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NOTE

Salinamide F, new depsipeptide antibiotic and inhibitor of bacterial RNA polymerase from a marine-derived *Streptomyces* sp.

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The marine environment is a rich source for unique actinomycete bacteria of significant biological and chemical diversity.[1,2] Marine-obligate and marine-derived actinomycetes have been shown to be the source of structurally diverse secondary metabolites, many of which have been shown to possess interesting anticancer properties.[3] The discovery of antibiotics from this source is far less developed, but promising antibiotics such as anthracimycin provide significant motivation to explore this source further.[4] Earlier, we showed that the marine-derived *Streptomyces* sp., strain CNB-091, produces a suite of at least five structurally unprecedented depsipeptides, salinamides A − E, that show significant antibacterial and anti-inflammatory properties.[5,6] Subsequently, salinamide A was shown to possess significant inhibitory activity against RNA polymerase (RNAP) from Gram-positive and Gram-negative bacteria.[7,8] As part of a collaborative program to further explore the chemical biology of the RNAP-inhibitory activity of the salinamides, we examined in more detail the extract and semi-purified fractions from the recultivation of *Streptomyces* sp. CNB-091 and report here the isolation of a new salinamide analog, salinamide F, which, like salinamide A, also possesses significant RNAP-inhibitory and antibacterial activity.

MATERIALS AND METHODS

Culture conditions and extraction

Streptomyces sp., strain CNB091, was isolated from a surface swab of a jellyfish (*C. xamachana*) collected in the Florida Keys in 1987 as previously described.[5] The producing organism was cultured in forty 2.8 l Fernbach flasks each containing 1 l of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3$-$4H_2O$, 100 mg KBr) by shaking at 230 r.p.m. at 27 °C for 7 days. At the end of the 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 and cell debris were filtered through cheesecloth, washed with deionized water and eluted with acetone. The acetone filtrate was concentrated under reduced pressure, and the resulting aqueous layer was extracted with ethyl acetate (3 × 500 ml). The ethyl acetate-soluble fractions were combined and reduced *in vacuo* to yield 3.5 g of extract from a 40 × 1 l culture.

Isolation and purification of salinamide F

The ethyl acetate extract (3.5 g), from cultivated strain CNB091, was subjected to silica vacuum flash chromatography using sequential mixtures of dichloromethane and methanol as eluents (elution order: 1, 2, 3, 4, 5, 10, 15, 20 and 30% methanol in dichloromethane, and 100% methanol). Guided by the results of bioassays and ^{1}H NMR analyses, the fractions containing bioactive metabolites were combined (210.0 mg) and purified using a C_8 reversed-phase semi-preparative HPLC column (YMC ODS-A column, 1 × 25 cm, 50% aqueous MeCN) to yield 13.2 mg of salinamide F (**1**) as pale yellow amorphous solid along with the previously described salinamides A and B and minor amounts of salinamides C–E.

Preparation of the acetonide 4

Salinamide F (**1**, 2 mg) was dissolved in dry acetone (1 ml), and $BF_3{\cdot}Et_2O$ (20 µl) was added at 0 °C. The reaction was allowed to stir for 3 h at 0 °C, and then quenched with saturated aqueous $NaHCO_3$. Next, the aqueous phase was extracted twice with ethyl acetate. The combined ethyl acetate solutions were concentrated under reduced pressure, and the residue was purified by C_8 reversed-phase semi-preparative HPLC (YMC ODS-A column, 1 × 25 cm, 50% aqueous acetonitrile) to yield 1.4 mg of the acetonide **4** as pale yellow solid.

