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Sungsanpin, a Lasso Peptide from a Deep-Sea Streptomycete

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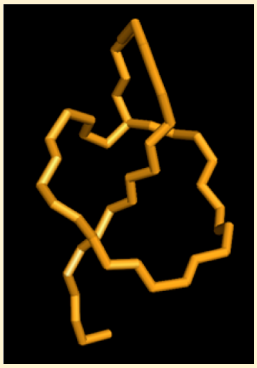
Supporting Information

ABSTRACT: Sungsanpin (**1**), a new 15-amino-acid peptide, was discovered from a *Streptomyces* species isolated from deep-sea sediment collected off Jeju Island, Korea. The planar structure of **1** was determined by 1D and 2D NMR spectroscopy, mass spectrometry, and UV spectroscopy. The absolute configurations of the stereocenters in this compound were assigned by derivatizations of the hydrolysate of **1** with Marfey's reagents and 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl isothiocyanate, followed by LC-MS analysis. Careful analysis of the ROESY NMR spectrum and three-dimensional structure calculations revealed that sungsanpin possesses the features of a lasso peptide: eight amino acids (-Gly[1]-Phe-Gly-Ser-Lys-Pro-Ile-Asp[8]-) that form a cyclic peptide and seven amino acids (-Ser[9]-Phe-Gly-Leu-Ser-Trp-Leu[15]) that form a tail that loops through the ring. Sungsanpin is thus the first example of a lasso peptide isolated from a marine-derived microorganism. Sungsanpin displayed inhibitory activity in a cell invasion assay with the human lung cancer cell line A549.



Marine microorganisms are widely recognized as prolific and relatively unexplored sources of structurally and biologically novel natural products.[1,2] Deep-sea habitats (>50 m depth as defined by Skropeta[3]) that are far from seashores and shallow waters are even less explored and potentially harbor phylogenetically and chemically novel microbes.[3] Despite the difficulty of collecting deep-sea microbes, pioneering chemical analyses have shed light on the biomedical potential of deep-sea microorganisms.[4] One example of the potential of deep-sea microorganisms is a new cytotoxic phenazine derivative from a *Bacillus* strain isolated at a depth of 5059 m.[5] Investigation of marine bacteria isolated from a deep-sea sediment core (~980 m below sea level) yielded the macrolactins, a novel class of antiviral and cytotoxic macrolides.[6] A *Streptomyces* strain from a sediment collected at a depth of 4680 m produced new antibiotic quinones.[7] The structurally novel chlorinated cyclopenta[*a*]indene glycosides, the cyanosporasides, were isolated from a culture of a deep-sea marine obligate actinomycete, *Salinispora pacifica*, which was collected at a depth of 500 m.[8] Recently, unprecedented halogenated dimeric pyrroles with potent antibiotic activity, the marinopyrroles, were discovered from a *Streptomyces* strain obtained from a 51 m deep-sea sediment.[9] The diversity and bioactivities of these compounds make them attractive targets for synthetic and biomedical studies.

In our continuing search for novel bioactive molecules, we collected deep-sea marine sediments off the coast of Sungsanpo on Jeju Island, Korea, and selectively isolated actinomycete strains growing on seawater-based agar plates. Cultivation in liquid media and chemical examination of the isolated actinobacterial strains by LC-MS revealed that one of the strains, SNJ013, a streptomycete isolated from sediment collected at a depth of 138 m, produced a metabolite with a large molecular ion at *m*/*z* 1592 and UV absorptions at 203 and 281 nm. Subsequent chemical analysis of cultures of this strain led to the identification of a new lasso peptide named sungsanpin (**1**), the first lasso peptide to be isolated from a marine-derived microorganism. Here, we report the structural determination of sungsanpin, including its three-dimensional structure as well as its inhibitory activity in a cell invasion assay with the lung cancer cell line A549.

RESULTS AND DISCUSSION

Sungsanpin (**1**) was isolated as a white powder by a combination of reversed-phase column (C_{18}) chromatography and HPLC. The molecular formula of **1**, $C_{77}H_{109}N_{17}O_{20}$, was established by an analysis of HRFABMS data and ^{1}H and ^{13}C NMR spectra. On the basis of the molecular formula, 32 degrees of unsaturation were deduced. The ^{1}H NMR spectrum displayed typical features of a peptide-derived compound, including 14 NH signals (δ_H 11.01, 10.20, 10.01, 9.70, 9.51, 9.16, 9.01, 8.97, 8.41, 8.35, 8.30, 8.09, 8.04, and 7.80) and 18 *α*-proton signals (δ_H 5.57, 5.43, 5.37 (2H, overlapped), 5.36, 5.17, 5.13, 5.10, 5.01, 4.96, 4.92, 4.77, 4.76, 4.75, 4.19, 4.15, 4.09, and 3.80). The ^{13}C NMR spectrum was also consistent with a peptide-derived compound and contained 16 amide/acid carbonyl signals (δ_C 179.7, 175.0, 174.5 (2C overlapped), 173.2, 173.1, 172.1, 171.6, 171.5, 171.4, 171.3, 171.0, 170.7, 170.6, 170.0, and 169.5) and 15 *α*-carbon signals (δ_C 61.3, 60.0,

