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Cyanosporasides A and B, Chloro- and Cyano-cyclopenta[*a*]indene Glycosides from the Marine Actinomycete "*Salinispora pacifica*"

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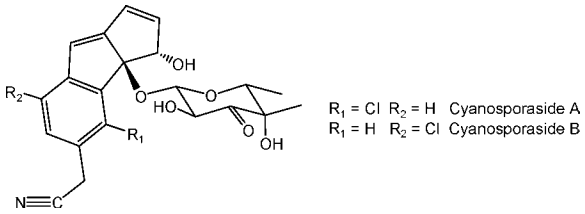
ABSTRACT



Two structurally novel cyclopenta[*a*]indene glycosides, cyanosporasides A and B (1 and 2) have been isolated from the culture broth of a new species of the obligate marine actinomycete genus *Salinispora*. The structures and absolute stereochemistries of these compounds were determined by spectral and chemical methods. The cyanosporasides possess a new 3-keto-pyranohexose sugar as well as a cyano- and chloro-substituted cyclopenta[*a*]indene ring system. The cyanosporasides are proposed to be cyclization products of an enediyne precursor.

Research conducted over the past decade has clearly demonstrated that the oceans are host to taxonomically unique and phylogenetically diverse populations of actinobacteria.[1] Although culturing the majority of these bacteria remains a major challenge, we recently reported the cultivation of the first marine actinomycete genus, *Salinispora*,[2] which requires seawater for growth. The potential of this genus to produce new chemical scaffolds is just starting to be realized, but already several compounds, for example, the salinosporamides and the sporolides, provide hints as to the chemical diversity that awaits discovery.[3] Our continued biological and chemical investigation of *Salinispora* strains has led to the discovery of a third species within this genus, for which we propose the name "*Salinispora pacifica*". Examination of the secondary metabolites produced by one "*Salinispora pacifica*" strain (designated CNS103), which was isolated from sediments collected at a depth of 500 m in Palau, has resulted in the isolation of two cyclopenta[*a*]-indene glycosides, cyanosporasides A (**1**) and B (**2**), which possess unique carbon skeletons.

LC-MS analysis of the culture extract of the *Salinispora* isolate CNS103 showed the presence of metabolites with unusual UV absorption spectra (λ_{max} 242, 298, and 324 nm). These molecules also showed MS isotopic patterns characteristic of monochlorinated secondary metabolites ([M +

(1) (a) Maldonado, L. A.; Stach, J. E. M.; Pathom-aree, W.; Ward, A. C.; Bull, A. T.; Goodfellow, M. *Antonie van Leeuwenhoek* **2005**, *87*, 11. (b) Montalvo, N. F.; Mohamed, N. M.; Enticknap, J. J.; Hill, R. T. *Antonie van Leeuwenhoek* **2005**, *87*, 27.

(2) (a) Mincer, T. J.; Jensen, P. R.; Kauffman C. A.; Fenical, W. *Appl. Environ. Microbiol.* **2002**, *68*, 5005. (b) Jensen, P. R.; Gontang, E.; Mafnas, C.; Mincer, T. J.; Fenical, W. *Environ. Microbiol.* **2005**, *7*, 1039. (c) Maldonado, L. A.; Fenical, W.; Jensen, P. R.; Kauffman, C. A.; Mincer, T. J.; Ward, A. C.; Bull, A. T.; Goodfellow, M. *Int. J. Syst. Evol. Microbiol.* **2005**, *55*, 1759.

(3) (a) Feling, R. H.; Buchanan, G. O.; Mincer, T. J.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Angew. Chem., Int. Ed.* **2003**, *42*, 355. (b) Williams, P. G.; Buchanan, G. O.; Feling, R. H.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *J. Org. Chem.* **2005**, *70*, 6196. (c) Buchanan, G. O.; Williams, P. G.; Feling, R. H.; Kauffman, C. A.; Jensen, P. R.; Fenical, W. *Org. Lett.* **2005**, *7*, 2731.

