

ORIGINAL ARTICLE

Actinoquinolines A and B, anti-inflammatory quinoline alkaloids from a marine-derived *Streptomyces* sp., strain CNP975

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Actinomycete bacteria of the common genus *Streptomyces* can be routinely isolated from shallow and deep ocean sediments. Although commonly considered a terrestrial genus, and most abundantly found in soil, *Streptomyces* strains are found that have distinct requirements for seawater and routinely do not show significant similarity, with terrestrial strains by 16S ribosomal DNA phylogenetic sequence comparisons. Our examination of the culture broth of a *Streptomyces* sp., strain CNP975, isolated from a local La Jolla, California sediment sample, resulted in the isolation of actinoquinolines A and B (1, 2), which show significant inhibition of the arachidonic acid pathway enzymes cyclooxygenases-1 and -2. The new compounds contain the 3-hydroxyquinaldic acid (3HQA) motif found in numerous peptide antibiotics. In the actinoquinolines, 3HQA forms an amide linkage with a linear six-carbon fragment, formally a 2, 6-diamino-1, 5-dihydroxyhexane unit, a component of likely amino acid reductive off-loading origin. Actinoquinoline A illustrated amide rotational isomerism leading to complex NMR spectral data. Actinoquinoline B was assigned as the C-13 aldehyde analog isolated as an intramolecular hemiacetal. Reduction of 2 with NaBH₄ yielded actinoquinoline A thus confirming the relative configurations of all centers in the actinoquinolines.

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INTRODUCTION

Exploration of diverse, poorly examined environments is of significant importance in the quest for the discovery of new microbial strains that produce bioactive natural products. Our program has focused on marine sediments, as a source of actinomycete bacteria that are genetically distinct from terrestrial strains.^{1–2} We have shown that marine-derived actinomycetes produce structurally unprecedented compounds, which have been illustrated to possess interesting anticancer,³ antibacterial⁴ and anti-inflammatory properties.^{5–7} As a continuation of our interest in marine bacterial alkaloids,⁸ we conducted a comprehensive LC–MS-based investigation of the crude extracts of numerous strains in our collection. Analysis of one actinomycete, our strain CNP975, revealed several unknown compounds with MWs in the range of 300–400 a.m.u. possessing long wavelength UV chromophores. A large-scale fermentation and extraction, followed by repeated purification of the organic extract, resulted in the isolation of two new quinoline alkaloids, actinoquinolines A (1) and B (2) of a unique alkaloid skeletal class possessing 3-hydroxyquinaldic acid, and a six-carbon fragment possibly derived from 5-hydroxylysine (Figure 1). The new compounds showed significant inhibition of the arachidonic acid inflammatory pathway

enzymes cyclooxygenases-1 and -2 (COX-1 and COX-2). Here we present the details of the isolation and characterization of these new alkaloids.

RESULTS AND DISCUSSION

Isolation and structure determination of actinoquinolines A (1) and B (2)

Actinoquinolines A and B were isolated as light green amorphous solids from a 40×1 liter cultivation by XAD-16 solid-phase resin extraction followed by reversed-phase flash and HPLC purification. High-resolution time of flight-MS (HR–TOF–MS) data for actinoquinoline A (1) showed adduct molecular ions at m/z 404.2186 [M+H]⁺ and 426.2004 [M+Na]⁺, which analyzed for the molecular formula C₂₁H₂₉N₃O₅. The molecular formula of 1 indicated nine degrees of unsaturation in the molecule. Proton and ¹³C NMR data revealed the presence of 10 aliphatic, 9 aromatic and 2 carbonyl carbons. From NMR and molecular formula data, the nine degrees of unsaturation were accounted for by a heteroaromatic bicyclic ring and two carbonyl groups. Proton,¹³C and HSQC NMR data, measured in CD₃OD (Table 1), showed five aromatic signals, H-4' δ_H 7.69 (1H, s) δ_C (119.8), H-5' δ_H 7.80 (1H, br d, *J* 8.0) δ_C (126.4), H-6' δ_H 7.57

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This paper is dedicated to Professor David Cane in recognition of his pioneering work in natural product biosynthesis.

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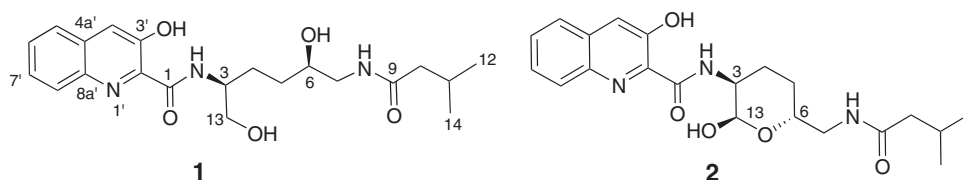


Figure 1 Structures of the actinoquinolines A (**1**) and B (**2**).