RNAP-inhibitory activity

Fluorescence-detected RNAP-inhibition assays were performed by a modification of the procedure previously described.[9] Reaction mixtures contained (20 µl): 0–100 µM test compound, 75 nM bacterial RNAP holoenzyme (75 nM *Escherichia coli* RNAP holoenzyme or 75 nM *Staphylococcus aureus* RNAP core enzyme and 300 nM *S. aureus* σ^A; (prepared as described in a previous paper[10]), 20 nM DNA fragment containing bacteriophage T4 N25 promoter (positions

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−72 to +367; prepared by PCR from plasmid pARTaqN25-340-tR2[11]), 100 μM ATP, 100 μM GTP, 100 μM UTP and 100 μM CTP in transcription buffer (50 mM Tris–HCl, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10 μg ml^{-1} bovine serum albumin, 5% methanol and 5.5% glycerol). Components other than DNA and NTPs were pre-incubated for 10 min at 37 °C. Reactions were carried out by addition of DNA and incubation for 15 min at 37 °C, followed by addition of NTPs and incubation for 60 min at 37 °C. DNA was removed by addition of 1 μl 5 mM $CaCl_2$ and 2 U DNase I (Ambion), followed by incubation for 90 min at 37 °C. RNA was quantified by addition of 100 μl Quant-iT RiboGreen RNA Reagent (Life Technologies, Carlsbad, CA, USA; 1:500 dilution in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA), followed by incubation for 10 min at 22 °C, and measurement of fluorescence intensity (excitation wavelength = 485 nm and emission wavelength = 535 nm; GENios Pro microplate reader (Tecan, Männedorf, Switzerland)).

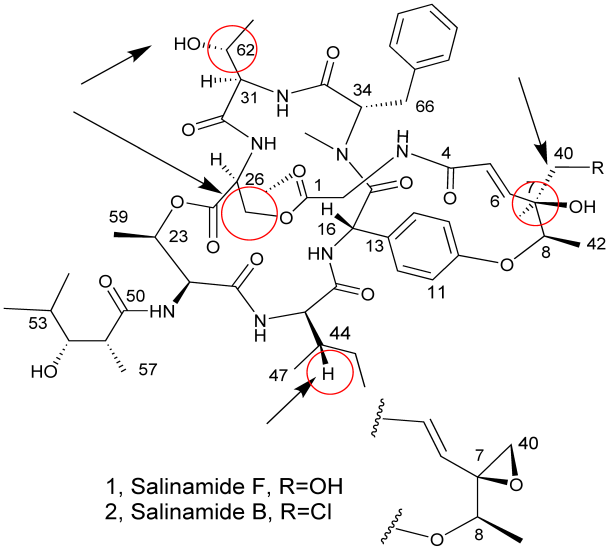
Antibacterial activity

Minimum inhibitory concentrations (MICs) were quantified using broth microdilution assays;[12] using a starting cell density of 2×10^5 c.f.u. ml^{-1}, LB broth[13] and an air atmosphere for *E.* D21f2tolC (*tolC:Tn10 rfa lac28 proA23 trp30 his51 rpsL173 ampC tsx81*; strain with cell-envelope defects resulting in increased susceptibility to hydrophobic agents, including salinamides[8,14]), *S. aureus* (ATCC 12600), *Enterococcus faecalis* (ATCC 19433) and *Enterobacter cloacae* (ATCC 13047); and using a starting cell density of 2×10^5 c.f.u. ml^{-1}, *Haemophilus* Test Medium broth,[15] and a 7% CO_2, 6% O_2, 4% H_2, 83% N_2 atmosphere for *Haemophilus influenzae* (ATCC 49247) and *Neisseria gonorrhoeae* (ATCC 19424).