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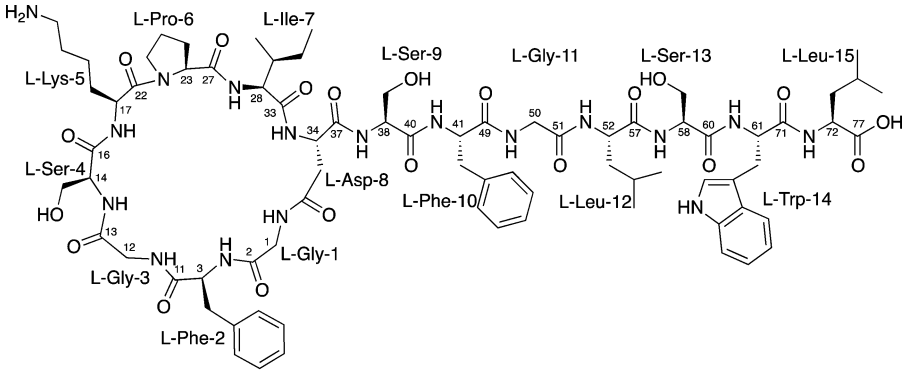
Chart 1



1

58.6 (2C, overlapped), 57.4, 56.3, 55.2, 54.5, 54.4, 54.2, 51.1 (2C, overlapped), 46.0, 45.2, and 44.3) (Table 1).

The 1D (^{1}H and ^{13}C) and 2D (COSY, TOCSY, HMQC, and HMBC) NMR data revealed 15 amino acid residues, including an aspartic acid, three glycines, an isoleucine, two leucines, a lysine, two phenylalanines, a proline, three serines, and a tryptophan. Because the 16 carbonyl signals, the signals from the aromatic rings of the two phenylalanine and tryptophan residues, and the proline ring accounted for 31 of the 32 degrees of unsaturation required by the molecular formula, we deduced that sungsanpin possessed an additional ring. The ring system inherently hampered the use of N-terminal sequencing to determine the structure of sungsanpin. The amino acid sequence was therefore established by extensive analyses of HMBC and ROESY NMR data.

First, the C-terminal residue was identified as Leu-15 based on the relatively downfield carbonyl carbon at δ_C 179.7. In the HMBC spectrum, NH-72 (δ_H 7.80) of Leu-15 correlated with the amide carbon C-71 (δ_C 170.6) belonging to Trp-14, securing the Leu-15 → Trp-14 connectivity. The amide NH (NH-61, δ_H 8.30) of the Trp-14 residue showed a long-range heteronuclear coupling with C-60 (δ_C 174.5), the carbonyl carbon of Ser-13, connecting Trp-14 to Ser-13. The HMBC correlation from the NH (δ_H 10.01) of Ser-13 to C-57 at δ_C 175.0 of Leu-12 established the connectivity of Ser-13 to Leu-12. The ^{1}H−^{13}C two-bond coupling between NH-52 (δ_H 10.20) of Leu-12 and C-51 (δ_C 170.0) of Gly-11 confirmed the Leu-12 → Gly-11 sequence. In the HMBC spectrum, the amide proton (δ_H 9.16) of Phe-10 correlated with C-40 (δ_C 171.0), the amide carbon of Ser-9, thus indicating the connectivity between Phe-10 and Ser-9. Aspartic acid was connected to Ser-9 by the ROESY correlation between NH-38 (δ_H 9.01) and H-34 (δ_H 5.57). The sequence of Asp-8 to Ile-7 was secured by the heteronuclear correlation between NH-34 (δ_H 9.51) and C-33 (δ_C 170.7) in the HMBC spectrum. The extension of the chain between Ile-7 and Pro-6 was established by the HMBC correlation from the α-proton of Ile-7 (δ_H 5.13) to C-27 (δ_C 174.5), the carbonyl carbon of Pro-6. The ^{1}H−^{13}C long-range coupling between the α-proton (δ_H 4.76) of Pro-6 and the amide carbon (δ_C 171.4) of Lys-5 allowed for the connectivity from Pro-6 to Lys-5. The ROESY correlation between H-23 and the amide proton of Lys-5 at δ_H 9.70 supported the connectivity of Pro-6 → Lys-5. The residue adjacent to Lys-5 was identified as Ser-4 based on the HMBC correlation from the amide proton of Lys-5 to C-16 (δ_C 171.4), the amide carbon of Ser-4.

