Salinamides, Antiinflammatory Depsipeptides from a Marine Streptomycete

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In addition to the previously reported antiinflammatory agents salinamides A and B from the marine isolate *Streptomyces* sp. CNB-091, three minor peptides are described. Their total structures were established using a combination of spectral and chemical techniques. Revised structures are presented for the bicyclic depsipeptides salinamides A and B on the basis of the analysis of the dansylated salinamide A hydrolysate by chiral capillary electrophoresis. The fermentation yield of salinamide D, which contains a D-valine residue in place of the D-isoleucine moiety in salinamide A, can be dramatically increased 30-fold by supplementing the growth media with L-valine. Salinamides C and E are monocyclic depsipeptides that are likely methylated byproducts of salinamide A biosynthetic intermediates.

Introduction

Actinomycetes are a proven source of structurally diverse secondary metabolites possessing broad ranges of biological activities. Examples include antibiotic (erythromycin and tetracycline), anticancer (mitomycin and daunomycin), immunosuppressant (rapamycin and FK506), and veterinary (thiostrepton and monensin) agents. As the frequency of novel bioactive compounds discovered from terrestrial actinomycetes decreases with time, however, academic and industrial programs are increasingly screening actinomycetes from diverse environments for their ability to generate new metabolites.

Streptomycetes isolated from the marine environment have been largely ignored until recently. Early reports suggested that marine actinomycetes were derived from terrestrial sources and that they existed as metabolically inactive spores.[1] Consequently, the examination of marine actinomycetes was predicted to lead to a high isolation rate of known compounds. Although known compounds are frequently encountered, novel compounds are being reported at a high frequency,[2,3] indicating that marine actinomycetes have the ability to produce novel metabolites that have the potential for development into useful commercial products.[4,5] Recent studies have shown that actinomycetes isolated from the marine environment are metabolically active[6] and that certain taxonomic groups have adapted to life in the sea.[7,8]

In a preliminary communication, we reported on the structures of the major depsipeptides, salinamides A and B, isolated from *Streptomyces* sp. CNB-091, an actinomycete isolated from the surface of the jellyfish *Cassiopeia xamachana* collected from the Florida Keys.[9] Salinamides A and B exhibit moderate antibiotic activity against Gram-positive bacteria and show potent topical antiinflammatory activity in the phorbol ester-induced mouse ear edema assay.[9] Recently, salinamide A was found in an edaphic *Streptomyces* strain (NRRL 21611), where it was shown to exhibit strong inhibitory activity against bacterial RNA polymerases.[10] In this paper, we describe the entire spectrum of salinamides isolated from *Streptomyces* sp. CNB-091 and amend the absolute configurations of salinamides A and B.

Results and Discussion

Fermentation of *Streptomyces* sp. CNB-091 in seawater-based media followed by double EtOAc extraction of the whole-broth suspension, vacuum-flash chromatography, and reversed-phase HPLC afforded predominantly salinamide A (**1**) in approximately 9% yield based on the dry extract. Additional fermentations yielded salinamides B (**2**), D (**3**), C (**4**), and E (**5**) in addition to the major **1**.

Salinamide A (**1**, Figure 1) is a pale yellow noncrystalline solid having the molecular formula $C_{51}H_{69}N_7O_{15}$ based on high-resolution fast atom bombardment mass spectrometry (HRFABMS). Analyses by ^1H (Table 1) and ^{13}C (Table 2) NMR indicated that **1** is a depsipeptide composed of seven amino acids and two non-amino acid residues. The structures of all of the amino acids (glycine,

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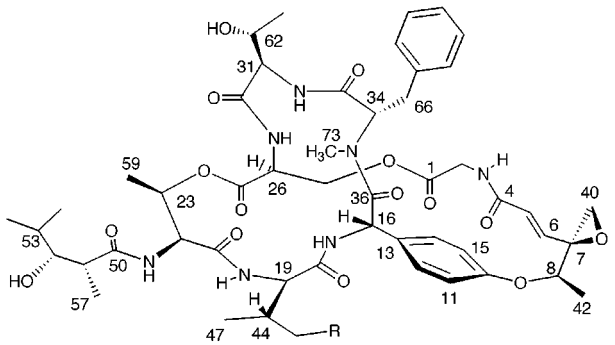
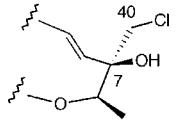


salinamide A (**1**) R = Me
salinamide D (**3**) R = H



salinamide B (**2**) R = Me

Figure 1. Structures of salinamides A (**1**), B (**2**), and D (**3**). The numbering system is based on the X-ray structure of **2**[9] and includes all oxygens and nitrogens.

