Inhibition of Bacterial RNA Polymerases. Peptide Metabolites from the Cultures of *Streptomyces* sp.

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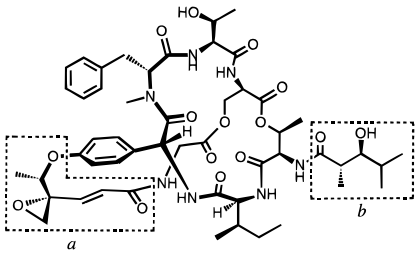
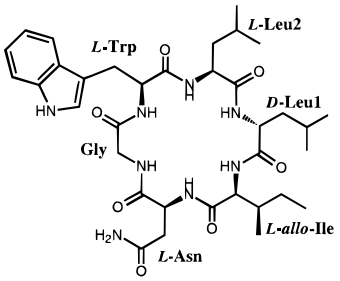
The fermentation culture of a soil strain of *Streptomyces* has been chemically investigated for its inhibitory activity against RNA polymerases from the bacteria *Escherichia coli* and *Staphylococcus aureus*. The active component was found to be the unusual bicyclodepsipeptide salinamide A (**1**) recently discovered from a marine Streptomycete. The same extract also yielded a new cyclohexapeptide natural product, desotamide (**2**), the structure elucidation of which was based on amino acid analysis, detailed spectroscopic data interpretation, and peptide synthesis.

Although there are a large number of antibiotics currently in clinical use, many of them are becoming less effective as a result of drug resistance developed by some pathogenic strains of bacteria.[1] One such antibiotic is rifampin (rifampicin), an antituberculosis drug derived from rifamycins, which exhibits its antibacterial activity through inhibition of the bacterial RNA polymerase.[2] Mutations affecting the β subunit of RNA polymerase can confer bacterial resistance to rifampin.[3] In our effort to search for new bacterial RNA polymerase inhibitors that may be used as broad-spectrum antibiotics, we have chemically investigated the bioactive organic extract of the liquid culture of a *Streptomyces* strain (NRRL 21611) isolated from a soil sample collected in DeSoto Falls, GA. The polymerase inhibitory component in this extract was found to be salinamide A (**1**), a recently-reported[4] unusual bicyclic depsipeptide from a Streptomycete that was found on the surface of a marine jellyfish. From the extract of this Georgia soil strain, we also isolated a new cyclohexapeptide, which we gave the name desotamide (**2**). In this paper, we report the bioactivity of salinamide A and the structure elucidation of desotamide.



Salinamide A (1)



Desotamide (2)

Bioassay-guided fractionation of the crude EtOAc extract of the bacterial culture on C18 silica gel (flash column and repeated HPLC) yielded pure, active compound **1**. The FABMS spectrum of **1** revealed a molecular weight of 1020. The ^{1}H NMR spectrum in DMSO-d_6 clearly suggested the depsipeptide nature of **1**. The ^{13}C NMR spectrum showed 51 carbons (9 × CH_3, 5 × CH_2, 13 × CH, 1 × C, 11 × =CH (only 4 signals were observed for 8 of the 11 =CH's due to local phenyl ring symmetries), 3 × =C, 9 × C=O). Extensive structural studies by using COSY, HMQC, HMBC, and NOE experiments, combined with an online literature search, revealed that **1** was salinamide A. All spectroscopic aspects of compound **1** were in agreement with the literature data reported recently by Fenical *et al.*[4] The final confirmation of the identity of compound **1** was provided by its HPLC coelution with an authentic sample of salinamide A. Some structural features in salinamide A, such as the 5-hydroxy-5-methyl-4-oxiranylpentenoyl residue (fragment a) and the 3-hydroxy-2,4-dimethylpentanoyl residue (fragment b), are unprecedented among natural products. Our report represents the first finding of this unusual depsipeptide from a terrestrial source. In our polymerase assay, salinamide A exhibited strong inhibitory activity (IC_{50} = 0.5 μM) against bacterial polymerases while not affecting the human polymerase.

