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JBIR-31, a new teleocidin analog, produced by salt-requiring *Streptomyces* sp. NBRC 105896 isolated from a marine sponge

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The marine environment has recently been described as a source of novel chemical diversity for drug discovery,[1] as many bioactive substances are isolated from marine organisms including phytoplankton, algae, sponges, tunicates and mollusks.[2,3] Our group has also reported the isolation of azaspiracid-2[4] and JBIR-44[5] from marine sponges. Microorganisms from marine habitats,[6,7] especially actinobacteria, also constitute a promising untapped resource of novel compounds and are receiving special attention. Compared with higher organisms, microorganisms can be easily maintained under laboratory conditions ensuring a constant and inexpensive supply of their secondary metabolites. Moreover, compounds originally thought to be produced by a marine organism were later found to be produced by host-associated microorganisms.[8] A significant body of work has emerged in the past 10 years on the isolation of actinobacteria from marine habitats and their screening has yielded several novel bioactive compounds.[9–11]

Our group recently engaged in the isolation of microorganisms from marine sources including fungi and actinobacteria. Some of the isolated fungi were found to produce novel compounds, namely, JBIR-27, -28,[12] -15,[13] -37 and -38.[14] Among actinobacteria, many novel members of the genus *Streptomyces* were isolated from a marine sponge *Haliclona* sp.[15] Interestingly, many of the isolated strains required salt for their optimal growth. The requirement of salt by these strains may indicate their marine origin. One such salt-requiring strain *Streptomyces* sp. NBRC 105896 was isolated from *Haliclona* sp. When tested for the production of novel secondary metabolites, *Streptomyces* sp. NBRC 105896 produced a novel teleocidin analog designated as JBIR-31 (**1**) with eight known compounds. This paper describes the fermentation, isolation, structure elucidation and briefly the biological activity of **1**.

To isolate *Streptomyces* sp. NBRC 105896 from *Haliclona* sp. collected from the sea shore at Tateyama, Chiba Prefecture, Japan, starch casein nitrate (SCN) agar[16] was used. A colony that appeared on SCN plates was selected and was further purified using ISP-2 medium[17] prepared in 50% (v/v) artificial seawater (Sealife, Marine-tech, Tokyo, Japan). Interestingly, NBRC 105896 required 50–75% (v/v) seawater for optimal growth and the growth in the absence of sea salts was very weak. A comparison of partial 16S rRNA sequences (AB498723) for NBRC 105896 with the DNA Data Bank of Japan database (http://www.ddbj.nig.ac.jp/) showed that this strain shared a 100% 16S rRNA sequence similarity with strain *Streptomyces* sp. TP-A0873 (AB449972). The closest validly published species was *Streptomyces bambergiensis* (EF654096), with only 98% sequence similarity. Marine actinobacteria are a preeminent source of novel secondary metabolites, but their existence *per se* was questionable. Recently, not only has their existence been confirmed by the isolation of obligate marine actinobacteria[18] by showing their presence in the deepest marine environment[19] but they have also been shown to produce a number of novel metabolites.[6,7,9–11] A character that differentiates marine actinobacteria from terrestrial bacteria is the requirement of salt. During this study, a salt-requiring strain of *Streptomyces* sp. NBRC 105896 isolated from a marine sponge *Haliclona* sp. may be considered as indigenous to the marine habitat.

To examine for the production of secondary metabolites by the strain NBRC 105896, the strain was cultivated in 500-ml Erlenmeyer

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flasks containing 100 ml of the production medium containing 2.5% starch (Kosokagaku, Tokyo, Japan), 1.5% soybean meal (Nisshin Oillio, Tokyo, Japan), 0.2% dry yeast (Mitsubishi Tanabe Pharma, Osaka, Japan), 0.4% $CaCO_3$ (Kozaki Pharmaceutical, Tokyo, Japan) and 1.75% Sealife (pH 6.2 before sterilization), and cultured on a rotary shaker (180 r.p.m.) at 27 °C for 5 days. The structures of secondary metabolites by NBRC 105896 were determined by HR-electrospray ionization-MS and UV spectroscopic data. Interestingly, a total of nine compounds, including a novel compound **1**, were purified from the culture of NBRC 105896. These include indolactam V,[20] mocimycin,[21] 5,6-dihydromocimycin,[22] *N*-demethylteleocidin A_1,[23] **1** and teleocidin A_1[24] from the mycelial extract, and factumycin[25] and kirrothricin[22] from the supernatant extract of the culture. Levorin A_2[26] was also detected in the culture grown in a production medium containing 1% HP-20 (Mitsubishi Chemical, Tokyo, Japan).