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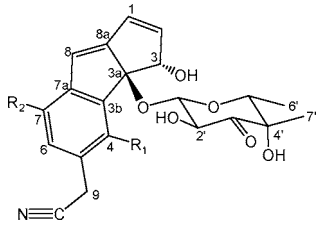
$Na]^+$:$[M + Na + 2]^+ = 3:1$). Large-scale saline fermentation (25 L), followed by solid-phase extraction with Amberlite XAD-7 resin, yielded 8.1 g of culture extract. The crude extract was fractionated by normal-phase silica gel vacuum column chromatography, and the fraction eluting with ethyl acetate was subsequently purified by silica gel HPLC (ethyl acetate isocratic) to yield 18 mg of cyanosporaside A (**1**). The next column fraction (eluting in 10:1 ethyl acetate/methanol) was also further purified by repeated silica gel HPLC (5:1 ethyl acetate/toluene isocratic) to give 5 mg of cyanosporaside B (**2**).



1 R_1 = Cl R_2 = H
2 R_1 = H R_2 = Cl

Cyanosporaside A (**1**)[4] was obtained as an oil, which had a molecular formula of $C_{21}H_{20}{}^{35}ClNO_6$ (12 degrees of unsaturation) as determined by ESI high-resolution mass spectrometry (obsd $[M + Na]^+$ at *m*/*z* 440.0872, calcd $[M + Na]^+$ 440.0871). This molecular formula was corroborated by ^1H and ^{13}C NMR spectral data (Table 1). The ^{13}C NMR spectrum of **1** displayed 21 carbon signals including one carbonyl, 11 carbons in the olefinic region, six oxygenated carbons between 70 and 100 ppm, and three aliphatic carbons.

Analysis of the combined 1D and 2D spectral data (see Table S2 in Supporting Information) established that cyanosporaside A possessed five olefinic methine, four oxygenated methine, nine quaternary, and two methyl carbons, in addition to three D_2O exchangeable protons and one aliphatic methylene carbon. The methylene proton signal that was observed as a singlet at 3.89 ppm correlated to an upfield carbon at 22.1 ppm. This distinctive chemical shift combination strongly indicated that this carbon was directly adjacent to a triple bond (C≡C or C≡N), since its anisotropy shields the adjacent carbon (α-position nucleus) but has little effect on the corresponding protons (*β*-position nuclei).[5] A nitrile functionality was suggested by the presence of a quaternary carbon at 118.6 ppm in the ^{13}C NMR spectrum of **1** and an odd number of carbon resonances in the olefinic region. This structural fragment was confirmed by an IR absorption at 2249 cm^{-1}. The 12 degrees of unsaturation inherent in the molecular formula of **1**, coupled with data showing the presence of one carbonyl, one nitrile, and 10 olefinic carbons (8 degrees of unsaturation), indicated that cyanosporaside A must possess four rings.

Table 1. ^1H and ^{13}C NMR Spectral Data for **1** and **2** in CD_3CN

	cyanosporaside A (**1**)		cyanosporaside B (**2**)	
C/H	δ_H,[a] mult (*J*[b])	δ_C,[c] H	δ_H,[a] mult (*J*[b])	δ_C,[c] H
1	6.69, d (6.0)	130.4, CH	6.74, d (6.0)	130.6, CH
2	6.63, dd (6.0, 2.5)	145.8, CH	6.63, dd (6.0, 2.5)	146.1, CH
3	4.81, dd (6.5, 2.5)	76.2, CH	4.67, dd (6.0, 2.5)	76.3, CH
3-OH	2.93, d (6.5)		3.01, d (6.0)	
3a		97.1, C		97.1, C
3b		140.1, C		144.7, C
4		132.3, C	7.37, d (0.5)	125.2, CH
5		127.0, C		130.8, C
6	7.46, d (7.5)	132.6, CH	7.28, d (0.5)	130.1, CH
7	7.17, d (7.5)	121.5, CH		127.0, C
7a		152.3, C		147.4, C
8	6.40, s	121.0, CH	6.52, s	119.0, CH
8a		159.1, C		159.6, C
9	3.89, s	22.1, CH_2	3.83, s	23.4, CH_2
10		118.6, C		119.2, C
1′	3.62, d (8.5)	99.9, CH	3.69, d (8.0)	100.1, CH
2′	4.28, dd (8.5, 5.0)	75.5, CH	4.27, dd (8.0, 4.5)	75.5, CH
2′-OH	3.12, d (5.0)		3.28, d (4.5)	
3′		206.8, C		206.8, C
4′		76.9, C		76.9, C
4′-OH	3.45, s		3.45, s	
5′	3.04, q (6.5)	75.0, CH	3.09, q (6.5)	74.9, CH
6′	1.11, d (6.5)	13.1, CH_3	1.09, d (6.5)	13.2, CH_3
7′	1.02, s	18.1, CH_3	1.03, s	18.1, CH_3