threonine (2), serine, *N*-methylphenylalanine (MePhe), isoleucine, and a *p*-alkoxyphenylglycine unit) were determined by 2D-NMR, including HMQC, HMBC, COSY, TOCSY, and ROESY. A partial sequence of the amino acids could be determined independently by ROESY and HMBC experiments and formed the fragment Gly-COO-Ser/Thr/MePhe/*p*-alkoxyphenyl-Gly/Ile/Thr. The amide connectivities of the above fragment could be readily assigned by an HMBC experiment utilizing the respective NH protons and their correlations to the carbonyls of the vicinal amino acid. The ester linkage between serine and glycine was established by an HMBC correlation between the serine methylene hydrogen (H27b) at 4.66 ppm and the glycine carbonyl (C1, 169.7 ppm). In addition, the glycine residue formed an amide bond to a seven-carbon non-amino acid moiety as revealed by HMBC correlation of its NH (6.23 ppm) to the C5 olefin at 120.1 ppm. This residue (C4−C8, C40, and C42) contained an α,*β*-unsaturated amide and a 1,1-dialkyloxirane. The trans configuration of the C5/C6 olefin of the α,*β*-unsaturated system was based on the large coupling of the olefinic hydrogens (14.8 Hz), and the 5.4 Hz coupling of the C40 methylene hydrogens (2.44 and 2.95 ppm) was indicative of the 1,1-dialkyloxirane. Key HMBC correlations from the H8 quartet (4.91 ppm) to the quaternary carbons C7 and C10 linked this methine to the epoxide and to the phenylglycine moiety via an aromatic ether linkage. A second seven-carbon non-amino acid fragment (C50−C58) was identified as 3-hydroxy-2,4-dimethylpentanoate (HDMP) largely by COSY and HMBC correlations and attached via an amide bond to the threonine NH (7.95 ppm) from HMBC correlations from H22 and H49 to C50. The structure of **1** at this point was incomplete only in stereochemical assignments and in the ester connectivity between the serine carbonyl (C25, 168.6 ppm) and the threonine C23 oxygen.

A subsequent fermentation in which the final pH was several units lower than the first fermentation fortuitously yielded roughly equal portions of **1** and a new crystalline compound, salinamide B (**2**, Figure 1). Salinamide B analyzed for $C_{51}H_{70}N_7O_{15}Cl$ by HRFABMS and is related to the epoxide **1** as the chlorohydrin. The structure of **2** was established by single-crystal X-ray diffraction analysis.[9] The NMR assignments (Tables 1 and 2) were established through HMQC and TOCSY experiments and comparisons with those for **1**.

The core of **2** is a bicyclic hexadepsipeptide with two ester linkages involving serine (glycine to serine at C1−O28 and serine to threonine at C25−O24) and an aromatic ether linkage (C8−O9−C10). The connectivity of **2** severely limits the flexibility of the central hexapeptide core, a feature that complicates the NMR analysis by increasing relaxation times. The X-ray analysis indicated that the aromatic ring of the alkoxyphenylglycine moiety participates in two important interactions: a $\pi-\pi$ stacking with the C5−C6 double bond and a face-to-edge aromatic stack with MePhe.[9] The tight turn in this region prevents rotation of the alkoxyphenylglycine ring about the O9−C16 axis. These aromatic protons are barely visible as broad humps in the room temperature ^{1}H NMR spectrum but become pronounced signals at lower temperature (Figure 2). The lack of flexibility can be appreciated by noting in the −30 °C ^{1}H and ^{13}C NMR spectra that distinct shifts are seen for this ring. A pair of broad AB doublets at 6.62 and 6.82 ppm with a coupling of 8 Hz correspond to H11 and H12, respectively, whereas the second pair of aromatic protons (H14 and H15) are nearly chemical shift equivalent and reside at 7.19 ppm. The signals for H11 and H12 are dramatically shifted upfield in relation to those for H14 and H15, as **1** likely adopts a conformation in $CDCl_3$ at −30 °C similar to that in the crystal structure, resulting in the orientation of the C11−C12 bond under the shielding effect of the phenylalanine aromatic ring. Conversely, H15, which is ortho to the C10 ether, is shifted downfield to a chemical shift similar to that for H14, because H15 resides in the deshielding region of the C5−C6 double bond.

Upon treatment with HCl, **1** is converted to the chlorohydrin **2**, which is identical in all respects to naturally occurring **2**, thus confirming the structure of **1**. We suspect, however, that **2** is an artifact arising from **1** during fermentation. Salinamide A, which accumulates in the media during growth, is slowly converted to the chlorohydrin **2** upon treatment with the high salt A1 growth media.

Because single-crystal X-ray diffraction analysis of **2** established only the relative stereochemistry, the absolute configuration was originally determined by chiral GC analysis on the hydrolytic threonine units, providing D-threonine and L-*allo*-threonine.[9] However, reexamination of the absolute configuration of **1** by capillary electrophoresis (CE) indicated that the originally published structure is the enantiomer.

Salinamide A was subjected to mild acid hydrolysis, and the resulting acid hydrolysate was treated with dansyl chloride to derivatize the individual amino acids to their *N*-dansylates. Analysis by CE using *β*-cyclodextrin in the running buffer as the chiral recognition agent permitted us to reassign the configurations of the serine and two threonine residues and thus reset the absolute configuration of **1**. Co-injections of the dansylated **1** amino acids with those of authentic *N*-dansyl L-, D-, L-*allo*-, and D-*allo*-threonine standards clearly identified the **1** threonine residues as L-threonine and D-*allo*-threonine, opposite to those earlier reported. CE provided baseline separation of all four threonine isomers (see