To isolate a novel compound **1**, the fermentation broth (1000 ml) was centrifuged, and the collected mycelial cake was extracted with acetone (250 ml). The extract was concentrated *in vacuo*, and the residual aqueous concentrate was extracted with EtOAc and evaporated to dryness. The dried residue (507 mg) was subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60, Moritex, Tokyo, Japan) using an *n*-hexane-EtOAc linear gradient system (0–10% EtOAc), followed by a chloroform-MeOH linear gradient system (0–20% MeOH). The fractions including **1** were collected by LC-MS monitoring. The eluate (2–5% MeOH, 8.9 mg) was subjected to preparative reversed-phase HPLC using a PEGASIL-ODS column (20 i.d.×150 mm; Senshu Scientific, Tokyo, Japan) developed using 75% aqueous MeOH containing 0.125% formic acid (flow rate, 10 ml min^{-1}) to yield teleocidin A_1 (0.7 mg, retention time (Rt)=10.3 min) and JBIR-31 (**1**, 0.8 mg, Rt=34.2 min).

Compound **1** was obtained as a colorless amorphous solid (melting point, 58–62 °C, optical rotation, $[\alpha]_D^{25}$ −226.3, *c* 0.02, in MeOH), and its molecular formula was determined to be $C_{27}H_{39}N_3O_3$ (found: 454.3073 $[M+H]^+$, calculated: 454.3070) on the basis of HR-electrospray ionization-MS analysis. The presence of an oxindole chromophore was deduced from its UV and IR spectra (λ_{max} (MeOH) 243 (ε=12 490), 309 (ε=1600) nm and ν_{max} (KBr) 1709 cm^{-1}). The structure of **1** was mainly determined by NMR spectral analyses and the tabulated ^{13}C and ^1H NMR spectral data for **1** are shown in Table 1. A comparison of the ^{13}C and ^1H NMR data of **1** with those of

Table 1 ^{13}C (150 MHz) and ^1H (600 MHz) NMR data for teleocidin A_1 and 1

	Teleocidin A_1[24]		***1***	
No.	δ_C	δ_H *(multiplicity, J=Hz)*	δ_C	δ_H *(multiplicity, J=Hz)*
1		8.50 (br, s)		7.79 (s)
2	120.7	6.81 (br, s)	178.2	
3	121.4		43.6	3.90 (dd, 10.9, 5.3)
3a	114.1		116.0	
4	146.2		148.4	
5	106.1	6.44 (d, 8.0)	111.0	6.52 (d, 8.8)
6	119.7	6.96 (d, 8.0)	127.2	7.09 (d, 8.8)
7	118.4		119.5	
7a	137.2		140.2	
8	33.7	3.11 (dd, 17.5, 2.0)	29.0	2.47 (m)
		3.05 (dd, 17.5, 3.5)		1.58 (m)
9	55.7	4.32 (br, s)	53.3	4.17 (m)
10		7.77 (br, s)		6.31 (d, 5.9)
11	174.7		173.1	
12	70.6	4.33 (d, 12.0)	67.2	4.07 (d, 8.5)
14	64.7	3.72 (dd, 11.5, 3.0)	65.8	3.70 (m)
		3.56 (dd, 11.5, 8.5)		3.53 (m)
15	28.1	2.55 (m)	29.7	2.48 (m)
16	21.5	0.89 (d, 6.5)	21.1	1.03 (d, 6.5)
17	19.3	0.62 (d, 6.5)	19.4	0.80 (d, 6.8)
18	32.9	2.87 (s)	33.5	2.86 (s)
19	43.0		42.8	
20	25.3	1.44 (s)	24.4	1.35 (s)
21	148.3	6.15 (dd, 18.0, 10.0)	146.6	6.03 (dd, 17.8, 10.6)
22	112.1	5.30 (dd, 18.0)	113.3	5.26 (d, 10.6)
		5.26 (dd, 10.0)		5.22 (d, 17.8)
23	38.3	1.89 (td, 12.0, 12.0, 3.0)	38.3	1.73 (m)
		1.80 (td, 12.0, 12.0, 3.0)		
24	22.6	1.93 (br, m)	22.8	1.90 (m)
		1.70 (br, m)		1.72 (m)
25	124.4	5.06 (br, m)	123.9	5.07 (t, 6.8)
26	131.3		131.6	
27	23.6	1.47 (br, s)	25.5	1.65 (s)
28	17.2	1.63 (br, s)	17.3	1.49 (s)

NMR spectra were obtained using the Varian NMR system 600 NB CL (Palo Alto, CA, USA) in MeOH-d_4 (CD_3OD), and the solvent peak was used as an internal standard (δ_H 3.35 and δ_C 49.0 p.p.m.).