Salinamide F (**1**), a new bicyclic depsipeptide, was isolated in addition to the known salinamides A (**3**) and B (**2**) (Figure 1), as well as salinamides C–E, which were produced in minor amounts but not purified. Analysis of salinamide F by HRTOFMS showed quasi-molecular ions at *m/z* 1038.51940 $[M+H]^+$ and 1060.50454 $[M+Na]^+$, which analyzed for the true molecular formula $C_{51}H_{71}N_7O_{16}$. The molecular weight of **1** was larger than salinamide A (**3**) by 18 mass units, which suggested the addition of one molecule of water. The structure could be fully defined by comprehensive analysis of 1D and 2D NMR data, including ^1H,^{13}C NMR, COSY, HSQC and HMBC experiments (Table 1). A loss of the C-40 signals in both the ^1H and ^{13}C NMR spectra at δ_H 2.44 (d, 5.4), 2.95 (d, 5.4) and δ_C 55.4, as well as the appearance of new signals δ_H 3.47 (m) and δ_C 66.0, in addition to the downfield shift of C-8 by δΔ +20 p.p.m. suggested that the epoxide ring had been opened (C-7-O-41-C-40)

Table 1 ^1H and ^{13}C NMR data for salinamide F (1) in $CDCl_3$

Position	δ_H(*J in Hz*)[a]	δ_C[b]
1	—	170.4 (C)
2	3.66 (1H, d, *J* 20.0) 4.87 (1H, dd, *J* 9.0, 20.0)	40.9 (CH_2)
3	7.12 (1H, d, *J* 8.0)	—
4	—	165.9 (C)
5	6.05 (1H, d, *J* 15.0)	123.6 (CH)
6	6.20 (1H, d, *J* 15.0)	147.7 (CH)
7	—	79.3 (C)
8	4.61 (1H, m)	80.7 (CH)
10	—	161.3 (C)
11	6.9–7.1 (1H, m)	127.8 (CH)
12	6.9–7.1 (1H, m)	123.8 (CH)
13	—	126.1 (CH)
14	6.9–7.1 (1H, m)	129.2 (CH)
15	6.9–7.1 (1H, m)	123.4 (CH)
16	5.13 (1H, d, *J* 2.0)	56.5 (CH)
17	8.55 (1H, br s)	—
18	—	169.9 (C)
19	4.99 (1H, dd, *J* 10.0, 5.0)	54.6 (CH)
20	6.60 (1H, br d, *J* 10.0)	—
21	—	168.0 (C)
22	4.87 (1H, m)	52.7 (C)
23	5.45 (1H, dq, *J* 6.1, 2.0)	73.2 (CH)
25	—	169.4 (C)
26	4.64, (1H, m)	53.0 (CH)
27	4.43 (1H, dd, *J* 10.0, 5.0) 4.74 (1H, d, *J* 10.0)	65.8 (CH_2)
29	7.48 (1H, br d, *J* 5.0)	—
30	—	169.4 (C)
31	4.33 (1H, d, *J* 10.0)	61.9 (CH)
32	7.24 (1H, d, *J* 10.0)	—
33	—	170.1 (C)
34	3.84 (1H, dd, *J* 10.0, 5.0)	69.6 (CH)
36	—	170.2 (C)
40	3.47 (1H, m)	66.0 (CH)
42	1.34 (3H, d, *J* 6.5)	14.7 (CH_3)
44	1.73 (1H, m)	40.0 (CH)
45	1.19 (1H, m) 1.28 (1H, m)	26.4 (CH_2)



3, Salinamide A

Figure 1 Structures of salinamides F (**1**), B (**2**) and A (**3**).

Table 1 (Continued)

Position	δ_H(*J* in Hz)[a]	δ_C[b]
46	0.91 (3H, t, *J* 7.5)	11.8 (CH_3)
47	0.88 (3H, d, *J* 6.5)	14.7 (CH_3)
49	7.81 (1H, d, *J* 10.0)	—
50	—	178.0 (C)
51	2.78 (1H, m)	42.7 (CH)
52	3.30 (1H, m)	79.6 (CH)
53	1.70 (1H, m)	32.7 (CH)
54	0.94 (3H, d, *J* 7.0)	18.2 (CH_3)
55	1.01 (3H, d, *J* 6.5)	20.1 (CH_3)
57	1.39 (3H, d, *J* 7.0)	16.8 (CH_3)
58	3.31 (1H, m)	—
59	1.42 (3H, d, *J* 6.5)	16.0 (CH_3)
62	4.33 (1H, m)	68.8 (CH_2)
63	1.62 (3H, d, *J* 6.0)	21.6 (CH_3)
64	5.78 (1H, br s)	—
66	3.29 (1H, dd, *J* 15.0, 10.0) 3.62 (1H, dd, *J* 15.0, 5.0)	35.0 (CH_2)
67	—	137.9 (C)
68	7.01 (1H, m)	129.4 (CH)
69	7.05 (1H, m)	128.8 (CH)
70	7.07 (1H, m)	126.9 (CH)
71	7.06 (1H, m)	128.8 (CH)
72	7.01 (1H, m)	129.4 (CH)
73	2.69 (3H, s)	40.2 (CH_3)