Table 1. Proton NMR Data for Salinamides A−E (1−5)

no.	1[a]	2[a]	3[a]	4[b]	5[b]
2	3.66 (d, 19.1)	3.62 (d, 18.8)	3.69 (d, 18.6)	3.98 (d, 16.1)	
	4.90 (dd, 19.1, 9.0)	4.90 (dd, 18.8, 10.0)	4.94 (m)	5.22 (m)	
3	6.23 (d, 9.0)	7.02 (br d, 10.0)	6.23 (m)	6.18 (br d, 8.6)	
5	5.64 (d, 14.8)	5.92 (d, 15.6)	5.66 (d, 14.8)	5.58 (d, 14.0)	
6	6.21 (d, 14.8)	6.30 (d, 15.6)	6.22 (d, 14.8)	6.52 (d, 14.0)	
8	4.91 (q, 6.2)	4.78 (m)	4.88 (m)	4.54 (q, 7.5)	
11	6.62 (br d, 8)	6.9−7.1 (m)	6.65 (br d, 8)	6.75 (d, 8.6)	7.02 (d, 8.3)
12	6.82 (br d, 8)	6.9−7.1 (m)	6.83 (br d, 8)	7.09 (d, 8.6)	7.35 (d, 5.6)
14	7.19 (m)	6.9−7.1 (m)	7.20 (m)	7.09 (d, 8.6)	7.35 (d, 5.6)
15	7.19 (m)	6.9−7.1 (m)	7.20 (m)	6.75 (d, 8.6)	7.02 (d, 8.3)
16	5.17 (d, 3.2)	5.05 (d, 2.0)	5.17 (d, 3.1)	5.18 (d, 2.0)	5.17 (d, 3.0)
17	8.41 (d, 3.2)	8.71 (br s)	8.52 (br s)	9.14 (d, 2.0)	7.02 (br s)
19	5.05 (dd, 10.4, 4.0)	4.95 (dd, 10.6, 3.2)	4.83 (m)	5.25 (m)	4.81 (m)
20	6.51 (br d, 10.4)	6.50 (d, 10.8)	6.59 (br d, 9.9)	6.56 (d, 10.7)	6.75 (d, 9.7)
22	4.83 (dd, 9.7, 2.2)	4.84 (m)	4.82 (m)	4.84 (dd, 9.2, 2.0)	4.74 (dd, 9.3, 1.9
23	5.45 (dq, 6.1, 2.2)	5.44 (dq, 6.4, 2.0)	5.43 (dq, 6.2, 2.4)	5.52 (dq, 7.5, 2.0)	5.61 (m)
26	4.64 (m)	4.68 (br s)	4.62 (m)	4.67 (br s)	4.33 (m)
27	4.42 (dd, 10.8, 3.2)	4.50 (br d, 10.0)	4.41 (dd, 10.5, 2.0)	4.54 (m, 2H)	3.97 (br d, 11.7)
	4.66 (d, 10.8)	4.74 (q, 8.6, 6.0)	4.67 (d, 10.5)		4.17 (m)
28					4.60 (br s)
29	7.23 (br d, 6.1)	7.73 (br s)	7.25 (br d, 5.0)	7.28 (d, 6.5)	7.07 (d, 7.3)
31	4.32 (d, 7.2)	4.38 (d, 7.6)	4.30 (d, 7.4)	4.24 (d, 7.0)	4.29 (dd, 6.8, 3.9)
32	7.21 (br d, 7.6)	7.23 (br d, 9.6)	7.16 (m)	7.20 (db, 9.6)	7.09 (d, 6.9)
34	3.92 (dd, 11.2, 3.6)	3.73 (dd, 11.6, 2.0)	3.93 (dd, 11.7, 4.3)	3.67 (m)	3.73 (dd, 11.3, 3.9)
40	2.44 (d, 5.4)	3.20 (m)	2.45 (d, 5.4)	1.76 (s, 3H)	
	2.95 (d, 5.4)	3.37 (d, 11.2)	2.97 (d, 5.4)		
42	1.32 (d, 6.1, 3H)	1.47 (d, 5.6, 3H)	1.34 (d, 6.2, 3H)	1.82 (d, 7.5, 3H)	
44	1.77 (m)	1.72 (m)	1.97 (m)	1.73 (m)	1.66 (m)
45	1.14 (m)	1.14 (m)		1.20 (m)	1.09 (m)
	1.28 (m)	1.28 (m)		1.28 (m)	1.37 (m)
46	0.87 (t, 7.9, 3H)	0.88 (t, 8.4, 3H)	0.86 (d, 6.2, 3H)	0.86 (t, 8.4, 3H)	0.87 (t, 7.3, 3H)
47	0.83 (d, 7.2, 3H)	0.83 (d, 6.8, 3H)	0.86 (d, 6.2, 3H)	0.84 (d, 6.8, 3H)	0.80 (d, 6.9, 3H)
49	7.95 (br d, 9.7)	7.84 (d, 8.6)	7.91 (br d, 9.5)	7.94 (d, 10.7)	7.70 (d, 9.2)
51	2.76 (dq, 6.8, 4.3)	2.74 (m)	2.76 (m)	2.80 (m)	2.82 (dq, 6.9, 4.4)
52	3.18 (dd, 7.0, 3.5)	3.19 (m)	3.16 (br d, 7.5)	3.28 (m)	3.39 (m)
53	1.62 (m)	1.65 (m)	1.67 (m)	1.69 (m)	1.68 (m)
54	0.96 (d, 6.8, 3H)	0.94 (d, 6.4, 3H)	0.96 (d, 6.5, 3H)	0.99 (d, 6.4, 3H)	0.99 (d, 6.9, 3H)
55	0.88 (d, 6.5, 3H)	0.88 (d, 6.6, 3H)	0.90 (d, 6.2, 3H)	0.94 (d, 6.6, 3H)	0.95 (d, 6.8, 3H)
57	1.38 (d, 6.8, 3H)	1.42 (d, 6.8, 3H)	1.38 (d, 6.8, 3H)	1.37 (d, 8.6, 3H)	1.37 (d, 7.3, 3H)
58	3.46 (br d, 8.6)	3.51 (m)		3.30 (m)	3.53 (br s)
59	1.30 (d, 6.1, 3H)	1.38 (d, 6.4, 3H)	1.28 (d, 6.8, 3H)	1.32 (d, 6.4, 3H)	1.27 (d, 6.9, 3H)
62	4.33 (m)	4.34 (br q, 7.5)	4.33 (m)	4.30 (br q, 7.5)	4.29 (m)
63	1.56 (d, 6.1, 3H)	1.64 (d, 6.0, 3H)	1.56 (d, 6.2, 3H)	1.58 (d, 7.5, 3H)	1.56 (d, 6.4, 3H)
64	5.84 (br d, 1.8)	5.90 (br s)		5.76 (br s)	5.13 (br s)
66	3.34 (dd, 14.4, 11.2)	3.25 (m)	3.33 (br t, 13.0)	3.21 (d, 11.3)	3.15 (dd, 13.7, 11.7)
	3.63 (dd, 14.4, 3.6)	3.42 (m)	3.63 (dd, 14.0, 3.0)	3.54 (dd, 11.3, 2.0)	3.52 (m)
68,72	7.05 (m)	6.91 (m)	7.05 (m)	6.74 (d, 8.0)	6.62 (d, 7.4)
69,71	7.10 (m)	6.97 (m)	7.05 (m)	6.90 (t, 8.0)	6.90 (t, 7.8)
70	7.10 (m)	6.99 (m)	7.05 (m)	7.02 (d, 8.0)	7.02 (m)
73	2.67 (s, 3H)	2.60 (s, 3H)	2.65 (s, 3H)	2.63 (s, 3H)	2.73 (s, 3H)
74				3.72 (s, 3H)	3.90 (s, 3H)