The connectivity between Ser-4 and Gly-3 was deduced by the HMBC coupling from NH-14 (δ_H 8.97) to C-13 (δ_C 169.5), which belongs to the Gly-3 residue. Phe-2 was then connected to Gly-3 by the long-range heteronuclear correlation between NH-12 (δ_H 8.35) and C-11 (δ_C 171.5). This sequence was supported by the ROESY correlation between NH-12 and H-3 (δ_H 4.15), the α-proton of Phe-2. The last residue of the peptide, Gly-1, was located next to Phe-2 based on the HMBC correlation from NH-3 (δ_H 11.01) to the carbonyl carbon (δ_C 171.6) of Gly-1. The macrocyclic ring connecting Gly-1 to Asp-8 was deduced by the ROESY correlation between H-35a (δ_H 3.83) and the amide proton NH-1 (δ_H 8.41). The amino acid sequence of the chain portion of the molecule and the existence of the macrocyclic ring bearing Gly-1, Phe-2, Gly-3, Ser-4, Lys-5, Pro-6, Ile-7, and Asp-8 were well supported by the ESIMS/MS data (Figure 1), which confirmed that the planar structure of sungsanpin is composed of an eight-amino-acid macrocyclic ring and a seven-amino-acid chain.

To determine the absolute configuration, sungsanpin (**1**) was subjected to acid hydrolysis with 6 N HCl at 115 °C. Although conventional acid hydrolysis is usually performed as an overnight reaction, we performed the reaction for one hour[10] to preserve the fragile tryptophan residue. The hydrolysate was derivatized with Marfey's reagents, 1-fluoro-2,4-dinitrophenyl-L- and -D-alanine amide (L- and D-FDAA).[11] The derivatives were analyzed by LC-MS to determine the absolute configurations of the α-carbon of each of the amino acid residues, all of which corresponded to the L-amino acids. The configuration of the additional stereogenic center of Ile was established by comparing the retention time of its GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate, derivative with the retention times of the GITC derivatives of authentic standards of *allo*-L-Ile and L-Ile.[12]

After establishing the absolute configuration of each stereocenter, the ROESY NMR spectrum was examined to deduce the three-dimensional structure of sungsanpin (**1**). Interestingly, aside from the through-space correlations confirming the amino acid sequence, additional ROESY cross-peaks indicating that the tail part of **1** is folded into the ring were also observed (Figure 2a). On the basis of the observed ROESY correlations, the three-dimensional structure of sungsanpin (**1**) was calculated (Figure 2b). The structure clearly shows that eight amino acids (-Gly^1-Phe-Gly-Ser-Lys-Pro-Ile-Asp^8-) form a macrocyclic ring and seven amino acids (-Ser^9-Phe-Gly-Leu-Ser-Trp-Leu^{15}) form a tail that loops through the ring. This result demonstrates that sungsanpin

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Table 1. NMR Data for Sungsanpin (1) in Pyridine-d_5[a]

	position	δ_C, type	δ_H, mult (J in Hz)		position	δ_C, type	δ_H, mult (J in Hz)
Gly-1	1-NH		8.41, br s	Ser-9	38-NH		9.01, br s
	1a	45.2, CH_2	5.10, d (5.5)		38	58.6, CH	4.77, m
	1b		3.80, m		39a	62.0, CH_2	4.08, m
	2	171.6, C			39b		3.85, dd (10.5, 8.5)
Phe-2	3-NH		11.01, d (7.0)		40	171.0, C	
	3	58.6, CH	4.15, br dd (15.0, 7.0)	Phe-10	41-NH		9.16, d (10.0)
	4	35.0, CH_2	3.92, m		41	54.5, CH	5.43, ddd (12.0, 10.0, 4.0)
	5	141.3, C			42a	39.0, CH_2	2.92, dd (15.0, 4.0)
	6/10	130.2, 2C	7.23, d (7.5)		42b		2.69, dd (15.0, 12.0)
	7/9	129.1, 2C	7.25, dd (7.5, 7.5)		43	139.2, C	
	8	126.9, CH	7.19, m		44/48	129.5, 2CH	7.12, d (7.0)
	11	171.5, C			45/47	128.8, 2CH	7.15, dd (7.0, 7.0)
Gly-3	12-NH		8.35, d (6.0)		46	126.8, CH	7.08, dd (7.0, 7.0)
	12a	46.0, CH_2	4.96, m		49	172.1, C	
	12b		4.19, m	Gly-11	50-NH		8.09, d (6.5)
	13	169.5, C			50a	44.3, CH_2	5.01, m
Ser-4	14-NH		8.97, d (9.0)		50b		4.09, m
	14	57.4, CH	5.36, m		51	170.0, C	
	15a	64.5, CH_2	4.41, dd (10.5, 5.5)	Leu-12	52-NH		10.20, d (7.5)
	15b		4.36, dd (10.5, 3.0)		52	54.2, CH	4.75, m
	16	171.4, C			53a	39.3, CH_2	2.56, br dd (13.5, 13.5)
Lys-5	17-NH		9.70, d (8.0)		53b		1.55, m,
	17	51.1, CH	5.37, m		54	15.3, CH	1.75, m
	18a	31.9, CH_2	2.10, m		55	23.6, CH_3	0.90, d (6.5)
	18b		1.87, m		56	20.1, CH_3	0.86, d (6.5)
	19a	26.6, CH_2	1.82, m		57	175.0, C	
	19b		1.62, m	Ser-13	58-NH		10.01, d (9.0)
	20a	20.1, CH_2	1.74, m		58	55.2, CH	5.37, m
	20b		1.62, m		59a	64.5, CH_2	4.42, m
	21a	38.9, CH_2	3.14, m		59b		4.05, dd (11.0, 4.0)
	21b		3.06, m		60	174.5, C	
	22	173.2, C		Trp-14	61-NH		8.30, d (7.0)
Pro-6	23	61.3, C	4.76, m		61	56.3, CH	5.17, m
	24a	30.4, CH_2	2.16, m		62a	27.4, CH_2	4.09, m
	24b		2.09, m		62b		3.82, m
	25a	26.7, CH_2	2.10, m		63	110.8, C	
	25b		1.78, m		64	126.1, CH	8.35, br s
	26a	48.4, CH_2	3.93, m		64-NH		11.75, s
	26b		3.62, m		65	137.8, C	
	27	174.5, C			66	111.9, CH	7.47, d (7.5)
Ile-7	28-NH		8.04, br s		67	121.8, CH	7.20, m
	28	60.0, CH	5.13, m		68	119.5, CH	7.18, m
	29	43.4, CH	2.24, m		69	120.9, CH	8.20, d (7.5)
	30a	25.8, CH_2	2.04, m		70	129.7, C	
	30b		1.56, m		71	170.6, C	
	31	12.9, CH_3	0.99, t (7.0)	Leu-15	72-NH		7.80, d (9.0)
	32	16.0, CH_3	1.04, d (6.5)		72	54.4, CH	4.92, m
	33	170.7, C			73a	43.9, CH_2	1.41, m
Asp-8	34-NH		9.51, d (8.0)		73b		1.03, m
	34	51.1, CH	5.57, m		74	25.2, CH	1.50, m
	35a	37.5, CH_2	3.83, m		75	23.2, CH_3	0.82, d (6.0)
	35b		3.69, dd (17.0, 3.0)		76	23.1, CH_3	0.73, d (6.5)
	36	171.3, C			77	179.7, C	
	37	173.1, C					