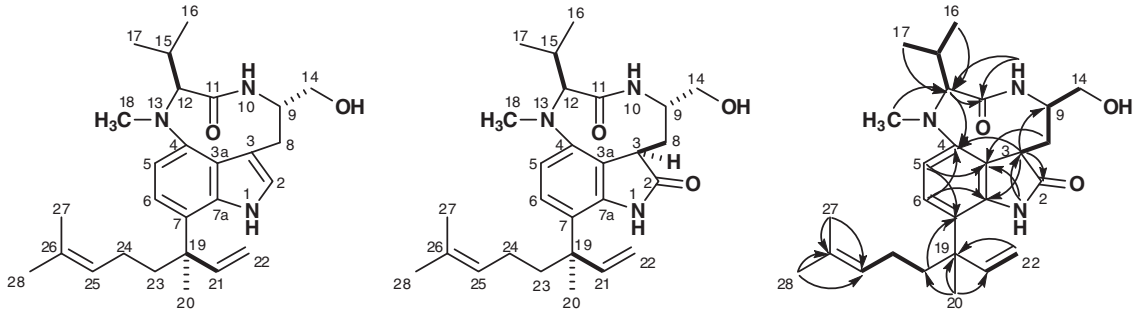

Figure 1 Structures of teleocidin A_1 and JBIR-31 (**1**), and key correlations in the double quantum-filtered correlation spectroscopy (bold line) and heteronuclear multiple bond coherence (arrow) spectra of **1**.

teleocidin A_1, together with 2D NMR spectral analyses such as ^{1}H-^{1}H double quantum-filtered correlation spectroscopy and heteronuclear multiple bond coherence spectra, the key correlations of which are summarized in Figure 1, revealed that **1** consisted of a skeleton similar to that of teleocidin A_1. In the ^{1}H NMR spectrum of **1**, all proton signals that are observed in teleocidin A_1, except for an olefinic proton 2-H, were observed; moreover, a methine proton 3-H (δ_H 3.90) was also newly observed. This methine proton was determined to be 3-H in the oxindole ring by the sequence from 3-H to hydroxymethylene protons 14-H (δ_H 3.70, 3.53) through to methylene protons 8-H (δ_H 2.47, 1.58) and a methine proton 9-H (δ_H 4.17). Furthermore, the ^{13}C NMR spectrum of **1** exhibited a carbonyl carbon C-2 (δ_C 178.2) instead of olefinic carbons C-2 (δ_C 120.7) and C-3 (δ_C 121.4) in teleocidin A_1. The ^{1}H-^{13}C long-range couplings from 3-H to C-2, C-4 (δ_C 148.4), C-7a (δ_C 140.2) and C-9 (δ_C 53.3) also confirmed the oxindole moiety. From these observations, **1** was deduced to be 2-oxo-teleocidin A_1 (Figure 1). The similarity of ^{1}H NMR signals of **1** to those of teleocidin A_1 indicated that **1** has the same configuration as teleocidin A_1, except C-3. In comparison with the ^{1}H NMR spectrum and an optical rotation of (−)-2-oxo-indlactam V,[27] the absolute configuration of **1** at C-3 could be *R* (Figure 1). Although the 2-oxo teleocidin derivatives of (−)-indolactam V,[20] such as (−)-2-oxo-indolactam V,[27] and blastmycetin B and C[28,29] have already reported, **1** is the first 2-oxo derivative of teleocidin.

Cytotoxic activities of **1** against human cervical carcinoma HeLa cells and human malignant pleural mesothelioma (MPM) ACC-MESO-1 cells[30,31] were tested by WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) colorimetric assay (Cell Counting Kit, Dojindo, Kumamoto, Japan). As a result, **1** and teleocidin A_1 showed cytotoxic effects against HeLa (IC_{50}=49 and 35 μM, respectively) and ACC-MESO-1 (IC_{50}=88 and 11 μM) cells for 48 h. Although the cytotoxic activities of these compounds were weak, these compounds showed cytotoxic effects for MPM cells that confer resistance to clinical anticancer drugs. It is therefore interesting to study the mechanism of cytotoxicity of **1** and teleocidin A_1. Teleocidin is a potent tumor promoter on mouse skin and activates protein kinase C, which has crucial roles in the signal-transduction pathways affecting a variety of physiological activities.[32] Recently, it has been reported that a teleocidin derivative, 14-*O*-(*N*-acetylglucosaminyl) teleocidin A, induces the translocation of protein kinases Cα and θ, and sensitizes the release of excitatory neuropeptide substance P induced by capsaicin from primary-cultured dorsal root ganglion neurons of the rat.[33] Because teleocidin analogs possess various biological activities, studies on detailed biological activities against MPM cells by **1** and teleocidin A_1 are now underway.

In conclusion, we found that the strain *Streptomyces* sp. NBRC 105896 produces several secondary metabolites including a novel teleocidin analog. Results presented in this paper and those of previous studies contributed by our group[12–14] strongly suggest that marine microorganisms are a promising source of novel secondary metabolites.

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