Desotamide (**2**) was isolated through repeated reverse-phase chromatography as a white amorphous solid. The HRFABMS revealed a molecular formula of $C_{35}H_{52}N_8O_7$, requiring 14 sites of unsaturation. The ^{1}H NMR spectrum in DMSO-d_6 indicated that **2** was an oligopeptide rich in leucines/isoleucines. The best dispersion of the amide signals and aromatic signals was achieved in DMSO-d_6 at 35 °C. Among the eight amide signals, there were one triplet attributed to the Gly-NH, five doublets, and two mutually coupled broad singlets attributed to a C(O)NH_2 group. The ^{13}C NMR spectrum showed all of the 35 carbons (6 × CH_3, 6 × CH_2, 8 × CH, 5 × =CH, 3 × =C, 7 × C=O). Signals for seven carbonyls were observed between 169 and 173 ppm.

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Table 1. NMR Data for Desotamide (**2**)[a]

	no.	δ_H (400 MHz)	δ_C (100 MHz)	COSY
Trp	1		170.9	
	2	4.36m	55.4	H3a,b, 2NH
	3	a: 3.13dd, 14.7, 5.0	27.3	H2
		b: 2.96dd, 14.7,10.1		
	2-NH	8.24d, $J = 8.3$		H2
	1′ (NH)[b]	10.76s		
	2′	7.11s	123.5	
	3′		110.0	
	3′a		127.1	
	4′	7.49d, $J = 7.7$	118.1	H5′
	5′	6.96t, $J = 7.7$	118.3	H4′,6′
	6′	7.04t, $J = 7.7$	120.9	H5′,7′
	7′	7.30d, $J = 8.1$	111.3	H6′
	7′a		136.1	
Gly	1		169.1	
	2	a: 3.89dd, 15.8,6.2	43.3	H2b, NH
		b: 3.28dd, 15.8,4.6		H2a, NH
	NH	7.90t, $J = 5.5$		H2a,b
Asn	1		171.0	
	2	4.56m	49.4	H3a,b
	3	a: 2.79dd, 16.0, 5.5	36.9	H2, 3b
		b: 2.61dd, 16.0, 5.5		H2, 3a
	4		172.0	
	2-NH	7.59d, $J = 8.4$		H2
	NH_2	7.56s, 7.00s		
Ile	1		171.0	
	2	4.16dd, $J = 7.5,4.5$	56.6	H3, NH
	3	2.00h, $J = 7$	35.2	H2, H4, 3-Me
	4	1.2m	25.6	H3, 5
	5 (Me)	0.81t (3H), $J = 7$	11.5	H4
	3-Me	0.82d (3H), $J = 7$	14.5	H3
	NH	8.19d, $J = 7.5$		H2
Leu1	1		173.3	
	2	4.34m	51.8	H3, NH
	3	1.45m[d]	39.3	
	4	1.54m[d]	24.2	
	5 (Me)	0.82d[c], $J = 7$	22.1[c]	H4
	4-Me	0.82d[c], $J = 7$	22.4[c]	H4
	NH	8.20d, $J = 6$		H2
Leu2	1		171.9	
	2	4.35m	50.8	H3, NH
	3	1.45m[d]	41.4	
	4	1.54m[d]	24.4	
	5 (Me)	0.88d[c], $J = 7$	22.5[c]	H4
	4-Me	0.88d[c], $J = 7$	22.6[c]	H4
	NH	7.65d, $J = 7.8$		H2

[a] Recorded in DMSO-d_6 at 35 °C. [b] 1′−7a′: the standard numbering system for indoles, with 1′ being the NH. [c,d] Assignments may be interchanged within the same column.

Standard amino acid analysis revealed that **2** contained 2 × Leu, 1 × Ile, 1 × Gly, 1 × Asp/Asn[5]. A tryptophan residue that was visible in the ^{1}H NMR spectrum was not detected in the amino acid analysis as it did not survive the acid hydrolysis.