[a] ^{1}H and ^{13}C data were recorded at 900 and 225 MHz, respectively.

(1) is one of the rare examples of a lasso peptide. Lasso peptides are ribosomally synthesized peptides consisting of 16–23 amino acids that have an N-terminal eight- or nine-residue ring with a linear C-terminal tail threaded through the ring.[13] A literature search revealed that 11 lasso peptide series (microcin J25,[14] anantin,[15] capistruin,[16] lariatin,[17] propeptin,[18] RES 701,[19] aborycin,[20] siamycin,[21] BI-32169,[22] caulosegnin,[23] and astexin[24]) have been reported. We compared the sequence of **1** with

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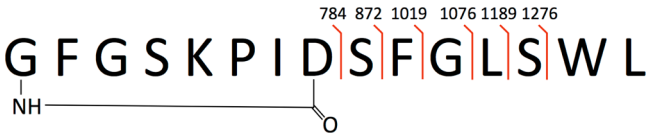

Figure 1. MS/MS data for **1** confirming the sequence of the amino acids in the tail.

the sequences of the 11 known lasso peptide series (Figure 3). By forming the macrocyclic ring through the Gly (G) and Asp (D) residues, sungsanpin (**1**) is similar to anantin, propeptin I, and RES 701-1. However, the rings of propeptin I and RES 701-1 contain nine amino acid residues, whereas the ring of sungsanpin (**1**) contains only eight residues. Even though anantin contains an eight-residue ring, the amino acid sequence of sungsanpin (**1**) contains only 15 residues and is quite different from that of anantin. In addition, sungsanpin (**1**) is the shortest lasso peptide, which would revise the previous range for the number of amino acid residues in lasso peptides. Thus, sungsanpin (**1**) is a new and structurally unique member of the lasso peptide family.

The known lasso peptides have a variety of biological activities. For example, microcin, aborycin, siamycin (MS-271), and propeptin are known to inhibit RNA polymerase,[25] HIV-1 protease,[26] HIV fusion[27] and myosin light chain kinase,[28] and prolyl endopeptidase,[29] respectively. Lariatin has antimycobacterial activity,[17] and capistruin has antibacterial activity.[30] In addition, anantin is a peptide antagonist of the atrial natriuretic factor.[31] RES-701 and BI-32169 were reported as antagonists of endothelin receptor B[32] and the glucagon receptor,[22] respectively. Sungsanpin (**1**) was initially evaluated in cytotoxicity and antimicrobial assays, but did not exhibit significant activity. We then tested sungsanpin (**1**) for immunosuppressive activity in an NO production assay and also observed no activity.