[a] Spectra were obtained at −30 °C and 500 MHz and were recorded in $CDCl_3$. [b] Spectra were obtained at 25 °C and 500 MHz and were recorded in $CDCl_3$. Chemical shifts are reported in δ (ppm). Coupling constants are presented in Hertz. Unless otherwise noted, all proton signals integrate to 1H.

Supplementary Information). This result was corroborated by the identification of L-serine, which was assigned the D configuration in the original communication.

With the structures of **1** and **2** firmly in hand, we turned our attention to the minor salinamides. These salinamides were all isolated by reversed-phase HPLC from the same flash chromatographic fraction that contained **1** and **2**, furnishing salinamides D (**3**), C (**4**), and E (**5**).

Inspection of the NMR spectral data for salinamide D (**3**, Figure 1) suggested that it possessed essentially the same gross structure as **1**. Salinamide D analyzed for a molecular formula of $C_{50}H_{67}N_7O_{15}$ by HRFABMS, which differs from **1** by a methylene group. Careful examination of the ^{1}H NMR spectrum revealed a change in the chemical shifts of H19 from 5.05 to 4.83 ppm and of H44 from 1.77 to 1.97 ppm (Table 1). The methylene protons of C45 were completely missing, leading to the conclusion that the **1** isoleucine unit had been replaced in **3** by a valine residue.

By supplementing the A1 growth media with L-valine (3 g/L), **3** was produced by *Streptomyces* sp. CNB-091 as the major salinamide. The yield of **3** dramatically increased from approximately 0.35 to 10 mg/L, nearly a 30-fold increase. Additional **3** permitted us to completely assign the ^{1}H (Table 1) and ^{13}C (Table 2) NMR data by HMBC and to confirm its structure. The valine β-hydrogen H44 showed HMBC correlations to two methyl carbons [C46 (17.7 ppm) and C47 (19.3 ppm)], as well as the C19 α-carbon (55.8 ppm), thus verifying its structure. The remainder of the HMBC correlations were consistent with those for **1**. CE analysis of the hydrolyzed **3** amino acids derivatized as their *N*-dansylates indicated that the absolute configuration of the valine moiety was D and that the remaining amino acid residues had the same configurations as those in **1**. An additional salinamide

Table 2. Carbon NMR Data for Salinamides A−E (1−5)[a]

no.	1[b]	2[b]	3[b]	4[c]	5[c]
1	169.7	170.2	169.3	170.2	
2	40.8	40.7	40.7	41.5	
4	165.3	165.2	165.2	167.1	
5	120.1	123.3	120.0	116.3	
6	142.9	146.9	143.0	146.9	
7	59.6	81.3	59.8	135.7	
8	78.9	80.8	78.8	133.1	
10	159.3	160.9	159.0	160.2	161.0
11	127.7	128.4	127.7	114.6	115.1
12	125.4	123.0	125.3	130.2	130.0
13	125.0	124.0	124.8	122.8	122.9
14	130.8	131.1	130.8	130.2	130.0
15	120.5	118.1	120.6	114.6	115.1
16	56.4	56.6	56.4	56.6	56.1
18	173.6	173.7	172.9	174.3	174.4
19	53.6	54.3	55.8	54.1	55.0
21	167.5	167.8	167.3	167.9	168.7
22	55.6	56.2	55.6	56.1	56.6
23	72.4	73.6	72.5	72.6	71.4
25	168.6	168.9	168.5	168.6	169.4
26	52.0	53.1	52.0	52.4	55.2
27	65.6	65.5	65.6	65.6	62.7
30	169.1	170.1	169.0	169.1	169.2
31	61.3	61.6	61.3	61.5	61.6
33	168.9	170.1	168.9	169.4	168.8
34	68.7	69.3	68.4	69.9	70.2
36	169.9	170.2	169.4	170.6	171.8
40	55.4	47.9	55.4	14.5	
42	14.7	14.5	14.8	11.8	
44	39.5	39.8	32.8	39.9	39.7
45	26.1	26.3		26.4	26.2
46	11.5	11.5	17.7	11.6	11.7
47	14.2	14.4	19.3	14.2	14.1
50	177.6	177.9	177.4	177.9	177.7
51	41.6	42.1	41.5	42.1	42.1
52	79.4	79.7	79.6	79.5	79.5
53	32.2	32.4	32.3	32.2	32.2
54	18.6	18.5	18.3	18.2	18.1
55	19.7	19.9	19.8	19.8	19.7
57	16.7	16.7	16.8	16.7	16.6
59	15.6	15.8	15.2	15.8	15.9
62	68.1	68.9	68.0	68.6	68.3
63	21.1	21.4	21.4	21.2	21.3
66	34.6	34.8	34.5	34.1	33.5
67	137.1	137.7	136.8	137.9	137.5
68,72	128.8	129.2	128.8	129.0	128.9
69,71	128.4	128.5	128.4	128.3	128.5
70	126.6	126.4	126.7	126.4	126.5
73	40.1	40.0	40.1	40.0	40.4
74				54.9	55.6

