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Salternamide E from a Saltern-derived Marine Actinomycete *Streptomyces* sp.

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Abstract – Comprehensive chemical analysis of extracts and fractions of marine actinomycete strains led to the discovery of a new minor secondary metabolite, salternamide E (**1**), from a saltern-derived halophilic *Streptomyces* strain. The planar structure of salternamide E (**1**) was elucidated by a combinational analysis of spectroscopic data including NMR, MS, UV, and IR. The absolute configuration of salternamide E (**1**) was determined by circular dichroism spectroscopic analysis. Salternamide E displayed weak cytotoxicity against various human carcinoma cell lines.

Keywords – Marine actinomycete, Saltern, LC/MS analysis, Salternamide E, Cytotoxicity

Introduction

Marine actinomycetes are now generally considered talented chemical synthesizers that produce structurally and pharmaceutically interesting secondary metabolites.[1] In search of new bioactive compounds from these chemically prolific marine actinomycetes, detailed chemical analysis of microbial culture extracts provides an effective strategy to discover minor and thus easily neglected but structurally new compounds.[2,3] Applications of LC/MS screening of microbial secondary metabolites from marine actinomycetes have been particularly efficient in prioritizing chemically interesting strains.[3-5] By analyzing LC/MS profiles of marine actinomycetes, which are composed of chromatograms, UV, and mass spectra, we recently discovered new bioactive compounds belonging to diverse structural classes. Representatively, the first marine lasso-peptide, sungsanpin, was isolated from a deep-sea streptomycete bacterium.[6] New cytotoxic and antimicrobial cyclic peptides, ohmyungsamycins A and B, were discovered from a seashore-derived actinomycete.[7] Our LC/MS-based chemical analysis also led to the discovery of a new benzofuran glycoside and indole alkaloids from a sponge-associated *Amycolatopsis* sp.[8] Novel dilactone-tethered pseudo-dimeric peptides, mohangamides A and B, which inhibit *Candida albicans* isocitrate lyase, were discovered from a mudflat-derived *Streptomyces* sp.[9] More recently, we investigated secondary metabolites produced by halophilic actinomycetes that inhabit extremely saline environments, salterns. LC/MS screening of saltern-derived actinomycetes identified that a strain isolated from the topsoil of Shinui Island saltern in the Republic of Korea, #HK10, produced chlorine-bearing compounds and their analogues. We reported a series of compounds, including the chlorinated compound salternamide A, as the first secondary metabolites from actinomycetes inhabiting a saltern.[10] Further chemical analysis of the bacterial culture extract and fractions for minor chemical components by detailed LC/MS analysis finally led to the discovery of a new compound, salternamide E (**1**), in the series of salternamides. Here, we report the structure and biological activity of salternamide E.

Experimental

General experimental procedures – Optical rotations were measured on a Jasco P-1020 polarimeter with a 1-cm cell. UV spectra were obtained with a 1-cm cuvette using a PerkinElmer Lambda 35 UV/VIS spectrophotometer. CD spectra were recorded on an Applied Photophysics Chirascan-plus circular dichroism detector. IR spectra were obtained on a Thermo NiCOLET iS10 spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY experiments, were acquired on a Bruker Avance 500 MHz spectrometer at the NCIRF (National Center for Interuniversity Research Facilities at Seoul National University) operating at 500 MHz (^{1}H)

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and 125 MHz (^{13}C) with chemical shifts given in ppm (δ). Electrospray ionization (ESI) low-resolution LC/MS data were collected on an Agilent Technologies 6130 quadrupole mass spectrometer coupled with an Agilent Technologies 1200 series HPLC using a reversed-phase C_{18} (2) column (Phenomenex Luna, 5 μm, 100 × 4.6 mm). High-resolution fast atom bombardment (HRFAB) mass spectra were obtained using a JMS-700 mass spectrometer at the NCIRF (National Center for Interuniversity Research Facilities at Seoul National University). Semi-preparative HPLC was conducted using a Gilson 305 pump with a Gilson UV/VIS-155 detector and a C_{18} column (Kromasil, 5 μm, 250 × 10 mm).