Table 1 ^1H and ^{13}C NMR data for actinoquinolines A (**1**) and B (**2**) in CD_3OD

Position	1		2	
	δ_{H} (J in Hz) ^a	δ_{C} ^b	δ_{H} (J in Hz) ^a	δ_{C} ^b
1	—	168.8 (C) 168.7 ^c	—	168.7 (C) 167.8 ^c
2-NH	—	—	—	—
3	4.21 (1H, m)	51.1 (CH) 51.7 ^c	4.22 (1H, m)	48.4 (CH) 47.0 ^c
4	1.84 (1H, m) 1.95 (1H, m)	26.9 (CH ₂) 26.8 ^c	ax1.90 (1H, m) eq2.22 (1H, m)	26.2 (CH ₂) 26.1 ^c
5	1.51 (1H, m) 1.62 (1H, m)	30.9 (CH ₂) 31.2 ^c	ax1.89 (1H, m) eq2.28 (1H, m)	21.5 (CH ₂) 21.7 ^c
6	3.66 (1H, m)	69.7 (CH) 70.3 ^c	4.23 (1H, m)	75.1 (CH) 67.1 ^c
7	3.71 (2H, m)	45.0 (CH ₂) 45.1 ^c	3.41 (2H, m)	43.5 (CH ₂) 43.3 ^c
8-NH	—	—	—	—
9	—	174.7 (C) 174.7 ^c	—	174.6 (C)
10	3.18 (2H, m)	45.1 (CH ₂) 45.2 ^c	3.38 (2H, m)	45.2 (CH ₂)
11	1.92 (1H, m)	26.8 (CH) 26.9 ^c	2.25 (1H, m)	26.1 (CH) 26.3 ^c
12	0.83 (3H, m)	21.4 (CH ₃)	0.86 (3H, m)	21.5 (CH ₃) 21.7 ^c
13	3.72 (2H, d, J 4.1)	63.9 (CH ₂) 63.7 ^c	5.16 (1H, br s) 5.00 ^c	92.5 (CH) 94.9 ^c
14	0.85 (3H, m)	21.5 (CH ₃)	0.90 (3H, m)	22.8(CH ₃) 23.2 ^c
1'-N	—	—	—	—
2'	—	135.2 (C)	—	135.0 (C) 134.8 ^c
3'	—	153.8 (C)	—	153.8 (C)
4'	7.69 (1H, s)	119.8 (CH) 119.9 ^c	7.71 (1H, d, J 5.3)	120.2 (CH) 120.0
4a'	—	132.2 (C)	—	132.3 (C)
5'	7.80 (1H, br d, J 8.0)	126.4 (CH) 126.2 ^c	7.80 (1H, br d, J 8.0)	126.4 (CH) 126.3 ^c
6'	7.57 (1H, m)	128.5 (CH) 128.6 ^c	7.59 (1H, m)	135.0 (CH) 134.8 ^c
7'	7.57 (1H, m)	127.1 (CH) 127.3 ^c	7.59 (1H, m)	132.3 (CH) 132.3 ^c
8'	8.03 (1H, br d, J 8.0)	129.3 (CH) 129.4 ^c	8.06 (1H, br d, J 8.0)	129.3 (CH) 129.4 ^c
8a'	—	141.6 (C)	—	141.6 (C)

^aRecorded at 500 MHz.

^bRecorded at 125 MHz.

^cThe chemical shifts of the other major rotamer.

(1H, m) δ_{C} (128.5), H-7' δ_{H} 7.57 (1H, m) δ_{C} (127.1) and H-8' δ_{H} 8.03 (1H, br d, J 8.0) δ_{C} (129.3). The presence of a quinoline ring was established by analysis of gCOSY and HMBC NMR correlation data.