[a] Spectra taken in $CDCl_3$ at 125 MHz; chemical shifts are reported in ppm from TMS. [b] Spectra taken at −30 °C. [c] Spectra taken at 25 °C.

that was tentatively assigned as the chlorohydrin of **3** was also observed in the valine supplementation experiment but was inseparable from **1** by normal and reversed-phase HPLC.

Salinamide C (**4**), $C_{52}H_{73}N_7O_{14}$, is a monocyclic depsipeptide that was isolated only once from a 12 L fermentation (Scheme 1). The most notable feature of its NMR spectrum was its clarity at room temperature, and thus all of the NMR data (Tables 1 and 2) for **4** were acquired at 25 °C. Indeed, the restricted alkoxyphenylglycine ring in **1**, **2**, and **3** is relaxed in **4**, as the C8−C10 ether connectivity is broken, rendering **4** monocyclic and the aromatic ring free to rotate. Structural differences between **4** and **1** were limited to the alkoxyphenylglycine moiety of **1**: the epoxide signals were absent and additional olefinic and methoxy signals were present. The methoxy singlet (3.72 ppm) showed an HMBC correlation to the quaternary aromatic C10 carbon, establishing the methyl ether connectivity in the *p*-methoxyphenylglycine

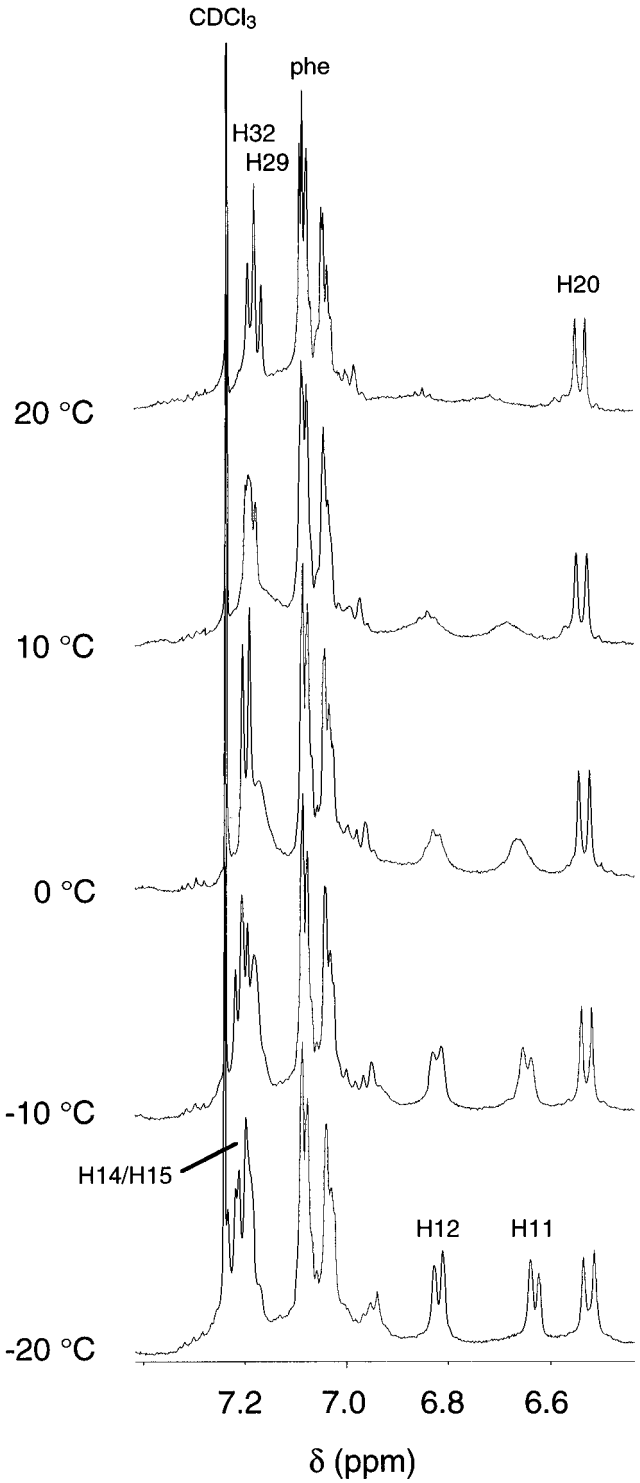
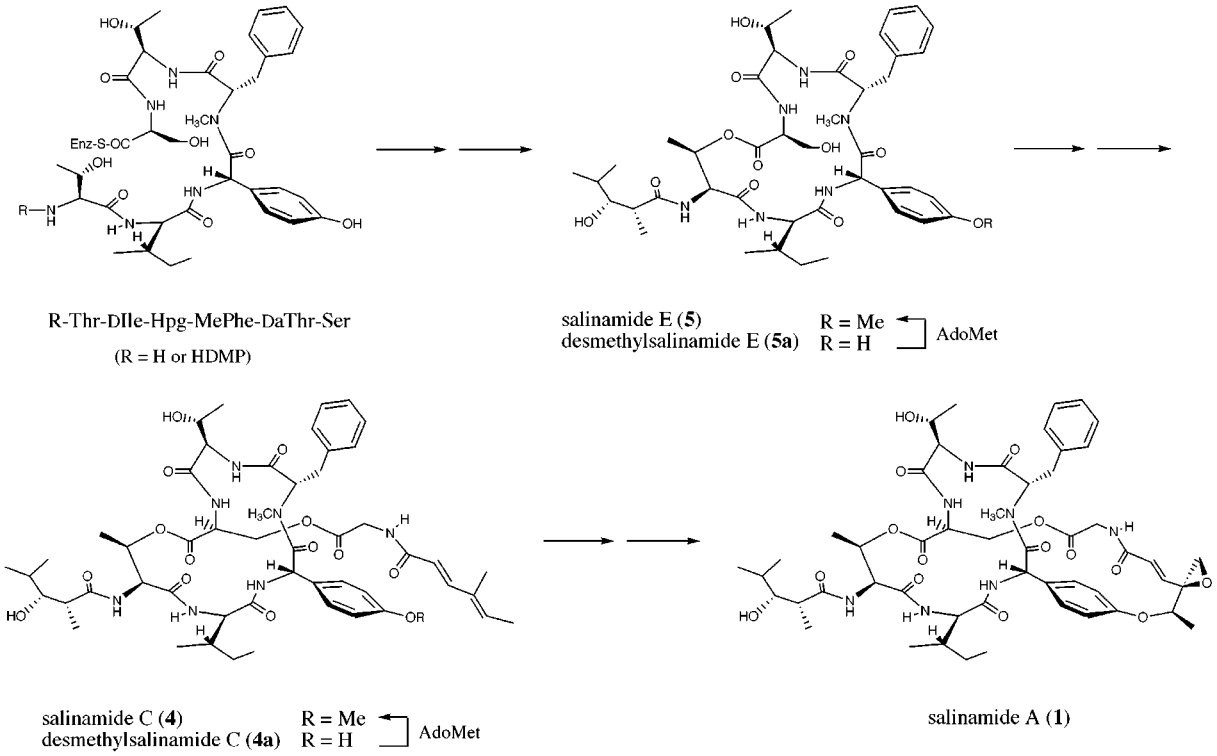