A combination of TOCSY and COSY experiments readily identified the spin systems of six amino acid residues present in **2**, including the tryptophan not detected by amino acid analysis. The ambiguity between Asp and Asn was resolved by analysis of the HMBC data; the fact that the two mutually coupled broad singlets showed HMBC correlations to the C3 (δ 36.9) of the Asp/Asn system suggested that this residue was an asparagine (Asn). A combination of HMQC and HMBC experiments was used to complete the assignment of carbon chemical shifts in each amino acid (aa) residue. These ^{1}H and ^{13}C NMR assignments (Table 1) agree well with the literature data for aa residues in peptides.

The sequence of the cyclohexapeptide was established by analysis of the NOE data and HMBC correlations between the amide proton in aa residue *n* and the carbonyl carbon of aa residue $n + 1$ (Figure 1). The two-bond HMBC correlations from Ile-NH (δ 8.19) to Leu1-C1 (δ 173.3), from Leu1-NH (δ 8.20) to Leu2-C1 (δ 171.9), from Leu2-NH (δ 7.65) to Trp-C1 (δ 170.9), and from Trp-NH (δ 8.24) to Gly-C1 (δ 169.1) clearly established the linear Ile-Leu1-Leu2-Trp-Gly sequence. The overlapping ^{13}C signals for Asn-C1 and Ile-C1 prohibited the proper placement of Asn based on the HMBC data. However, the nuclear Overhauser enhancements between Gly-NH and Asn-H2 as well as from Asn-NH to Leu-H2 unequivocally placed Asn between Gly and Leu, therefore establishing the complete cyclic sequence of desotamide. This established sequence is also in complete agreement with the other observed NOE correlations.

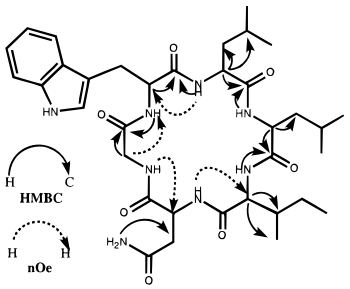

Figure 1. Key HMBC and NOE correlations for desotamide (**2**).

Marfey's method[6] was employed to determine the absolute stereochemistry of desotamide. After hydrolysis of desotamide with 5% thioglycolic acid[7] in 6 N HCl, the liberated amino acids were derivatized with L-FDAA (Marfey's reagent) and analyzed on HPLC. Retention time comparison with the L-FDAA-derivatized amino acid standards indicated that desotamide contained L-Trp, L-Asn, L-Ile, L-Leu and D-Leu. Since both L-Leu and D-Leu were present in the same molecule, the unambiguous assignments of the absolute configurations of Leu1 and Leu2 were resorted to the total syntheses of the two possible stereoisomers (L-Leu1/D-Leu2 and D-Leu1/L-Leu2).

Two linear peptide stereoisomers (Gly-L-Trp-L-Leu2-D-Leu1-L-Ile-L-Asn and Gly-L-Trp-D-Leu2-L-Leu1-L-Ile-L-Asn) were synthesized using solid-phase Fmoc chemistry with HBTU/HOBt[8] activation.[9,10] The linear peptides were cyclized head to tail on the resin using DIC/HOBt activation and coupling methodology[10] to produce the two cyclic hexapeptide stereoisomers (**3**, with L-Leu2-D-Leu1; **4**, with D-Leu2-L-Leu1). When analyzed on reverse phase HPLC, stereoisomer **3** and desotamide (**2**) showed identical retention times and coeluted as one single peak when the two compounds were co-injected. On the contrary, stereoisomer **4** displayed a HPLC retention time different from that of desotamide. However, the ^{1}H and ^{13}C NMR data of stereoisomer **3** showed some slight difference from those of desotamide in the Ile signals. This evidence combined with the possibility that the L-Ile in desotamide may actually be an L-*allo*-Ile[11] prompted us to synthesize the cyclic hexapeptide containing L-Leu2-D-Leu1-L-*allo*-Ile (**5**). The fact that stereoisomer **5** displayed identical chromatographic and spectroscopic properties as desotamide (**2**) unequivocally established the absolute configurations of the two leucine residues and the Ile residue in desotamide as L-Leu2, D-Leu1, and L-*allo*-Ile.