Bacterial isolation – A saltern topsoil sample was collected on Shinui Island (445-9, Sangtaeseo-ri, Shinui-myeon, Shinan-gun, Jollanam-do) in the Republic of Korea. The strain HK10 was isolated on actinomycete isolation agar as previously described.[10] Based on 16S rDNA sequence analysis, HK10 was identified as *Streptomyces* sp., which is most closely related to *Streptomyces radiopugnans* (99% identity) (GenBank accession number: LC013479).

LC/MS chemical analysis – The seed of the HK10 strain for LC/MS chemical screening was inoculated from 50 mL of YEME medium (4 g of yeast extract, 10 g of malt extract, and 4 g of glucose in 1 L of artificial seawater) to 200 mL of A1+YPM medium (6 g of yeast extract, 4 g of peptone, 10 g of starch, and 4 g of mannitol in 1 L of artificial seawater). During cultivation, 10 mL of the culture broth was extracted with 10 mL of EtOAc every two days. After separating the organic layer from the aqueous layer, anhydrous sodium sulfate was added to completely remove residual water. The EtOAc layer was fully concentrated to yield dry material. The dry extract material was dissolved in 1 mL of methanol to prepare a sample for LC/MS analysis. Then, a screening sample was analyzed with LC/MS using a gradient analytic system (90% H_2O:10% acetonitrile 100% acetonitrile with 0.1% formic acid in 20 min, Phenomenex Luna C_{18} (2), 5 μm, 100 × 4.60 mm). Under these conditions, Salternamide E was detected at 15 min in the extract.

Cultivation, extraction and isolation – The culture of the HK10 strain was initiated in 50 mL of YEME medium (4 g of yeast extract, 10 g of malt extract, and 4 g of glucose in 1 L of artificial seawater) contained in a 125-mL Erlenmeyer flask. After incubating the culture for 3 days (200 rpm shaking at 30 °C), the culture was scaled up to 1 L by inoculating 10 mL of the culture to 1 L of YPM+starch medium (6 g of yeast extract, 4 g of peptone, 10 g of starch, and 4 g of mannitol in 1 L of artificial seawater) in a 2.8-L Fernbach flask. Sixty 1 L cultures were prepared in total and incubated at 180 rpm at 30 °C. After 5 days, the entire culture was extracted twice with EtOAc. The EtOAc layer was concentrated to yield 5 g of dried material. The dried extract was resuspended in MeOH with celite and powdered by drying. The dried powder containing the extract was loaded onto a 20 g C_{18} resin and fractionated with 200 mL each of 20%, 40%, 60%, 80% and 100% MeOH in H_2O and 1:1 MeOH/CH_2Cl_2. Salternamide E (**1**) was found in the 80% MeOH/H_2O fraction. To isolate **1**, the 80% fraction was subjected to reversed-phase HPLC on a C_{18} column (Kromasil, 5 μm, 250 × 10 mm) under isocratic conditions (55:45 CH_3CN/H_2O, UV detection at 254 nm, flow rate of 2 mL/min). Finally, salternamide E (**1**) (2 mg) was purified with a retention time of 31 min.

Salternamide E (1) – Colorless powder. $[\alpha]_D^{20}$: +30.1 (*c* 0.1, MeOH); IR (ZnSe) ν_{max} cm^{-1}: 3701, 3377, 2926, 1672, 1640, 1516, 1404, 1052, 752; UV (MeOH) λ_{max} (log ε) 227 (3.75), 272 (3.57) nm; CD (*c* 2.4×10^{-4} M, MeOH) λ_{max} (Δε) 206 (2.36), 258 (–2.92), 319 (2.00); ^1H, ^{13}C NMR: see Table 1.; ESIMS *m/z* 446 $[M+Na]^+$; HR-FABMS *m/z* 446.2511 $[M+Na]^+$; (calcd for $C_{23}H_{37}NO_6$ Na, 446.2519).