Assignments by gHMQC and DEPT experiments. [a] 500 MHz. [b] Coupling constants in Hz. [c] 125 MHz.

Further analysis of $^1H-^1H$ COSY and HMBC NMR spectra provided two distinctive units. The first was clearly a pyranohexose sugar moiety. Three-bond $^1H-^1H$ couplings of H-2′ with 2′-OH and H-1′ connected 2′-OH, C-2′ and C-1′. A homonuclear correlation between the H-5′ and H-6′ and the long-range heteronuclear couplings from 4′-OH to C-4′ and from H-7′ to C-5′ established the other half of the pyranose ring. HMBC correlations from H-7′ and H-2′ to C-3′, from H-5′ to C-1′, and from H-1′ to C-5′ established a pyranohexose ring system. The second part was the aglycone, which contained the aromatic chromophore. A suite of COSY correlations showed the isolated spin system of H-1, H-2, H-3, and 3-OH. The small coupling constant (6.0 Hz) between the vinyl proton signals, H-1 and H-2, indicated that this spin system belonged to a five- rather than a six-membered ring. The two-bond and three-bond HMBC couplings from H-1 to C-8a and C-3a and from H-3 to C-3a and C-8a revealed a cyclopentene moiety. A $^1H-^1H$ coupling (7.5 Hz) between H-6 and H-7 and the three-bond HMBC correlations from H-6 to C-4 and C-7a, as well as from H-7 to C-3b and C-5, established the benzenoid aromatic ring system. The connection between the five- and the six-membered rings was secured by the long-range couplings observed from H-8 to C-7a, C-8a, C-3a, C-3b, C-1, and C-7. The strong HMBC correlation from the methylene protons (H-9) to C-10 and C-5 showed the location of the nitrile functionality at C-10 (118.6 ppm). The attachment of the chlorine at C-4 completed the structural assignment of the aglycone. Finally, a three-bond correlation between H-1′ and C-3a linked the pyranose ring and the aglycone to complete the planar structure of **1**.

(4) Cyanosporaside A (**1**): oil, $[\alpha]_D$ +25 (*c* 0.19, CH_3CN); UV (CH_3CN) λ_{max} (log ϵ) 242 (4.1), 298 (3.9), 324 (3.6) nm; CD (CH_3CN) ($\Delta\epsilon$) 309 (+4.3), 242 (−17.1), 226 (+9.3); IR ν_{max} 3436, 2942, 2249, 1731, 1607, 1378, 1249, 1131, 1072 cm^{-1}; NMR spectral data, see Table S2; HR-ESIMS *m*/*z* 440.0872 (calcd for $C_{21}H_{20}{}^{35}ClNO_6$ $[M + Na]^+$, 440.0871).

(5) Packer, M. J.; Zonta, C.; Hunter, C. *J. Magn. Reson*. **2003**, *162*, 102.