Biological activity for **1** was discovered in a cell invasion assay. We selected a human non-small-cell lung cancer cell line, A549, for the assay because lung cancer is one of the most malignant cancers, and non-small-cell lung cancer accounts for 75−80% of all cases.[32] In addition, the five-year survival rate for lung cancer patients is only 8−14% due to early metastasis and the lack of efficient therapies for the metastasis, which emphasizes the importance of inhibition of metastasis for successful lung cancer treatment.[33,34] Cancer metastasis, which involves the acquisition of cell motility, surface adhesion properties, and activity of extracellular proteases, is the major reason for poor clinical outcomes or death in various cancer patients.[35,36] Cell invasion through extracellular matrix, which is initiated by adhering to the blood vessel wall, is an important step in tumor metastasis. To examine the biological activity of **1** in a cell invasion assay, we first examined the cytotoxicity of sungsanpin (**1**) against A549 cells. Sungsanpin (**1**) did not show serious cellular damage for 24 h at concentrations up to 50 μM (Figure 4a). In the cell invasion assay with A549 cells, the number of cells invading the lower chamber was inhibited by exposure to 5 and 50 μM sungsanpin (**1**) (25% and 47%, respectively, versus the control in Figure 4b).

To further explore the mechanisms behind the inhibitory effect of sungsanpin (**1**) against cell invasion, we assessed matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitor of metalloproteinases-1 (TIMP-1), and TIMP-2 mRNA levels, which are important enzymes related to cell invasion processes. During the invasion process, proteolytic enzymes including matrix metalloproteinases (MMPs), which are regulated by TIMPs, degrade most extracellular matrix (ECM) components, forming the basal membrane surrounding the blood vessels, thereby allowing cancer cells to invade.[37] Sungsanpin (**1**) considerably increased TIMP-1 and TIMP-2 mRNA levels in a concentration-dependent manner (Figure 4c), whereas MMP-2 and MMP-9 mRNA levels were not altered (data not shown). This result suggests that the enhancement of both TIMP-1 and TIMP-2 expression by sungsanpin (**1**), which inhibit MMPs' degradation of the extracellular matrix, eventually decreases the invasiveness of A549 cells.

Lasso peptides have been isolated from phylogenetically diverse bacteria such as *Streptomyces*, *Rhodococcus*, *Burkholderia*, *Caulobacter*, and Enterobacteriaceae.[14−24] Every previously reported lasso peptide producer has originated from a terrestrial

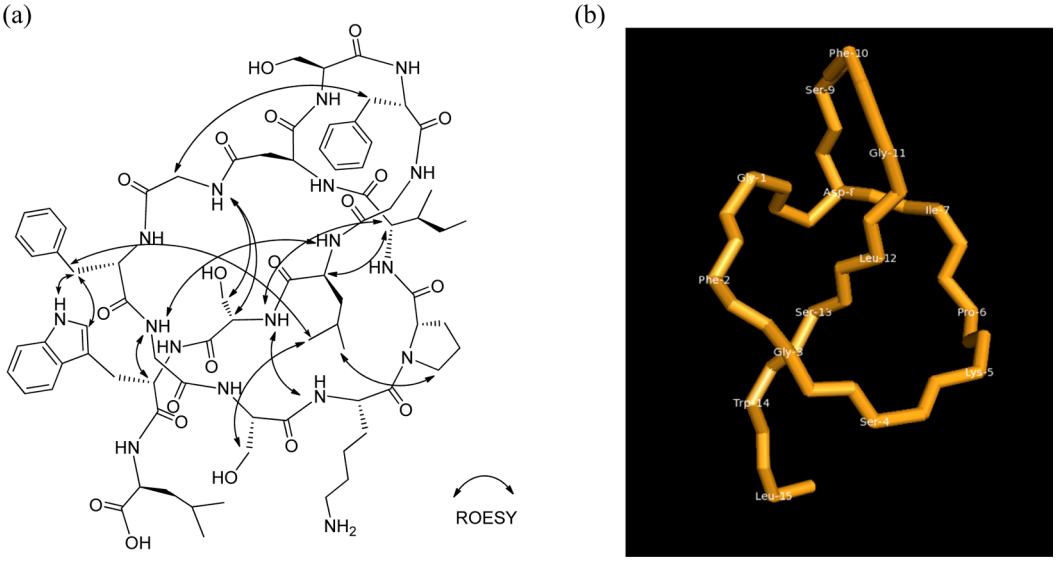

Figure 2. (a) Key ROESY correlations of sungsanpin (**1**). (b) Three-dimensional structure of **1**, calculated on the basis of observed ROESY correlations.