Cell culture – Human lung cancer (A549), colon cancer (HCT116), stomach cancer (SNU638), leukemia (K562), liver cancer (SK-HEP-1), and breast cancer (MDA-MB-231) cells were provided by the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in medium (RPMI 1640 medium for A549, HCT116, SNU638, and K562 cells; DMEM for SK-HEP-1 and MDA-MB-231 cells) supplemented with 10% heat-inactivated FBS and antibiotic-antimycotic solution (100 U/mL penicillin G sodium, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell proliferation assay – Six human cancer cell lines (A549, HCT116, SNU638, K562, SK-HEP1, and MDA-MB231; 3.5×10^4 cells/mL) were treated with several concentrations of salternamide E (**1**) for 3 days. After treatment, cells were fixed with 10% TCA solution, and cell viability was determined using a sulforhodamine B (SRB) assay. The results are expressed as percentages relative to solvent-treated control incubations, and IC_{50} values were calculated using nonlinear regression analysis (percent survival versus concentration). Etoposide was used as a positive control, providing IC_{50} values of 0.68 μM, 14 μM, 0,57 μM, 8.7 μM, 5,4 μM, and 1.5 μM against A549, HCT116, SNU638, SK-HEP1, MDA-MB-231, and K562 cell lines, respectively.

Results and Discussion

During the comprehensive LC/MS analysis of the 80% MeOH/H_2O fraction of HK10, which previously yielded salternamides A-D, a minor component with a UV spectrum similar to salternamide C was detected. Further chemical analysis of this minor compound revealed that its molecular mass is slightly greater than that of salternamide C by two daltons, indicating that this molecule is an analogue of the salternamides. Subsequent purification of the compound by HPLC led to the identification of a new derivative of the salternamides,[10] which is named salternamide E (**1**).

Salternamide E (**1**) was obtained as a colorless powder. The molecular formula was determined to be $C_{23}H_{37}NO_6$ by the molecular ion peak $[M+Na]^+$ at *m/z* 446.2511 (calcd for $C_{23}H_{38}NO_6Na$, 446.2519) in the positive ion mode of HR-FABMS. The IR spectrum of **1** indicated the presence of hydroxy (3377 cm^{-1}) and carbonyl functional groups (1672 and 1640 cm^{-1}). The ^1H NMR spectrum of **1** in CD_3OD displayed signals for four methyl groups at δ_H 1.05 (d, J = 6.5 Hz), 0.87 (d, J = 6.5 Hz), 0.86 (d, J = 6.5 Hz), and 0.85 (d, J = 6.5 Hz), an oxymethine proton signal at δ_H 3.98 (dd, J = 8.0, 4.0 Hz), three olefinic proton signals at δ_H 7.58 (s), 6.71 (dd, J = 15.5, 8.0 Hz), and 6.15 (dd, J = 15.5, 1.0 Hz), ten aliphatic protons between δ_H 2.82~1.01 (Table 1). In the ^{13}C NMR spectrum, 23 carbon signals appeared, including four methyl carbons at δ_C 23.7, 22.7, 20.9, and 20.0, an oxygenated methine carbon at δ_C 70.9, four olefinic carbons at δ_C 153.2, 133.8, 133.1, and 123.5, a quaternary carbon at δ_C 73.5, and three carbonyl carbons at δ_C 194.2, 178.3, and 167.2 (Table 1).