[a]Recorded at 500 MHz.
[b]Recorded at 125 MHz.

(Table 1). The HMBC NMR spectrum showed a ^2J correlation of H-6 (δ_H 6.19, d, $J=15.0$) and H-8 (δ_H 4.61, m) with C-8 (δ_C 80.7), as well as ^3J correlation between H-40 (δ_H 3.47, m) and C-6 (δ_C 147.7) as well as C-8 (δ_C 79.6) (Figure 2) supporting this suggestion. The remaining ^{1}H and ^{13}C NMR signals for **1** were virtually identical to those of salinamide A (**3**).[5,6]

The relative configuration at C40 was assigned by analysis of 2D ROESY NMR data derived from **1** and its acetonide derivative **4** (Figure 3). For salinamide F (**1**), NOE correlations between H_2-40 (δ_H 3.47, m), H-6 (δ_H 6.19, d, $J=15.0$) and H-8 (δ_H 4.61, m) also suggested the opening of the epoxide ring (C-7-O-41-C-40) with retention of configuration at the quaternary center. To confirm this, the acetonide derivative **4** was prepared and its relative configuration evaluated by ROESY NMR experiments using methods alrerady applied in similar systems.[16] Strong NOE correlations were observed between the H_2-40 (δ_H 3.64, m), and both H-66 methylene protons [δ_H 3.29, dd, $J=15.0$, 10.0), 3.62, dd, $J=15.0$, 5.0] and an acetonide methyl (δ_H 1.26, s), as well as NOE correlation between H-8 (δ_H 4.61,m) and H-66 (δ_H 3.62, dd, $J=15.0$, 5.0). Slight differences in the bond angles of derivative **4**, apparently derived by formation of the semi-planar ketal ring decreases the spatial distance between the H_2-40 protons and the benzyl protons at C-66, thus confirming the spatial proximity of these protons.

Salinamide F showed potent inhibition of Gram-positive and Gram-negative bacterial RNAP, with IC50 = 4 μM for *S. aureus* RNAP and 2 μM for *E. coli* RNAP. Salinamide F exhibited significant antibacterial activity against Gram-positive and Gram-negative bacteria, showing $MIC_{50}=12.5\,\mu g\,ml^{-1}$ for *Enterococcus faecalis*, $100\,\mu g\,ml^{-1}$ for *S. aureus*, $12.5\,\mu g\,ml^{-1}$ for *H. influenzae*, $25\,\mu g\,ml^{-1}$ for *Neisseria gonorrhoeae*, $50\,\mu g\,ml^{-1}$ for *Enterobacter cloacae* and $0.20\,\mu g\,ml^{-1}$ for *E. coli* D21f2tolC. The comparable RNAP-inhibitory activities of salinamide F (**1**) and salinamides A (**3**) and B (**2**)[8] are consistent with the conclusion that the epoxide functionality in salinamide A and the

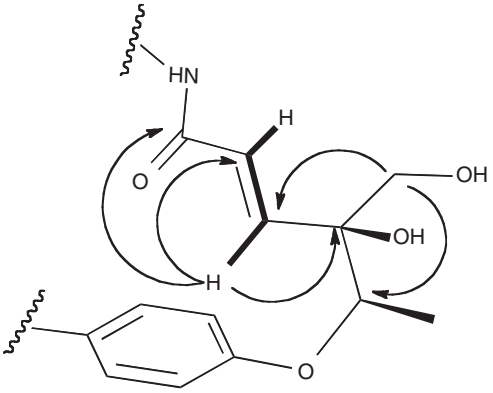


Figure 2 NMR ^{1}H-^{1}H COSY and HMBC correlations for salinamide F (1). COSY correlations are labeled by bold bonds; HMBC correlations shown as arrows.