The relative configuration of the pyranohexose ring was determined by analysis of $^1H-^1H$ coupling constants and by interpretation of 1D NOE experiments (Figure 1). The

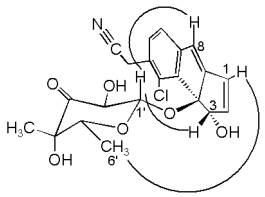


Figure 1. Key NOE correlations for cyanosporaside A (**1**).

magnitude of the $^1J_{CH}$ coupling constant for the anomeric proton (H-1′, 160 Hz) established the β-configuration of this center,[6] while a large diaxial coupling constant (8.5 Hz) between H-1′ and H-2′ indicated an axial configuration for H-2′ as well. NOE correlations between H-5′ and H-1′ and between H-7′ and H-5′ established their axial and equatorial positions, respectively. On the basis of the relative configuration, this unit was a modified β-fucopyranose (3′-oxo-4′-methyl-β-fucopyranose). Careful interpretation of 1D NOE data assigned the relative stereochemistry between the 3′-oxo-4′-methyl-β-fucopyranose and the cyclopenta[*a*]indene ring systems. The NOE correlations from H-8 to H-1′ and from H-1 to H-6′ established the relative orientation of the aglycone with respect to the 3′-oxo-4′-methyl-β-fucopyranose ring. A weak NOE correlation between H-1′ and H-3, due to rotational movement of the aglycone around the glycosidic bond, indicated that H-3 was oriented toward the pyranose ring and that the 3-OH is pointed away from it.

The absolute stereochemistry of cyanosporaside A (**1**) was assigned by application of the modified Mosher method.[7] Esterification of **1** with *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) yielded *S*- and *R*-bis-MTPA esters, respectively. The proton chemical shifts of these derivatives were assigned by interpretation of ^1H NMR and $^1H-^1H$ COSY spectral data. Calculation of $\Delta\delta_{S-R}$ values indicated the absolute configuration of **1** was as shown in Figure 2 (3*S*, 3a*R*, 1′*S*, 2′*S*, 4′*S*, and 5′*R*).

Cyanosporaside B (**2**),[8] which was isolated as an oil, had the molecular formula $C_{21}H_{20}{}^{35}ClNO_6$ (12 degrees of unsaturation) by interpretation of the high-resolution ESI mass spectral data (obsd $[M + Na]^+$ at *m*/*z* 440.0878, calcd $[M + Na]^+$ 440.0871), in combination with ^1H and ^{13}C NMR spectra.

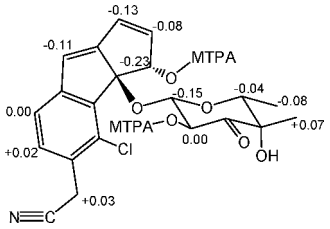


Figure 2. Delta values ($\Delta\delta_{S-R}$) in ppm for *S*- and *R*-bis-MTPA esters of cyanosporaside A (**1**) in CD_3CN.

The ^1H NMR spectrum of **2** was almost identical to that of cyanosporaside A (**1**) except for the two downfield aromatic signals that were a pair of mutually meta-coupled doublets [δ_H 7.28 (d, 0.5); 7.37 (d, 0.5)]. Comparing these doublet proton signals with the low field ortho-coupled signals in **1** [δ_H 7.17 (d, 7.5); 7.46 (d, 7.5)] indicated an alternate substitution pattern on the aromatic ring of **2**. Analysis of the two-dimensional COSY, HMQC, and HMBC NMR data for **2** led to the gross structure of cyanosporaside B as shown. The $^3J_{CH}$ coupling from H-4 to C-3a established the connectivity of C-3a, C-3b, and C-4. The C-7 chloro substitution was assigned on the basis of the meta-coupling observed between H-4 and H-6. The relative stereochemistry of **2** was determined by 1D NOE experiments and ^1H coupling constant analysis as in **1**. The absolute stereochemistry of cyanosporaside B was assigned as identical to **1** on the basis of the superimposable circular dichroism (CD) spectra observed for these two metabolites, which showed a distinct negative Cotton effect at 242 nm and a positive effect at 226 nm (see Supporting Information).

