH_2	a 4.14, m, 1H	H-12a/b, 13b	C-2, 12
		b 4.01, m, 1H	H-12a/b, 13a	C-2, 12
14	20.0 CH_3	2.07, s, 3H		C-2, 3, 4, 15
15	169.0 C			
NH		10.60, brs		C-2, 3, 4
OH		4.99, brs		

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[4] Crystal Data for **1**: $C_{15}H_{21}ClNO_4$, $M_r = 313.11$, monoclinic space group, $P21$, $a = 10.4805(6)$, $b = 24.2085(13)$, $c = 12.5163(7)$ Å, $\beta = 108.603(10)°$, $V = 3009.7(3)$ Å^3, $Z = 8$, $\rho_{calcd} = 1.385$ g cm^{-3}; $Mo_{K\alpha}$ radiation, $\lambda = 0.71073$ Å, $\mu = 0.269$ mm^{-1}, $T = -100$ K. 25627 data (13056 unique, $R_{int} = 0.0146$, $\theta < 27.52°$) were collected on a Bruker SMART APEX CCD X-ray diffractometer. The structure was solved by direct methods and refined by full-matrix least-squares on F^2 values of all data (G. M. Sheldrick, SHELXTL Manual) to give $wR2 = 0.0824$, conventional $R = 0.0313$ for F values of 12747 reflections, $S = 1.037$ and 773 parameters. Residual electron density max/min 0.448/ -0.232 e Å^{-3}. CCDC-183413 (**1**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

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Molybdenum–Silicon Multiple Bonds

Multiple Bonding Between Silicon and Molybdenum: A Transition-Metal Complex with Considerable Silylyne Character**

*Benjamin V. Mork and T. Don Tilley**

The 2p elements carbon, nitrogen, and oxygen readily form multiple bonds to many other elements, and such bonds contribute strongly to the chemical behavior of organic compounds. In contrast, the heavier main-group elements (with principal quantum numbers of three or greater) reluctantly participate in multiple bonding,[1] and this aspect of main-group chemistry has been the focus of considerable fundamental research. A number of stable compounds with

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