Table 2 Sal-resistant mutants: cross-resistance to SalF

Amino-acid substitution	*MIC ratio (MIC/MIC$_{wild\ type}$)*		
	Salinamide A	*Salinamide B*	*Salinamide F*
rpoB (RNAP β subunit)			
675 Asp → Ala	⩾8	⩾8	⩾8
677 Asn → Lys	⩾8	⩾8	⩾8
rpoC (RNAP β′ subunit)			
738 Arg → Pro	⩾8	⩾8	⩾8
779 Ala → Val	⩾8	⩾8	⩾8
782 Gly → Ala	⩾8	⩾8	⩾8

Abbreviations: MIC, minimum inhibitory concentration; RNAP, RNA polymerase.

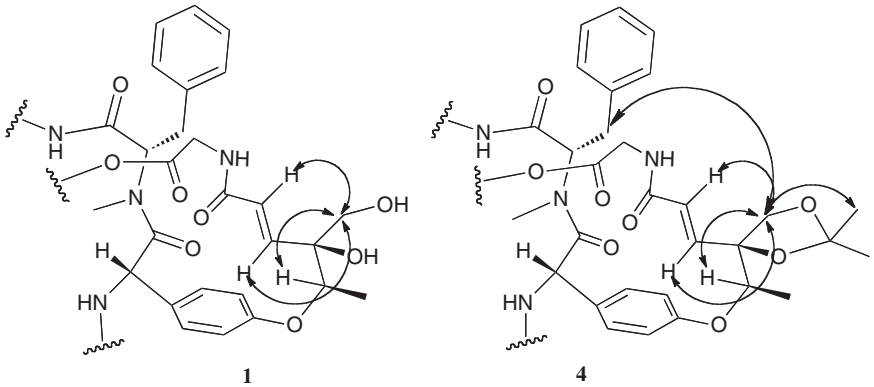


Figure 3 Illustration of the ROESY correlations observed for salinamide F (**1**) and the acetonide product **4**.

Table 3 Rif-resistant mutants: absence of cross-resistance to SalF

Amino-acid substitution	*MIC ratio (MIC/MIC$_{wild\ type}$)*	
	Rifampin	*Salinamide F*
rpoB (RNAP β subunit)		
516 Asp → Val	⩾8	1
526 His → Asp	⩾8	1
526 His → Tyr	⩾8	1
531 Ser → Leu	⩾8	1

Abbreviations: MIC, minimum inhibitory concentration; RNAP, RNA polymerase.

corresponding clorohydrin functionality in salinamide B are not essential for RNAP inhibition.[8] Substitutions of the RNAP β and β' subunits that confer high-level (⩾8-fold) resistance to salinamides A (**3**) and (**2**)[8] also confer high-level resistance to salinamide F (**1**) (Table 2). We infer that salinamide F inhibits RNAP through the same binding site on RNAP as salinamides A and B (i.e., the 'Sal target,' comprising residues of the 'F-loop' and 'link region' within RNAP β subunit and the 'bridge-helix N-terminal hinge' within RNAP β' subunit[8]). Substitutions of the RNAP β subunit that confer high-level resistance to the structurally unrelated RNAP inhibitor rifampin do not confer resistance to salinamide F (Table 3). We infer that salinamide F, like salinamides A and B,[8] does not interact with the rifampin binding site on RNAP.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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