The cyclopenta[*a*]indene carbon framework of the aglycone of **1** and **2** is similar to the cycloaromatization product from a naturally occurring enediyne, C-1027, isolated in 1988 by Marunaka from the culture broth of *Streptomyces globisporus*.[9] This raises the intriguing possibility that **1** and **2** are also derived from an enediyne precursor. Based on the chlorine substitution patterns in **1** and **2**, we hypothesize that the chloro-cyclopenta[*a*]indene ring system could arise by quenching of a Bergman diradical intermediate[10] with chlorine and hydrogen, respectively (see Figure 3).[11] LC-MS analysis of the crude fermentation broth showed that the ratio of cyanosporaside A to B was 1:1, supporting this proposed mechanism. The observation of trace amounts of a dihydro and two monobrominated analogues of **1** and **2** in the crude extract provides further evidence of the incorporation of halide after cycloaromatization. Unfortunately, attempts to isolate these trace components or their putative

(6) This value was measured from the $^1J_{CH}$ satellites in the gHMBC spectrum. The typical ranges of $^1J_{CH}$ values are 169−171 and 158−162 Hz for α- and β-linkages, respectively. See: Pretsch, E.; Bühlmann, P.; Affolter, C. *Structure Determination of Organic Compounds-Tables of Spectral Data*; Springer: New York, 2000; p 153.

(7) (a) Séco, J. M.; Quiñoa, E.; Riguera, R. *Tetrahedron: Asymmetry* **2001**, *12*, 2915. (b) Freire, F.; Séco, J. M.; Quiñoa, E.; Riguera, R. *J. Org. Chem.* **2005**, *70*, 3778.

(8) Cyanosporaside B (**2**): oil, $[\alpha]_D$ +21 (*c* 0.20, CH_3CN); UV (CH_3CN) λ_{max} (log ϵ) 244 (4.0), 301 (3.9), 328 (3.6) nm; CD (CH_3CN) ($\Delta\epsilon$) 309 (+4.2), 241 (−17.0), 225 (+8.9); IR ν_{max} 3448, 2938, 2255, 1733, 1606, 1374, 1246, 1130, 1074 cm^{-1}; NMR spectral data, see Table S3; HR-ESIMS *m*/*z* 440.0878 (calcd for $C_{21}H_{20}{}^{35}ClNO_6$ $[M + Na]^+$, 440.0871).

(9) (a) Hu, J.; Xue, Y.-C.; Xie, M.-Y.; Zhang, R.; Minami, Y.; Yamada, Y.; Marunaka, T. *J. Antibiot.* **1988**, *41*, 1575. (b) Minami, Y.; Yoshida, K.-I.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2633. (c) Yoshida, K.-I.; Minami, Y.; Azuma, R.; Saeki, M.; Otani, T. *Tetrahedron Lett.* **1993**, *34*, 2637.

(10) The alternative cyclization mechanism (Myer-Saito), which proceeds via an allene intermediate, would result in diradical formation at C-4 and C-8, inconsistent with halogenation at C-7.

(11) Bharucha, K. N.; Marsh, R. M.; Minto, R. E.; Bergman, R. G. *J. Am. Chem. Soc.* **1992**, *114*, 3120.

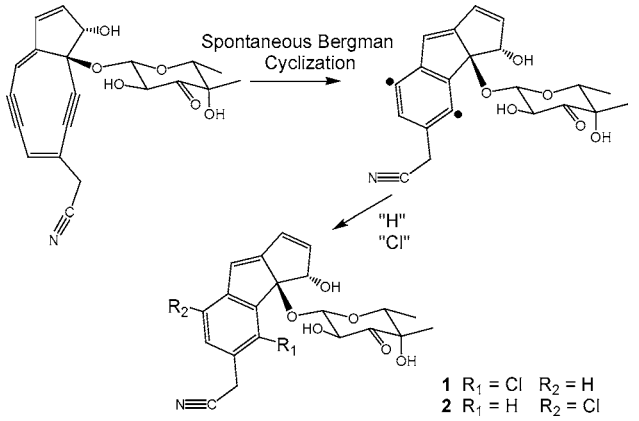
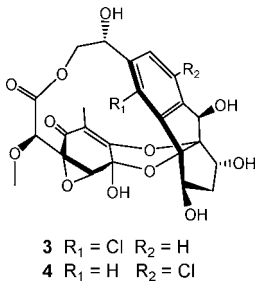


Figure 3. Proposed Cyclization Mechanism for **1** and **2**.

enediyne precursors proved unsuccessful. In the case of the latter, this is possibly attributed to the known instability of nine-membered enediynes, which are stabilized as chromoproteins, unlike the 10-membered enediynes. These enediynes rapidly cyclize once separated from their protein complex.[12] Although we hypothesize halogen trapping by the Bergman diradical intermediate, this reaction has not been reported in other systems.