Figure 2. Variable-temperature ^1H NMR spectra of salinamide A (**1**) in $CDCl_3$ from 20 to −20 °C (10 °C intervals) showing the sharpening of the *p*-alkoxyphenylglycine aromatic signals (H11−H15) as the temperature is lowered. At 20 °C, these aromatic signals are barely visible as broad humps but become strong signals (6.62, 6.82, and 7.19 ppm) at −20 °C. The H11 (6.62 ppm) and H12 (6.82 ppm) AB doublets (8 Hz) are resolved in the −20 °C spectrum, whereas the H14 and H15 signals are strongly coupled at 7.19 ppm. In the remainder of the partial spectrum, the H29 amide doublet shifts downfield as the temperature is lowered (7.19 → 7.23 ppm), whereas the H32 (7.21 ppm) and H20 (6.51 ppm) amide doublets and the phenylalanine aromatic protons (7.05−7.10 ppm) remain relatively unchanged.

residue of **4**. The 4-methylhexa-2,4-dienoyl unit attached

Scheme 1. Structures of Salinamides C (4) and E (5) and the Proposed Biosynthesis of Salinamide A (1) via the Hypothetical Intermediates Desmethylsalinamides E (5a) and C (4a)



to the glycine residue through an amide linkage was established on the basis of ^{1}H NMR chemical shifts, coupling constants, and key HMBC correlations between the H40 (singlet at 1.76 ppm) and H42 (doublet at 1.82 ppm) methyls to the olefinic C7 (135.7 ppm) and C8 (133.1 ppm) carbons. The 2*E*,4*E* geometry of the diene residue was based on the large coupling of the trans C5/C6 olefinic hydrogens (14 Hz) and the large upfield ^{13}C NMR shifts of the C40/C42 methyl carbons (Table 2), exhibiting γ effects of the cis methyl groups upon each other. The sequence of the remaining amino acids and the seven-carbon C50−C57 moiety in **4** was found to be identical with those in **1** by an HMBC experiment.

Salinamide E (**5**) was analyzed by HRFABMS for $C_{43}H_{62}N_6O_{12}$. Several features were immediately apparent by ^{1}H (Table 1) and ^{13}C (Table 2) NMR. The epoxide and olefinic signals of **1** were completely missing, the aromatic signals were sharp at room temperature (indicating that **5** is likely monocyclic), and a methoxy signal, such as that in **4**, was present (C74: δ_C 55.6, δ_H 3.90). Most of the correlations in the HMBC spectrum of **5** were analogous to those of **1**, except for the correlations of the C74 methoxyl (3.90 ppm) to C10 and of the exchangeable proton at 4.60 ppm to C26. These data indicated, respectively, that the hydroxyphenylglycine (Hpg) residue was methylated as in **4** and that the serine hydroxyl was free. Thus, the entire section from C1 to C8, including the glycine residue and the seven-carbon α,β-unsaturated amide, is absent in **5**.