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Name	Sequence	Length
Microcin J25	G - G A G H V P - E Y F - V G I G T P I S F Y G - -	21
Anantin	G - F I G W G N - D O F G - H Y S G - - D F - - - -	17
Capistruin	G - T P G F Q T P D A R - - V I S R - F G F N - - -	19
Lariatin A	G S Q L V Y R - - E W V - - G H S N - - V I K P - -	18
Propeptin	- - - - G Y P W W D Y R - D L F G G - H T F I S P -	19
RES 701-1	G N W H G T A P - D W - - - F F N Y - - Y W - - - -	16
Aborycin	C L G I G S C N - D F A G C G Y A V V C F W - - - -	21
Siamicin I	C L G V G S C N - D F A G C G Y A I V C F W - - - -	21
BI-32169	G L P W G C P S - D I P - - G W N T - - P W A C - -	19
Caulosegnin	G A F V G Q P E - A V N - - P L G R - - E I Q G - -	19
Astexin-1	G L S Q G V E P - D I G Q T Y F E E - - S R I N Q D	23
Sungsanpin (1)	**G - - F G S K P I D - - - - S F G L - - S W L - - -**	**15**

Figure 3. Alignment of the amino acid sequences of previously reported lasso peptides with the sequence of **1**. The peptide sequences were aligned using the ClustalW multiple alignment program with gap opening penalty of 9, gap extension penalty of 0.1, and scoring matrix of Blosum.

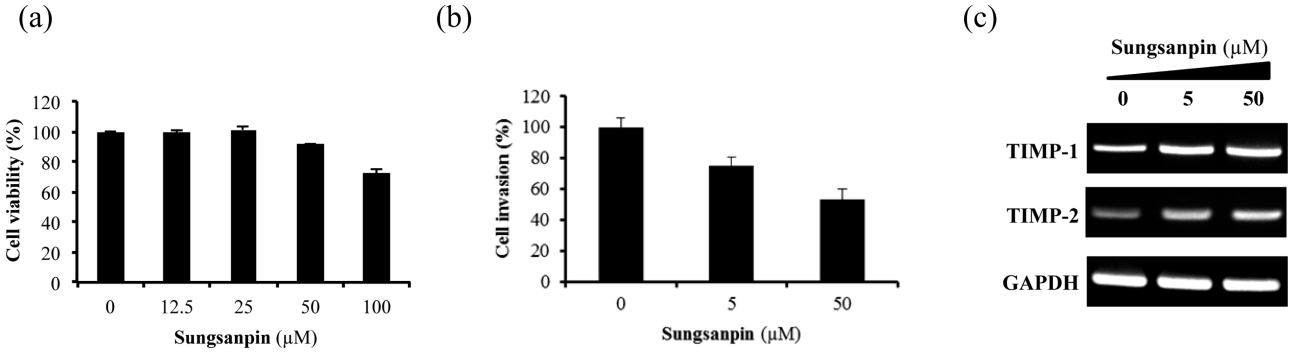

Figure 4. (a) Effects of sungsanpin on A549 cell viability. Cells were treated with different concentrations of sungsanpin for 24 h, and cell viability was measured using a CCK-8 assay. Each value represents the mean ± SD of three independent experiments. Control cells were treated with 0.5% (v/v) DMSO. (b) Effects of sungsanpin on the invasion of A549 cells. Cells were treated with the indicated concentrations of sungsanpin for 24 h. The cell invasion was measured with a cell invasion assay kit (Chemicon) according to the manufacturer's protocol. The invasion abilities were quantitated by dissolving stained cells in 10% acetic acid for colorimetric reading of OD at 560 nm. Each value represents the mean ± SD of three independent experiments. Control cells were treated with 0.5% (v/v) DMSO. (c) Effects of sungsanpin (**1**) on TIMP-1 and TIMP-2 mRNA levels in A549 cells. Cells were treated with the indicated concentrations of sungsanpin (**1**) for 24 h. The TIMP-1 and TIMP-2 mRNA levels were determined by RT-PCR. Control cells were treated with 0.5% (v/v) DMSO. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as an internal control.

or freshwater environment. Sungsanpin (**1**) is the first lasso peptide discovered from a marine environment, highlighting the still untapped chemical diversity of marine microorganisms. In addition, our discovery of a structurally novel bioactive lasso peptide from a deep-sea actinomycete indicates that the chemical investigation of deep-sea bacteria could provide unique natural products with therapeutic potential in the near future.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured on a JASCO P-1020 polarimeter with a 5 cm cell. UV spectra were recorded with a Perkin-Elmer Lambda 35 UV–vis spectrometer in a 1 cm cuvette. IR spectra were recorded on a JASCO FT-IR-4200 spectrometer. ^{1}H NMR (900 MHz) and ^{13}C NMR (225 MHz) spectral data were obtained on a Bruker 900 MHz NMR spectrometer at the Korea Basic Science Institute in Ochang. Low-resolution LC-MS data were acquired on an Agilent Technologies 1200 series HPLC coupled to an Agilent Technologies 6130 quadrupole mass spectrometer. High-resolution fast-atom bombardment (HR-FAB) mass spectrometry data were recorded with a Jeol JMS700 high-resolution mass spectrometer at the Korea Basic Science Institute in Daegu. MS/MS spectrometry data were acquired with a Finnigan LCQ mass spectrometer.