The structural features of the cyanosporasides are unique in numerous ways. The aglycone chromophore, which presumably originated from a polyketide biosynthetic pathway for the enediyne core, is an unusual 3,3a-dihydrocyclopenta[*a*]indene ring system. This structural architecture has only been reported in one other series of natural products, the sporolides (**3** and **4**), multicyclic chloroaromatic metabolites that we recently reported from a different species of the genus *Salinispora*.[3c] The chloro aromatic substitution in the cyclopenta[*a*]indene ring system of **3** and **4** resembles that of the cyanosporasides, providing circumstantial evidence that the sporolides might originate from a similar enediyne precursor as shown above.[13,14] The observation of the benzyl nitrile functionality is unusual. Even though benzyl nitriles are found in the nitrilosides from various plants,[15] the cyanosporasides are the first examples of the benzyl nitrile moiety in a multiple ring system. It has been reported that the nitrile functional group in borrelidin (from *Streptomyces parvulus)* is incorporated into the polyketide by enzymatic oxidation of a backbone carbon, transamination, aldoxime formation, and dehydration.[16] However, without further experimental evidence, the origin of the nitrile group in **1** and **2** remains uncertain.



In addition to the uniqueness of the chromophore, the sugar, 3′-oxo-4′-methyl-β-fucopyranose, has not been reported. We assume that this sugar is derived from oxidation of the 3′-hydroxyl group of 6′-β-deoxy-galactose followed by *S*-adenosylmethionine-dependent methylation of an enediolate intermediate at 4′ position, as has been previously proposed for a similar oxo-methyl sugar.[17]

Currently, the biological activity of these compounds is still under investigation. To date, limited testing has shown that **1** has weak cytotoxicity against human colon carcinoma HCT-116 (IC_{50} 30 μg/mL) but is inactive against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and amphotericin-resistant *Candida albicans*.

The discovery of the cyanosporasides provides additional evidence that taxonomically unique marine actinomycetes, such as *Salinispora*, have great potential as a source of secondary metabolites with novel structures. This result suggests that continued investigation of marine actinomycetes, especially those cultured from deep-sea sediments, represents a promising strategy for the discovery of structurally unique molecules.

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Supporting Information Available: ^{1}H, ^{13}C, DEPT, gHMQC, gCOSY, gHMBC, CD spectra, other NMR spectral data, and source and fermentation conditions for strain CNS103. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Shen, B.; Liu, W.; Nonaka, K. *Curr. Med. Chem*. **2003**, *10*, 2317.

(13) These questions about the origins of the cyclopenta[*a*]indene moieties in the sporolides will be addressed once the ongoing genomic sequence of *Salinispora tropica* is complete.

(14) Recent genetic evidence has suggested that enediynes are more prevalent in actinomycetes than original thought. See: Zazopoulos, E.; Huang, K.; Staffe, A.; Liu, W.; Bachmann, B. O.; Nonaka, K.; Ahlert, J.; Thorson, J. S.; Shen, B.; Farnet, C. M. *Nat. Biotechnol.* **2003**, *21*, 187.

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(16) Olano, C.; Moss, S. J.; Brana, A. F.; Sheridan, R. M.; Math, V.; Weston, A. J.; Mendez, C.; Leadlay, P. F.; Wilkinson, B.; Salas, J. A. *Mol. Microbiol*. **2004**, *52*, 1745.

(17) Chen, H.; Zhao, Z.; Hallis, T. M.; Guo, Z.; Liu, H.-W. *Angew. Chem., Int. Ed*. **2001**, *40*, 607.