The interpretation of 2D HSQC and HMBC NMR experiments allowed all of the protons and carbons to be assigned (Fig. 1). An analysis of COSY correlations revealed that salternamide E possesses three spin systems: one composed of the protons at C-5 and C-6, another built with the protons of the linear chain group from C-7 to C-10, and the last assembled with the protons of the C-2′ to C-12′ chain involving four methyl groups. These three spin systems are extended to three partial structures based on HMBC correlation. First, the HMBC correlations of [H-3/C-1, C-2, C-5], [H-5/C-3, C-4, C-6], and [H_2-6/C-1, C-2, C-4, C-5] elucidated a 6-membered ring system bearing an α,β-unsaturated ketone group, thus providing a cyclohexenone moiety bearing two hydroxy groups at C-4 and C-5. Second, an HMBC correlation from H_2-10 to C-11 (δ_C 178.3) assigned a pentanoic acid substructure from C-7 to C-11. Lastly, the third spin system from C-2′ to C-

Table 1. ^1H and ^{13}C NMR data of **1** in CD_3OD. (δ in ppm, 500 MHz for ^1H and 125 MHz for ^{13}C)[a]

Position	**1**		
	δ_H mult. (*J* in Hz)	δ_C	
1		194.2	C
2		133.8	C
3	7.58 s	133.1	CH
4		73.5	C
5	3.98 dd (8.0, 4.0)	70.9	CH
6a	2.82 dd (16.5, 8.0)	42.4	CH_2
6b	2.73 dd (16.5, 4.0)		
7a	1.87 m	39.4	CH_2
7b	1.71 m		
8a	1.52 m	24.6	CH_2
8b	1.50 m		
9a	1.67 m	26.9	CH_2
9b	1.65 m		
10	2.31 t (7.5)	35.4	CH_2
11		178.3	C
1'		167.2	C
2'	6.15 dd (15.5, 1.0)	123.5	CH
3'	6.71 dd (15.5, 8.0)	153.2	CH
4'	2.48 m	35.5	CH
5'a	1.37 m	45.5	CH_2
5'b	1.12 m		
6'	1.49 m	29.2	CH
7'a	1.09 m	48.5	CH_2
7'b	1.01 m		
8'	1.66 m	26.3	CH
9'	0.86 d (6.5)	23.7	CH_3
10'	0.85 d (6.5)	22.7	CH_3
11'	0.87 d (6.5)	20.0	CH_3
12'	1.05 d (6.5)	20.9	CH_3

[a]The assignments were based on ^1H-^1H COSY, HSQC, and HMBC experiments.

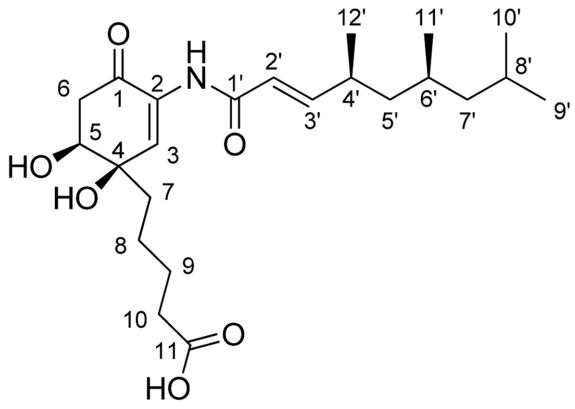

Fig. 1. The structure of salternamide E (**1**).

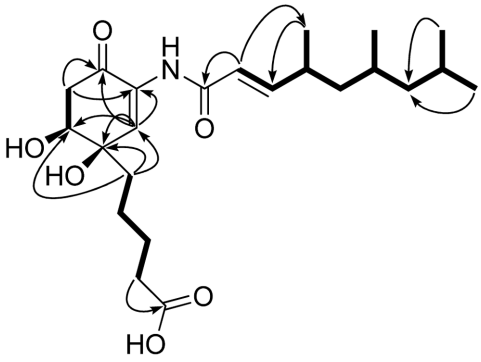

Fig. 2. Key COSY (▬) and HMBC (→) correlations of **1**.