Bacterial Isolation. Marine sediments (12 samples) were collected from the seafloor at depths of 108–138 m off the coast of Sungsanpo on Jeju Island in the Republic of Korea with a customized mud snapper in November 2010. The bacterium (strain SNJ013) was isolated from one of the sediment samples collected at 138 m below sea level (N33.15.620, E127.27.932). The following method was used for the isolation of SNJ013: before drying the sediment sample in a Petri dish, the sample was constantly agitated to ensure even spreading of the sediment. The sediment was then dried for two hours in a sterile environment. After drying, the sediment was pressed onto the surface of an isolation agar plate (18 g/L agar containing 3.4% seawater and 5 μg/mL kanamycin) with an autoclaved plug (1 cm in diameter). Colonies were repeatedly inoculated onto fresh agar plates to obtain single strains. On the basis of the 16S rDNA sequence (GenBank accession number: KC871171) of the strain SNJ013, the strain is most closely related to *Streptomyces* sp. I08A-00426 (99% identity), identifying the strain as a *Streptomyces* sp.

Cultivation and Extraction. The SNJ013 strain was cultured on solid YEME medium (4 g yeast extract, 10 g malt extract, 4 g glucose, and 18 g agar per 1 L of sterilized 3.4% seawater) at 30 °C. The culture was then transferred to liquid A1+C medium (10 g starch, 4 g yeast extract, 2 g peptone, and 1 g $CaCO_3$ per 1 L of sterilized 3.4% seawater) in a 500 mL Erlenmeyer flask containing 200 mL of medium. After incubation at 30 °C with shaking at 220 rpm for 2 days, 10 mL of the culture was used to inoculate each liter of A1+C in a 2.8 L Fernbach flask. The culture was cultivated for 3 days at 30 °C with shaking at 220 rpm. In total, 15 L of culture was prepared for the isolation of sungsanpin (**1**). The entire culture (15 L) was extracted twice with 30 L of distilled EtOAc using a separatory funnel. After adding anhydrous sodium sulfate to remove residual water, the organic phase was concentrated *in vacuo* to yield 3.2 g of extract.

Isolation of Sungsanpin (1). A quarter of the dried extract material was resuspended with Celite in MeOH and dried *in vacuo* to generate Celite-adsorbed extract. The Celite-adsorbed extract was loaded onto 2 g of prepacked C_{18} Sepak resin. The extract was fractionated by elution with a step gradient composed of H_2O and MeOH. Sungsanpin (**1**) eluted in the 80% MeOH fraction. To obtain

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pure sungsanpin (**1**), the material from the previous column was purified by preparative reversed-phase HPLC on a Phenomenex Luna 10 μm C_{18} (2) 250 × 21.20 mm column with a 68% aqueous MeOH solvent system (flow rate: 10 mL/min, detection: UV 280 nm). Sungsanpin (**1**) eluted at 35 min under these conditions. The two-column procedure was repeated with the remaining three portions of the extract for a total of four purification runs to yield 18.2 mg of sungsanpin (**1**).

Sungsanpin (1): white powder; $[\alpha]_D^{25}$ −7 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.68), 281 (2.50) nm; IR (neat) ν_{max} 3288, 2957, 1643, 1528, 1248 cm^{-1}; ^1H and ^{13}C NMR (pyridine-d_5) see Table 1; HRFABMS *m*/*z* 1592.8105 $[M + H]^+$ (calcd for $C_{77}H_{110}N_{17}O_{20}$, 1592.8113).

Determination of the Absolute Configurations of the Amino Acid Residues in Sungsanpin (1). A 1 mg quantity of sungsanpin (**1**) was hydrolyzed in 0.5 mL of 6 N HCl at 115 °C for 1 h; the reaction was then cooled rapidly by placing the reaction vial into ice water for 3 min. The reaction solvent was evaporated *in vacuo*, and residual HCl was completely removed by adding 0.5 mL of H_2O and removing the solvent three times. The dried hydrolysate was lyophilized for 24 h. The hydrolysate containing the free amino acids was divided into two portions, and each portion was transferred into an 8 mL vial. The hydrolysate was then dissolved in 100 μL of 1 N $NaHCO_3$. To each of the two vials containing the dissolved free amino acids, 50 μL of either 10 mg/mL L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) or D-FDAA (1-fluoro-2,4-dinitrophenyl-5-D-alanine amide) in acetone was added. The reaction mixtures were incubated at 80 °C for 3 min. A 50 μL aliquot of 2 N HCl was added to neutralize the reaction, followed by the addition of 300 μL of aqueous 50% CH_3CN. A 10 μL aliquot of each reaction mixture was analyzed by LC-MS with a gradient solvent system (20% to 60% CH_3CN containing 0.1% formic acid over 40 min, C_{18} reversed-phase column 100 × 4.6 mm, UV at 340 nm). L-FDAA derivatives eluted before D-FDAA derivatives for all amino acid residues in the hydrolysate. Therefore, the absolute configurations of all of the amino acid residues in **1** were determined as L (see Table S1).