The arrangement of the four aromatic protons H-5', H-6', H-7' and H-8' were assembled through analysis of gCOSY correlations, whereas H-4' was observed as an isolated singlet. HMBC NMR correlations from H-4' to C-3', C-2', C-4a', C-5' and C-8a', as well as correlations from H-6' to C-5', C-7' and C-8', and H-7' to C-5', C-6' and C-8' partially confirmed the quinoline ring structure. The position of the nitrogen between carbons C-2' and C-8a' was supported by their chemical shifts at δ_{C} 135.2 and 141.6, respectively. The chemical shift of C-3' at δ_{C} 153.8 indicated the site of a hydroxy group attachment to form a 3-hydroxyquinolaldic acid amide. The attachment of the amide side chain at C-1 (δ_{C} 168.8) was assembled by the interpretation of the gCOSY and the HMBC correlations, as well as the chemical shift of C-2' at δ_{C} 135.2 (Figure 2). gCOSY correlations linking H-3 through H-7, as well as the chemical shifts of H-3 (δ_{H} 4.21, 1H, m) and C-3 (δ_{C} 51.1), and H-7 (δ_{H} 3.71, 2H, m) and C-7 (δ_{C} 45.0) indicated its attachment to nitrogens N-2 and N-8, respectively, thus confirming the connectivity N-2/C-3/C-4/C-5/C-6/C-7/N-8. HMBC correlations further confirmed the location of the amide carbonyl at C-1 through correlation of H-3 to C-1, as well as the amide carbonyl at C-9 by observed correlations from H-7 and H-11 to C-9. The hydroxymethyl group, C-13, observed at δ_{H} 3.72 (2H, d, J = 4.1) and δ_{C} (63.9) was confirmed through gCOSY correlations to H-3, as well as HMBC correlations to C-3 and C-4. The terminal isobutyl group was positioned at C-9 by analysis of HMBC correlations between H₃-12 and H₃-14 to C-11 and C-10, as well as H₂-10 to C-9.

The HR-TOF-MS spectrum of actinoquinoline B (**2**) showed a protonated molecular ion at m/z 402.20283 $[\text{M}+\text{H}]^+$ and a sodium adduct ion at m/z 424.1848, which analyzed for the molecular formula $\text{C}_{21}\text{H}_{27}\text{N}_3\text{O}_5$, indicating an additional degree of unsaturation in comparison with **1**. Proton and ^{13}C NMR data, (Table 1), as well as UV absorption data, were similar to compound **1**, except for the disappearance of the oxymethylene signals from C-13 in **1**. In actinoquinoline B (**2**), a new oxymethine NMR signal at δ_{H} 5.16 (1H, br s) and δ_{C} (92.5) was observed for the C-13 carbon. On the basis upon the downfield chemical shifts of both C-13 and H-13, a cyclic hemiketal was constructed to account for the additional degree of unsaturation. Full NMR analyses confirmed the C-13 hemiketal by intramolecular cyclization of the C-6 hydroxy group to C-13.

Determination of the relative configurations of actinoquinolines A and B

An attempt was made to define the absolute configuration at C-6 using the Mosher Ester method.⁹ For reasons that were not clear, the reaction of compound **1** with the Mosher acid chlorides failed to yield C-6 OH Mosher esters. Consequently, the relative configuration of actinoquinoline B (**2**) was determined by the interpretation of ROESY NMR data (Figure 3). ROESY data indicated that the hemiketal ring in **2** adopted a classic chair configuration, thus allowing nuclear Overhauser enhancements to be readily observed and interpreted. ROESY NMR correlations between H-3ax (δ_{H} 4.22, 1H, m) H-5ax (δ_{H} 1.89, 1H, m) and H-13eq (δ_{H} 5.16, 1H, br s) indicated that they are on the same side of the hemiketal ring. Conversely, H-6ax

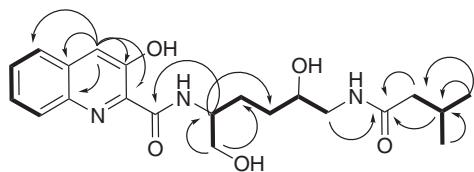


Figure 2 Key ^1H - ^1H gCOSY (bold) and HMBC (arrows) correlations for actinoquinoline A (1).

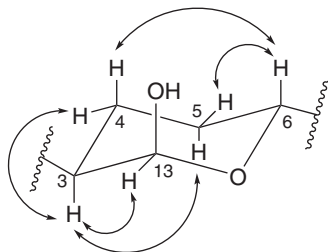


Figure 3 ROESY correlations of actinoquinoline B.

(δ_{H} 4.23, 1H, m) showed correlations with H-5eq (δ_{H} 2.28, 1H, m) and H-4ax (δ_{H} 1.90) indicated that they are on the opposite face of the ring. The relative configuration of actinoquinoline B (2) was thus assigned as $3S^*$, $6R^*$, $13R^*$.

Assigning the relative configuration of actinoquinoline A (1), which involved two remote centers, was not easily approached by NOE methods. Fortunately, treatment of 2 with NaBH_4 in THF^{10} resulted in an essentially quantitative conversion to actinoquinoline A (1), optical rotation of the converted one was determined as $[\alpha]_{\text{D}}^{25} +4.2$ (c 0.001, CHCl_3) similar to that of the original compound 1, which defined the relative configurations at C-3 and C-6 as $3S^*$, $6R^*$.