Naturally occurring cyclic hexapeptides are not very common. The two dozen or so natural cyclic hexapep-

tides can be categorized into three major groups. The majority belong to a group of siderophores[12−14] produced by *Aspergillus*, *Ustilago*, *Cryptococcus*, and *Micromonospora* species. Another group of cyclic hexapeptides, composed nearly exclusively of alanine residues and derivatives of tyrosine, are mostly found in the roots of *Rubia* species.[15−19] The third type, with L-156,373 as the sole member, has been found in *Streptomyces silvensis* as an oxytocin antagonist.[20] Desotamide is one of the very few cyclic hexapeptides from *Streptomyces* spp. and does not appear to belong to any of these three known groups of cyclic hexapeptides.

Desotamide (**2**) did not display any inhibitory activity in our bacterial RNA polymerase assay.

An inactive compound that was dominant in the crude extract of this Streptomycete was found to be herbicidin A, a polyether-type adenosine analog with herbicidal and antibacterial activities, which was isolated previously from *Streptomyces saganonensis*.[21]

Experimental Section

General Experimental Procedures. All NMR data were recorded on a Varian Gemini 400 NMR spectrometer, at 400 MHz for ^{1}H and 100 MHz for ^{13}C. A microtube from Shigemi Inc.[22] (Allison Park, PA), which employs a special glass material matching the magnetic susceptibility of the NMR solvent in use and enhances the NMR sample concentration by approximately 3-fold, was used for preparing the ^{13}C NMR samples. Analytical HPLC was performed on a Hewlett-Packard 1050 system equipped with a quaternary pump and a diode array detector, while the semipreparative HPLC was performed on a Waters 600 system equipped with a Waters 490E multiple wavelength detector. Standard amino acid analysis was done using the PTC amino acid analysis method on an Applied Biosystems Model 420H amino acid analyzer with online acid hydrolysis. FAB-MS and electrospray MS were performed on a VG Analytical ZAB 2-SE system and a VG Fison Platform single quadrupole system, respectively. An Applied Biosystems Model 394 DNA synthesizer was converted to perform the solid-phase peptide synthesis. The key chemicals used in the peptide synthesis were purchased from these commercial suppliers: Fmoc-Asp-ODmab, Novabiochem; HBTU, Anaspec Inc.; HOBt and DIC, Applied Biosystems; triisopropylsilane, Fluka.

Bacterial Material. The *Streptomyces* strain was isolated from a soil sample collected in DeSoto Falls, GA, identified by Dr. Dwight Baker and Mr. Vaughn Stienecker, and deposited as NRRL 21611 under the Budapest Treaty at the USDA National Center for Agricultural Utilization Research. The bacterium was cultured in 3.6 L (30 mL × 120) liquid media (glucose−sucrose−pharmamedia−L-histidine−$NaNO_3$−K_2HPO_4−KCl−$MgSO_4$·$7H_2O$, 20−50−20−1−0.5−0.7−0.014 g/L, pH 7.0) at 28 °C for 6 days.

Extraction and Isolation. The bacterial culture was extracted with ethyl acetate. Half of the organic extract was subjected to chromatography on a C18 flash column (60−100% MeOH/H_2O). The polymerase inhibitory activity was found mainly in fraction 6 (95% MeOH/H_2O) and, to a lesser degree, in fraction 3 (80% MeOH/H_2O). Fraction 6 contained mostly fat and was therefore discarded. Fraction 3 was subjected to chromatography on a preparative scale C18 HPLC column (21 × 250 mm, 0−100% MeOH/H_2O), and two of the resulting major peaks were individually further separated on a semi-preparative C18 HPLC column (10 × 250 mm, 0−60% CH_3CN/H_2O) to give pure salinamide A (**1**, 11 mg) and desotamide (**2**, 4 mg). Herbicidin A, the dominant component (65 mg) in the crude extract, was obtained from C18 HPLC separation (0−70% MeOH/H_2O, eluting at 50% MeOH) after the C18 flash column.

Bacterial RNA Polymerase Assay. Details of the RNA polymerase assay using the *E. coli* RNA polymerase and a G-less supercoiled DNA template have been published by us recently.[23] Fractions and the purified compounds were assayed either manually or using a Zymark XP robot.