The additional stereogenic center of Ile was established by comparing the retention time of its GITC derivative with the retention times of the GITC derivatives of the authentic standards of *allo*-L-Ile and L-Ile. The hydrolysate of sungsanpin (**1**) was dissolved in H_2O at a concentration of 1 mg/mL in a 8 mL vial. Then 100 μL of 6% triethylamine and 100 μL of 1% GITC in acetone were added to the vial. The reaction was conducted at room temperature for 15 min, and the reaction mixture was diluted with 100 μL of 5% acetic acid. A 10 μL aliquot of the reaction mixture was analyzed by LC-MS with a gradient solvent system (30% to 55% CH_3CN containing 0.1% formic acid over 80 min, C_{18} reversed-phase column 250 × 4.6 mm, UV at 254 nm). The GITC derivative was co-injected with each standard prepared from authentic *allo*-L-Ile (56.7 min) and L-Ile (58.8 min). Thus, Ile in **1** was determined to be L-Ile on that account.

Structure Calculation. The three-dimensional structure of the peptide was calculated using the CYANA 3.0 program with distance constraints obtained by analyzing the ROESY spectrum. The isopeptide bond between Gly-1 and Asp-8 was modeled by replacing one carboxylic oxygen with a dummy nitrogen atom in the Asp residue and adding a carbonyl group to Gly in the library file of the software (ASC8 and GLC1, respectively). In addition, the three atoms (C, N, and O) comprising the isopeptide bond in both residues were constrained to 0.1 Å with the highest constraint weight (10) to mimic the atypical bond. We did not use hydrogen bond constraints used in previous literature.[23] The actual structure calculation was performed using a standard simulated annealing procedure with 20 000 steps of torsion angle dynamics followed by 8000 conjugate gradient minimization steps. Diastereotopic and homotopic protons were treated with the R^{-6} summation approach. The presented structure was cleaned of the additional artificial atoms to model the atypical isopeptide bond. The NMR data were deposited in the Biological Magnetic Resonance Bank (accession number: BMRB 19154).

Cell Culture. The human non-small-cell lung cancer cell line A549 was purchased from the American Type Culture Collection (ATCC). Cells were grown in RPMI1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 25 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Analysis of Cell Viability. Cells were treated with different concentrations of sungsanpin (**1**) for 24 h, and the cell viability was measured using the cell counting kit-8 (CCK-8, Dojindo Laboratories) according to the manufacturer's recommendations. Paclitaxel was used as a positive control (data not shown).

Cell Invasion Assay. Cells were treated with the indicated concentrations of sungsanpin (**1**) for 24 h, and then *in vitro* invasiveness was determined using the cell invasion assay kit (Chemicon) according to the manufacturer's protocol. In brief, treated cells were seeded into the upper chamber thinly coated with ECM gel with 3 × 10^5 cells/well in 300 μL of serum-free media, and 500 μL of media containing 10% FBS (fetal bovine serum) was added to the lower chamber. After incubation at 37 °C in 5% CO_2, the ECM gel was removed using a cotton-tipped swab. Invasive cells on the lower surface of the membrane were stained and quantitated by scanning with a microplate reader at 560 nm.

Total RNA Extraction and Reverse Transcriptase (RT)-PCR Analysis. After exposure of A549 cells to the indicated concentrations of sungsanpin (**1**) for 24 h, total RNA was isolated using an RNeasy mini kit (Qiagen) and reverse transcribed into cDNA using Superscript RNase H^- reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. Subsequent PCR analysis was carried out with aliquots (100 ng) of the cDNA preparation using a GeneAmp PCR System 9700 (Applied Biosystems). The PCR conditions were as follows: predenaturation at 94 °C for 5 min, followed by 33 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C (MMP-2), 60 °C (MMP-9, TIMP-1, and GAPDH (glyceraldehyde 3-phosphate dehydrogenase)), or 62 °C (TIMP-2) for 30 s, and extension at 72 °C for 1 min. The PCR products were visualized in 2% agarose gels. The primer sequences were as follows: MMP-2, 5′-ggC CCT gTC ACT CCT gAg AT-3′ (sense), 5′-ggC ATC CAg gTT ATC ggg gA-3′ (antisense); MMP-9, 5′-CAA CAT CAC CTA TTg gAT CC-3′ (sense), 5′-Tgg gTg Tag AgT CTC TCg CT-3′ (antisense); TIMP-1, 5′-ATC CTg TTg TTg CTg Tgg CTg ATA g-3′ (sense), 5′-TgC Tgg gTg gTA ACT CTT TAT TTC A-3′ (antisense); TIMP-2, 5′-AAA CgA CAT TTA Tgg CAA CCC TAT C-3′ (sense), 5′-ACA ggA gCC gTC ACT TCT CTT gAT g-3′ (antisense); GAPDH, 5′-Cgg AgT CAA Cgg ATT Tgg TCg TAT-3′ (sense), 5′-AgC CTT CTC CAT ggT ggT gAA gAC-3′ (antisense).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra and the detailed analysis of the absolute configuration of **1** are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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