A sixth salinamide, which has the same structural relationship to salinamide E (**5**) as **3** has to **1**, was isolated in low yield. Proton NMR data clearly showed the presence of a valine unit in place of the isoleucine residue. Epoxide and olefinic signals were also missing in the NMR spectrum of this salinamide, and instead, methoxy and serine hydroxy signals that were observed for **5** were present. Complete structural analysis of this salinamide, however, was hampered as a result of low sample concentration.

Salinamides A and B were reported in the original communication to exhibit potent topical antiinflammatory activity and moderate antibiotic activity against Gram-positive bacteria.[9] Topically applied **1** and **2** effectively inhibited phorbol myristate acetate-induced edema in mouse ears at levels comparable to the nonsteroidal antiinflammatory agent indomethacin.[11] At the standard testing dose of 50 μg/ear, **1** and **2** respectively showed 84% and 83% inhibition of edema, whereas indomethacin showed a 72% inhibition. In addition, **1** and **2** exhibited moderate antibiotic activity against Gram-positive bacteria, including *Streptococcus pneumoniae* and *Staphylococcus pyrogenes* with MIC values of 4 μg/mL for **1** and 4 and 2 μg/mL for **2**. We are currently in the process of isolating sufficient quantities of the minor salinamides and of generating unnatural salinamide analogues through precursor-directed biosynthesis to evaluate their antiinflammatory properties.

The peptide core of **1** is probably derived from the hexapeptide Thr-DIle-Hpg-MePhe-DaThr-Ser (Scheme 1). Its biosynthesis probably involves a nonribosomal peptide synthetase pathway,[12,13] as the hexapeptide core contains two D-amino acids, an *N*-methyl amino acid, and the nonproteinogenic Hpg unit. This hypothesis is supported by the fact that the D-isoleucine unit of **1** was replaced with a D-valine residue to give **3** in the L-valine supplementation experiment. The proposed biosynthetic pathway proceeds through the hypothetical intermediates desmethylsalinamides E (**5a**) and C (**4a**). We speculate that the naturally occurring **4** and **5** are dead-end

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products of **4a** and **5a** whose Hpg phenolic groups have been methylated by *S*-adenosyl-L-methionine (AdoMet), thus preventing further conversion to **1**. We have recently determined the basic building blocks of **1** through feeding experiments with ^{13}C-labeled biosynthetic intermediates[14] and have cloned and sequenced a nonribosomal peptide synthetase from *Streptomyces* sp. CNB-091.

Experimental Section

Culture Conditions and Extraction. *Streptomyces* sp. strain CNB091[15] was isolated from a surface swab of a jellyfish (*C. xamachana*) collected in the Florida Keys in 1987. Fermentation 1: isolate CNB091 was cultured in a 12 L Virtis glass fermentor at 25 °C for 3 days in A1 medium consisting of 1% starch, 0.4% yeast extract, 0.2% peptone, 75% seawater, and 25% deionized water, buffered at pH 8.0 with 10 mL of 1.0 M Tris buffer. The entire fermentation broth was then double extracted with EtOAc. The extracts were combined and dried over Na_2SO_4, filtered, and evaporated in vacuo to yield 1.2 g crude organics. Fermentation 2: culture of CNB091 was scaled up from 10 mL (4 d) to 1 L (1 d) to 50 L (1 d) in the same media in a New Brunswick Scientific Fermatron fermentor with the following settings: air, 1.8 L/min; impellor speed, 200 rpm. The culture was filtered with Celite to remove the cells and then extracted using C18 resin, 1 L resin/5 L broth. The resin was eluted with a total of 20 L MeOH. The MeOH was evaporated in vacuo to approximately 500 mL and then partitioned between H_2O and $CHCl_3$. The dried $CHCl_3$ fraction was then partitioned again between MeOH and isooctane, yielding 3.4 g crude organics in the MeOH fraction.

Isolation of Salinamides A−E. The crude extracts were subjected to silica vacuum flash chromatography with isooctane/EtOAc mixtures followed by EtOAc/MeOH mixtures. The salinamide-containing fractions (100% EtOAc and 95:5 EtOAc/MeOH) were then purified by reversed-phase HPLC (C18 Dynamax, 10 mm × 250 mm or 25 mm × 500 cm, UV detection at 254 nm) using 75:25 MeOH/H_2O as the eluant. The large fermentation yielded salinamides A (**1**, 276.0 mg), B (**2**, 81.0 mg), D (**3**, 14.9 mg), and E (**5**, 20.3 mg). Salinamide C (**4**, 9 mg) was only isolated from the 12 L fermentation.

Salinamide A (**1**) was obtained as a pale yellow noncrystalline solid (12 mg/L fermentation yield): $[\alpha]_D$ −26° (*c* 0.97, $CDCl_3$); mp 221−225 °C dec; IR ($CDCl_3$) 3436, 3379, 1745, 1735, 1682, 1657, 1636 cm^{-1}; UV (MeOH) 281, 267, 224, 208 nm; HRFABMS *m/z* 1020.492 ($M^+ + H$) ($C_{51}H_{70}N_7O_{15}$, $\Delta = 0.98$ ppm); see Tables 1 and 2 for ^1H and ^{13}C NMR spectral data, respectively.