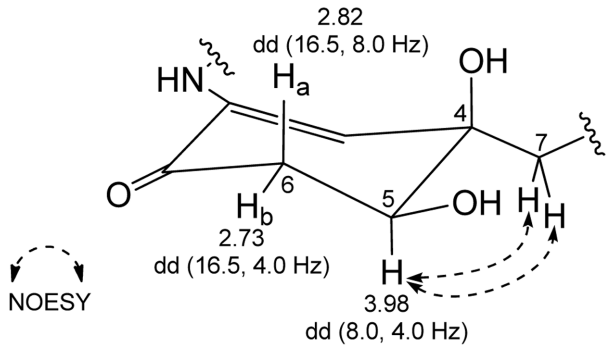

Fig. 3. ^{1}H-^{1}H coupling constants (*J* values) and key NOESY (⇠⋯⇢) correlations of **1**.

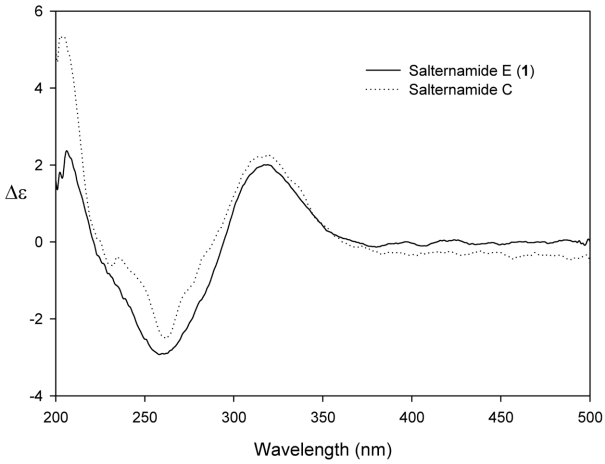

Fig. 4. The experimental ECD spectra of **1** and salternamide C.

12′ was extended to C-1′ by the HMBC correlation from H-2′ to the amide carbonyl carbon C-1′ (δ_C 167.2), completing a 4,6,8-trimethylnon-2-enamide chain. The geometry of the C-2′ double bond was assigned as *E* by a *trans* ^{1}H-^{1}H coupling constant between H-2′ and H-3′ ($J_{H2'H3'}$ = 15.5 Hz).

These three substructures were connected by the interpretation of the HMBC NMR spectrum. In particular, 2- and 3-bond ^{1}H-^{13}C couplings from H_2-7 to C-4, C-3, and C-5 assigned the pentanoic acid at C-4. Finally, the 4,6,8-trimethylnon-2-enamide chain was assembled to C-2 of the cyclohexenone moiety based on the molecular formula, thus elucidating the planar structure of salternamide E (**1**) (Fig. 2).

The relative configuration of the cyclohexenone system in **1** was determined by the analysis of the NOESY NMR spectrum and the ^{1}H-^{1}H coupling constants (*J* values) (Fig. 3). The carbinol proton H-5 (δ_H 3.98) showed a double-doublet with *J* values of 8.0 and 4.0 Hz. The large coupling constant (8.0 Hz) indicated that H-5 is located in a pseudoaxial position. H-6a (δ_H 2.82) showed a double-doublet splitting pattern with geminal coupling of 16.5 Hz and vicinal coupling of 8.0 Hz, which established the pseudoaxial position of H-6a. H-6b (δ_H 2.73) was assigned in a pseudoequatorial position based on the small vicinal coupling constant (4.0 Hz). In the NOESY spectrum of the salternamide E (**1**), the pentanoic acid chain was assigned in a pseudoequatorial position by the H-5/H_2-7 NOESY correlation, thus establishing the relative configuration of the ring (Fig. 3). The relative configuration of the two methyl groups at C-11′ and C-12′ in the 4,6,8-trimethylnon-2-enamide chain was deduced as *syn* based on the common biogenic origin of **1** with previously reported salternamides A-D.[10]

The structure of salternamide E (**1**) is similar to that of salternamide C. The difference is at the sp^3 bond between C-7′ and C-8′, whereas salternamide C has a sp^2 bond between these carbons and thus possesses a trimethyl nonadienamide chain. To determine the absolute configuration of salternamide E (**1**), we compared experimental ECD spectra of salternamide E and salternamide C,[10] of which the absolute configuration was unequivocally determined to be 4*R*, 5*S*, 4′*S*, and 6′*R* by chemical derivatizations. Based on the high similarity between the experimental ECD spectra of these compounds, the absolute configuration of salternamide E (**1**) was fully established to be 4*R*, 5*S*, 4′*S*, and 6′*S* (Fig. 4).