It is worthy to mention that actinoquinoline A (1) was consistently observed as a mixture of rotamers, which generated complex ^1H and ^{13}C NMR data depending on the solvent and other parameters. Table 1 and Supplementary Table S1 show the proton and carbon chemical shifts of the next major rotamer for 1.

The biosynthesis of the actinoquinolines appears to be similar to related compounds, such as thiocoraline,¹¹ sandramycin,¹² BE22179¹³ and quinaldopeptin¹⁴ all of which possess the 3-hydroxyquinaldic acid functionality. However, the extender chain, formally a 2, 6-diamino-1, 5-dihydroxyhexane unit, seems likely to be derived from amino acid modifications. Review of the literature provides ample evidence of reductive off-loading during biosynthesis, which could directly provide 5-hydroxylysine aldehyde.¹⁵ 5-Hydroxylysine is well known as a constituent of numerous proteins, including gelatine from which it was originally isolated.¹⁶ The aldehyde can then undergo an intramolecular cyclization to provide the hemiacetal observed in actinoquinoline B (2). Further reductive processing generates the primary alcohol in actinoquinoline A (1).

Biological activity

Actinoquinolines A (1) and B (2) showed moderately potent inhibition of COX-1 and COX-2, with inhibitory concentration with 50% inhibition (IC_{50}) values of 7.6 and 2.13 μM for 1, and 4.9 and 1.42 μM for 2 (Table 2). Actinoquinoline B (2) is the more potent inhibitor for both enzymes. These assays were conducted using indomethacin, as the positive control (IC_{50} values of 1.7 and 0.41 μM for COX-1 and COX-2) and celecoxib (IC_{50} values of 7.6 and 1.54 μM for COX-1 and

Table 2 Cyclooxygenase inhibition for actinoquinolines A and B (1, 2)

Compound	COX-1	COX-2
	IC_{50} μM^a	IC_{50} μM^a
1	7.6 \pm 0.35	2.13 \pm 0.19
2	4.9 \pm 0.29	1.42 \pm 0.13
Indomethacin (positive control)	1.7 \pm 0.11	0.41 \pm 0.04
Celecoxib (positive control)	7.6 \pm 0.35	1.54 \pm 0.10

^aThe IC_{50} was expressed as mean \pm s.e.

COX-2). Although both compounds were less active than indomethacin, compound 1 showed potent activity similar to celecoxib. On the other hand, they showed selectivity with COX-2/COX-1 comparable to the positive controls. Both compounds were 3.5 times more active against COX-2 than COX-1.

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured using a Rudolph Research Autopol III polarimeter (Hackettstown, NJ, USA) with a 10-cm cell and Jasco P-1020 polarimeter (Easton, MD, USA). UV spectra were recorded in a Varian Cary UV-visible spectrophotometer (Melbourne, VIC, Australia) with a path length of 1 cm, and IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer (Waltham, MA, USA). Proton and 2D NMR spectral data were recorded at 500 MHz (in CD_3OD solutions containing Me_4Si as internal standard) on a Varian Inova 500 MHz spectrometer. Carbon-13 NMR spectra were acquired at 125 MHz on the same instrument. High-resolution TOF mass spectral data were provided by The Scripps Research Institute, La Jolla, CA, USA by the MS facility at the Department of Chemistry and Biochemistry at the University of California, San Diego, La Jolla, CA, USA, and on an Agilent 6530 HR-TOF LCMS model 1100 LC/MS system (Santa Clara, CA, USA) with a reversed-phase C-18 column (Phenomenex Luna, Torrance, CA, USA, 4.6×100 mm, 5 μm) at a flow rate of 0.7 ml min^{-1} .

Isolation, microbe culture conditions and extraction

Streptomyces sp., strain CNP975 was isolated from a marine sediment sample collected in La Jolla, CA, USA by Alejandra Prieto Davó. *Streptomyces* sp., strain CNP975, was identified by 16S ribosomal DNA sequence analysis, which showed 99.5% similarity to *Streptomyces variabilis* strain DMS/RR/A (Genbank accession KP149559). The strain was cultured in 40 2.8 l Fernbach flasks each containing 1 liter of a natural seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO_3 , 40 mg $\text{Fe}_2(\text{SO}_4)_3 \cdot 4\text{H}_2\text{O}$ and 100 mg KBr) and shaken at 230 r.p.m. at 27 $^\circ\text{C}$. After seven days of cultivation, sterilized XAD-16 resin (20 g l^{-1}) was added to adsorb the organic metabolites, and the culture and resin were shaken at 215 r.p.m. for 2 h. The resin was filtered through cheesecloth, washed with deionized water and eluted with acetone. The acetone was removed under reduced pressure, and the resulting aqueous mixture was extracted with ethyl acetate (3×500 ml). The ethyl acetate-soluble fraction was dried *in vacuo* to yield 3.5 g of organic extract from a 40 liter culture.