Salinamide B (**2**) was obtained as an off-white crystalline solid (12 mg/L fermentation yield): $[\alpha]_D$ −65° (*c* 0.57, $CDCl_3$); mp 239−241 °C melts; IR ($CDCl_3$) 3445, 3347, 1745, 1734, 1683, 1651, 1636 cm^{-1}; UV (MeOH) 282, 268, 227, 209 nm; HRFABMS *m/z* 1056.470 ($M^+ + H$) ($C_{51}H_{70}N_7O_{15}Cl$, $\Delta = -1.89$ ppm); see Tables 1 and 2 for ^1H and ^{13}C NMR spectral data, respectively.

Salinamide D (**3**) was obtained as a pale yellow noncrystalline solid (0.35 mg/L fermentation yield): $[\alpha]_D$ −54.4° (*c* 0.853, $CDCl_3$); IR ($CDCl_3$) 3425, 3331, 1737, 1672, 1643, 1543, 1502, 756 cm^{-1}; HRFABMS *m/z* 1006.476 ($M^+ + H$) ($C_{50}H_{68}N_7O_{15}$, $\Delta = -1.7$ ppm); see Tables 1 and 2 for ^1H and ^{13}C NMR spectral data, respectively.

Salinamide C (**4**) was obtained as an off-white noncrystalline solid (0.75 mg/L fermentation yield): HRFABMS *m/z* 1020.525 ($M^+ + H$) ($C_{52}H_{74}N_7O_{14}$, $\Delta = -4.3$ ppm); see Tables 1 and 2 for ^1H and ^{13}C NMR spectral data, respectively.

Salinamide E (**5**) was obtained as an off-white noncrystalline solid (0.5 mg/L fermentation yield): $[\alpha]_D$ −93.4° (*c* 1.35, $CDCl_3$); IR ($CDCl_3$) 3389, 3354, 1731, 1672, 1643, 1537, 1508, 750 cm^{-1}; HRFABMS *m/z* 855.454 ($M^+ + H$) ($C_{43}H_{63}N_6O_{12}$, $\Delta = 3.6$ ppm); see Tables 1 and 2 for ^1H and ^{13}C NMR spectral data, respectively.

Acid Hydrolysis of 1 and 3. CE Analysis. Salinamides A (3 mg) and D (3 mg) were each dissolved in 3 M HCl (1 mL) and refluxed for 24 h. The freeze-dried hydrolysate in H_2O (1 mL) was adjusted to pH 8.5 with solid $NaHCO_3$ and treated with dansyl chloride (1 mg) in acetone (1 mL) at room temperature for 2 h.

CE analysis was performed on a Beckman P/ACE 5010 Series capillary electrophoresis system (UV detection at 214 nm, 14 °C). Polarity was reversed (cathode as inlet) on a 37 cm neutral-coated capillary using 10−15 mM β-cyclodextrin in High pH Buffer (eCAP Chiral Kit, Beckman). Samples were introduced by pressure injection, and standards were introduced by sequential co-injection. A separation voltage of 25 kV was applied for 20 min. Between injections, the column was prepared by sequentially rinsing with 1 N HCl, H_2O, and run buffer and then was further equilibrated by applying a 100 V/cm (3.7 kV) voltage for 5 min at room temperature. At 15 mM β-cyclodextrin concentration, the retention times for the dansyl amino acid standards relative to dansyl chloride were 2.8 min for dansyl L-*allo*-threonine, 3.0 min for dansyl L-threonine, 3.2 min for dansyl D-*allo*-threonine, 3.5 min for dansyl D-threonine, 3.8 min for dansyl L-serine, 4.2 min for dansyl D-serine, 5.2 min for dansyl L-isoleucine, and 6.2 min for dansyl d-isoleucine. The retention times for the dansylated amino acids from the **1** hydrolysate were 2.6, 3.0, 3.2, 3.8, 6.2, and 9.2 min. At 10 mM β-cyclodextrin concentration, the standard retention times were 6.1 min for dansyl L-valine and 7.0 min for dansyl D-valine relative to the dansyl chloride standard. Correspondingly, the retention time for dansyl D-valine from the **3** hydrolysate was 7.0 min.

Conversion of 1 to 2. Salinamide A (9.5 mg) was dissolved in THF and H_2O and stirred under N_2. Next, 6 N HCl was added dropwise, and the reaction was monitored by TLC for the disappearance of **1** (2 h). The organic products were back-extracted with $CHCl_3$ and then purified by reversed-phased HPLC. According to the rotation ($[\alpha]_D$ −56° (*c* 0.05, $CHCl_3$)) and the ^1H NMR spectrum of the single purified product, the reaction yielded **2** exclusively (20% overall with 80% starting material).

Production of 3 from L-Valine Enriched Culture Medium. *Streptomyces* sp. CNB091 was cultured in 1 L A1 media supplemented with 3 g L-valine (100 mL media/500 mL Erlenmeyer flask) for 3 d on a rotary shaker at 28 °C and 200 rpm. Salinamide D (**3**, 10 mg) was isolated as indicated above as the major salinamide in this fermentation.

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Supporting Information Available: Tables of NMR data of **1-5** in $CDCl_3$, ^1H NMR spectra for **1-6**, ^{13}C NMR and HMQC spectra for **1-5**, HMBC spectra for **1** and **3-5**, COSY spectra for **1** and **2**, ROESY spectra for **1** and selected CE electropherograms of the dansylated amino acids of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO9814391

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(15) TLC analyses of whole cell hydrolysates of CNB091 indicated the presence of the LL isomer of diaminopimelic acid and the sugars glucose and ribose, which are diagnostic for the genus *Streptomyces*. Fatty acid analysis (Microbial ID, Inc., Newark, DE) supported this identification with a similarity index of 0.44.