Because the salternamides A-D displayed cytoxicity against various cancer cell lines, the biological activity of **1** was evaluated against the cancer cell lines of A549, HCT116, SNU638, SK-HEP1, MDA-MB231, and K562.[10] Salternamide E exhibited weak cytotoxicities against A549, HCT116, SNU638, SK-HEP1, MDA-MB231, and K562 with IC_{50} values of 83, 85, 75, 91, 54, 70 μM, respectively.

In conclusion, detailed chemical analysis of the culture of saltern-derived *Streptomyces* sp. strain HK10 successfully identified a structurally new but minor compound, salter-

namide E. The discovery of this minor secondary metabolite, which could be easily neglected, by LC/MS-based chemical analysis indicates that coupling a unique source such as an extremely saline marine environment, a saltern, with a new strategy using LC/MS chemical analysis could be effective to further explore marine natural chemical diversity.

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References

(1) Fenical, W.; Jensen, P. R. *Nat. Chem. Biol.* **2006**, *2*, 666-673.
(2) Moon, K.; Chung, B.; Shin, Y.; Rheingold, A. L.; Moore, C. E.; Park, S. J.; Park, S.; Lee, S. K.; Oh, K.-B.; Shin, J.; Oh, D.-C. *J. Nat. Prod.* **2015**, *78*, 524-529.
(3) Hou, Y.; Braun, D. R.; Michel, C. R.; Klassen, J. L.; Adnani, N.; Wyche, T. P.; Bugni, T. S. *Anal. Chem*. **2012**, *84*, 4277-4283.
(4) Forner, D.; Berrué, F.; Correa, H.; Duncan, K.; Kerr, R. G. *Anal. Chim. Acta* **2013**, *805*, 70-79.
(5) Yang, J. Y.; Sanchez, L. M.; Rath, C. M.; Liu, X.; Boudreau, P. D.; Bruns, N.; Glukhov, E.; Wodtke, A.; de Felicio, R.; Fenner, A.; Wong, W. R.; Linington, R. G.; Zhang, L.; Debonsi, H. M.; Gerwick, W. H.; Dorrestein, P. C. *J. Nat. Prod.* **2013**, *76*, 1686-1699.
(6) Um, S.; Kim, Y.-J.; Kwon, H.; Wen, H.; Kim, S.-H.; Kwon, H. C.; Park, S.; Shin, J.; Oh, D.-C. *J. Nat. Prod.* **2013**, *76*, 873-879.
(7) Um, S.; Choi, T. J.; Kim, H.; Kim, B. Y.; Kim, S.-H.; Lee, S. K.; Oh, K.-B.; Shin, J.; Oh, D.-C. *J. Org. Chem.* **2013**, *78*, 12321-12329.
(8) Kwon, Y.; Kim, S.-H.; Shin, Y.; Bae, M.; Kim, B.-Y.; Lee, S. K.; Oh, K.-B.; Shin, J.; Oh, D.-C. *Mar. Drugs* **2014**, *12*, 2326-2340.
(9) Bae, M.; Kim, H.; Moon, K.; Nam, S.-J.; Shin, J.; Oh, K.-B.; Oh, D.-C. *Org. Lett.* **2015**, *17*, 712-715.
(10) Kim, S.-H.; Shin, Y.; Lee, S.-H.; Oh, K.-B.; Lee, S. K.; Shin, J.; Oh, D.-C. *J. Nat. Prod.* **2015**, *78*, 836-843.

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