Isolation and purification

The organic extract (3.5 g) from strain CNP975 was subjected to reversed-phase C-18 silica vacuum flash chromatography, using sequential mixtures of acetonitrile and water as eluents (elution order: 10, 15, 20, 30, 40, 50, 60, 70 and 100% methanol). Guided by the results of ^1H NMR and LC-MS analyses, the fractions containing the new alkaloids were combined (210.0 mg), and purified by C-8 reversed-phase semi-preparative HPLC (YMC ODS-A column, Kyoto, Japan, 1×25 cm, 30% aqueous MeCN) to yield 11.2 mg of actinoquinoline A (1) and 7 mg of actinoquinoline B (2), respectively, as pale green amorphous solids.

Actinoquinoline A (1). Pale green amorphous solid, $[\alpha]_{\text{D}}^{25} +4.4$ (c 0.001, CHCl_3); IR (NaCl) ν_{max} 3312, 2948, 2912, 2852, 1746, 1656, 1512, 1306 and

853 cm⁻¹; UV (MeOH) λ_{max} (log ϵ) 356, 298, 231 and 220 nm; ¹H and ¹³C NMR data, see Table 1; HR–TOF–MS m/z 404.21860 [M+H]⁺ (calcd for C₂₁H₃₀N₃O₅, 404.21854).

Actinoquinoline B (2). Pale green amorphous solid, [α]_D²⁵ -35 (c 0.001, CHCl₃); IR (NaCl) ν_{max} 3326, 2957, 2917, 2855, 1745, 1656, 1532, 1334, 865 cm⁻¹; UV (MeOH) λ_{max} (log ϵ) 351, 298, 231, 220 nm; ¹H and ¹³C NMR data, see Table 1; HR–TOF–MS m/z 402.20283 [M+H]⁺ (calcd for C₂₁H₂₈N₃O₅, 402.20289).

Reductive conversion of actinoquinoline B (2) to actinoquinoline A (1)

Finely powdered sodium borohydride (6 eq; 0.16 mmol) in tetrahydrofuran (THF) was added to actinoquinoline B (2 mg; 0.005 mmol) during a period of 15 min with stirring. Methanol (8 ml) was then added dropwise over 15 min with effervescence being observed. Stirring was maintained for a period of 60 min. The reaction was monitored by TLC using mixtures of ethyl acetate/*n*-hexane as eluants. After 60 min, the reaction mixture was further quenched with a saturated solution of NH₄Cl (1 ml) for an additional 1.5 h. For purification of the product, the organic layer was separated and the aqueous phase was extracted with ethyl acetate (2 × 2 ml). The organic extracts were combined and dried over anhydrous Na₂SO₄, and concentrated under vacuum. The reaction mixture was purified by semi-preparative C-18 silica HPLC to yield actinoquinoline A (1) in approximately quantitative yield. The actinoquinoline A isolated was also a pale green amorphous solid, [α]_D²⁵ +4.2 (c 0.001, CHCl₃), and showed identical NMR data alone and when mixed 1:1 with natural 1.

Evaluation of cyclooxygenase inhibition activities

In vitro COX-1 and COX-2 inhibition was evaluated for actinoquinolines A and B (1, 2), using a commercially available colorimetric COX (ovine) inhibition assay kit (Cayman Chemicals, Ann Arbor, MI, USA). The assay determines the peroxidase component of COX through colorimetric determination by monitoring the appearance of oxidized *N,N,N',N'*-tetramethyl-*p*-phenylenediamine at 590 nm. The background wells were prepared by addition of 160 μ l of the assay buffer and 10 μ l of heme to three wells. Initial activity wells (100% enzyme activity) were prepared by addition of 150 μ l of the assay buffer, 10 μ l of heme, and 10 μ l of each enzyme COX-1 and COX-2 in three wells. Compounds 1 and 2 were then added in different concentrations 10 μ l each, as well as 10 μ l of the dissolved solvent to the 100% initial activity wells and background wells. The plate was shaken carefully for a few seconds then incubated for 5 min at 25 °C, 20 μ l of the colorimetric substrate solution was added to the wells, as well as 20 μ l of arachidonic acid. The plate was then shaken for a few seconds and incubated at 25 °C, after which the absorbance was recorded at 590 nm using a plate reader.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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