Desotamide (2): amorphous powder; UV (MeOH) λ_{max} 223 (17 000), 281 (4200), 290 (3400) nm; IR ν_{max} 3258, 1631, 1070 cm^{-1}; $[\alpha]_D = -6.7°$ (*c* 0.35, MeOH); FABMS: *m*/*z* 719 (100, MNa^+), 697 (60, MH^+); HR-FABMS on the MH^+ peak 697.3992, calcd 697.4037 for $C_{35}H_{53}N_8O_7$; ^{1}H NMR and ^{13}C NMR, see Table 1.

Hydrolysis of Desotamide (2) and HPLC Analysis of the Marfey's Derivatives. About 100 μg of desotamide was mixed with 100 μL of 5% thioglycolic acid/6 N HCl. The mixture was degassed with N_2 and heated at 110 °C overnight in a tightly capped reaction vial. After cooling, the solvent was removed under N_2, and the majority of thioglycolic acid was removed under high vaccum overnight. The remaining residue was mixed with 50 μL L-FDAA solution (10 mg/mL in acetone) and 100 μL 1 M $NaHCO_3$ solution. The mixture was heated at 80 °C for 5 min in a tightly capped reaction vial. After cooling, 50 μL of 2 N HCl and 500 μL of 1:1 CH_3CN/H_2O were added. The mixture was analyzed on HPLC (Inertsil ODS3 4.6 × 150 mm, 0−50% CH_3CN/50 mM $(NH_4)_2CO_3$, $\lambda = 340$ nm). Each HPLC peak was identified by comparing its retention time and UV spectrum with those of the L-FDAA derivative of the pure amino acid standard.

Syntheses of Cyclic Hexapeptides 3, 4, and 5. *N*-α-Fmoc-L-aspartic acid α-Dmab ester was attached to Rink-MBHA-Amide Resin via the side-chain carboxylic acid of Asp. After the linear chain was synthesized on the resin on an automatic synthesizer with the relevant amino acids using solid-phase Fmoc chemistry with HBTU/HOBt activation,[9,10] the Dmab protecting group on the C-terminus of the aspartic acid was removed by 2% hydrazine in dimethylformamide.[24] The linear peptides were cyclized head to tail on the resin using DIC/HOBt activation and coupling methodology.[10] The cyclic peptides were cleaved from the solid support and deprotected using a cocktail of 2% triisopropylsilane, 2% water, and 96% trifluoroacetic acid for 2 h at room temperature. The peptides were purified on reverse phase HPLC (0.1% TFA/CH_3CN/H_2O). HPLC retention times on reverse phase HPLC (Inertsil ODS3 4.6 × 150 mm, 0−100% CH_3CN/H_2O): $t_R = 13.8$ min for compounds **3**, **5**, and desotamide (**2**), $t_R = 13.5$ min for compound **4**.

Cyclic hexapeptide 5: obtained as a white amorphous powder; electrospray MS $MH^+ = 697$; ^{1}H NMR (DMSO-d_6, 35 °C) δ 10.80 (s), 8.25 (d, 8.2), 8.20 (d, 7.5), 8.18 (d, 6), 7.90 (t, 5), 7.67 (d, 7.5), 7.60 (d, 8), 7.58 (s), 7.49 (d, 7.7), 7.30 (d, 8.1), 7.11 (s), 7.04 (t, 7.7), 7.00 (s), 6.96 (t, 7.7), 4.56 (m), 4.35 (m, 3H), 4.16 (dd, 7.5, 4.5), 3.88 (dd, 15.8, 6.0), 3.28 (dd, 15.8, 4.5), 3.13 (dd, 14.7,

5.0), 2.97 (dd, 14.7, 10.0), 2.78 (dd, 16.0, 5.5), 2.62 (dd, 16.0, 5.5), 2.00 (m), 1.45 (m, 6H), 1.25 (m), 1.15 (m), 0.88 (d, 6H), 0.82 (d, 9H), 0.81 (t, 3H) ppm.

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