The Journal of Antibiotics (2014) 67, 831–834

www.nature.com/ja

ORIGINAL ARTICLE

Actinopyrone D, a new downregulator of the molecular chaperone GRP78 from *Streptomyces* sp.

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A new downregulator of the molecular chaperone GRP78, actinopyrone D, was isolated together with a known related compound, PM050463, from *Streptomyces* sp. RAG92. The molecular formula of actinopyrone D was established as $C_{25}H_{36}O_4$ by high-resolution FAB-MS. NMR spectroscopic analysis revealed the structure of actinopyrone D, which consists of an α-methoxy-γ-pyrone ring and a C_{17} side chain containing a *cis* olefin moiety. Actinopyrone D and PM050463 dose-dependently inhibited 2-deoxyglucose-induced luciferase expression in HT1080 human fibrosarcoma cells transfected with a luciferase reporter plasmid containing the GRP78 promoter. Actinopyrone D inhibited GRP78 protein expression and induced cell death under endoplasmic reticulum stress.
The Journal of Antibiotics (2014) **67,** 831–834; doi:10.1038/ja.2014.76; published online 18 June 2014

INTRODUCTION

Most solid tumors are rather resistant to anticancer drugs. Poor blood vessel formation in solid tumors makes the chemotherapy more difficult. Under such conditions, low glucose supply causes endoplasmic reticulum (ER) stress.[1] Although ER stress induces the accumulation of unfolded protein and cell death, many solid tumors are known to express the molecular chaperone GRP78 to protect cells from ER stress-induced death.[2,3] As GRP78 is regulated by the promoter ERSE (ER-stress element),[4] downregulators of GRP78 expression under ER stress are expected to show antitumor activity selectively against solid tumors. In the course of screening for transcriptional regulators of GRP78 using luciferase reporter assay, an actinomycete strain RAG92 was found to produce two GRP78 downregulators, one of which was a new metabolite, actinopyrone D (**1**, Figure 1). This paper describes the fermentation, isolation, structure elucidation and biological activity of **1**.

RESULTS

Fermentation and isolation

A seed medium (pH 7.2) consisted of 1% soluble starch (Kanto Chemical, Tokyo, Japan), 1% peptone (Nihon Pharmaceutical, Tokyo, Japan), 1% molasses (Organic Land, Fukuoka, Japan) and 1% meat extract (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). The producing organism was cultured in 30-ml tubes containing 7.5 ml of the medium on a reciprocal shaker at 27 °C for 2 days. One-ml portions of the seed culture were inoculated into 200-ml Erlenmeyer flasks containing 30 ml of a medium (pH 6.2 before autoclaving) consisting of 2.5% glucose, 1.5% soybean meal (Nisshin Oillio Group, Tokyo, Japan), 0.2% dry yeast (Asahi Food & Healthcare, Tokyo, Japan) and 0.4% precipitated calcium carbonate (Kozakai Pharmaceutical, Tokyo, Japan). The fermentation (45 × 30 ml) was carried out three times at 27 °C for 4 days on a rotary shaker.

The culture (4 liter) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract was subjected to silica gel TLC (six plates, PLC Silica Gel 60 F_{254} 0.5 mm, Merck Millipore, Darmstadt, Germany) with chloroform–methanol (70:1). The active fraction was chromatographed on an ODS column (PEGASIL ODS, 20 i.d. × 250 mm, Senshu Scientific, Tokyo, Japan) with 80% methanol. The major active peak was identified as PM050463 (**2**, 57 mg)[5] from the NMR data (Table 1). A minor active fraction was further purified by ODS-HPLC (PEGASIL ODS) with 65% acetonitrile. Finally, a single peak fraction was obtained by HPLC using the same column with 75% methanol. Removal of solvent by evaporation yielded a colorless oil of **1** (0.52 mg).

Structure elucidation

The molecular formula of **1** was determined to be $C_{25}H_{36}O_4$ by high-resolution FAB-MS. The IR spectrum showed absorption peaks at 3390 and 1660 cm^{-1} due to hydroxy and conjugated carbonyl groups. ^{13}C and ^{1}H NMR data for **1** resembled those for **2** (Table 1). All one-bond ^{1}H-^{13}C connectivities were confirmed by an HMQC[6] experiment.

Three separate proton spin systems in **1**, depicted in Figure 2 by bold lines, were identified by a COSY experiment and were expanded to a C_{17} side chain moiety based on ^{1}H–^{13}C long-range couplings from four singlet methyls (8-Me, 12-Me, 14-Me and 16-Me) to the relevant carbons in the HMBC[7] spectrum (Figure 2). An oxygen substituent was located on C-15 from the chemical shifts (δ 82.8).

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Received 24 March 2014; revised 9 May 2014; accepted 19 May 2014; published online 18 June 2014

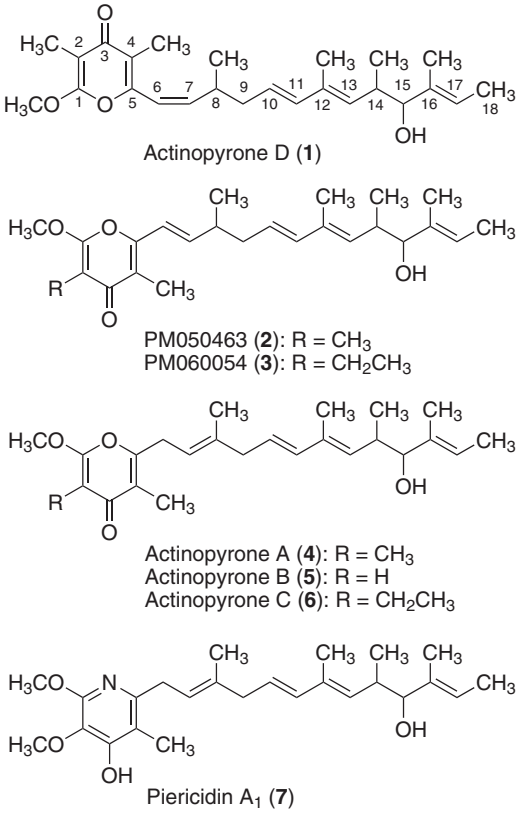

Figure 1 Structures of actinopyrone D (**1**) and related compounds.

The remaining one oxygen and five sp^2 carbons including a carbonyl group (δ 180.8) were required to construct a pyrone ring. The γ-pyrone structure and its substituents were confirmed by ^{1}H–^{13}C long-range correlations from two methyl groups (2-Me and 4-Me) and a methoxy group (1-OMe) to the ring carbons (Figure 2). The geometrical configurations were determined to be 6*Z*, 10*E*, 12*E* and 16*E* from coupling constants ($J_{6-7} = 12.0$ Hz, $J_{10-11} = 16.5$ Hz) and high-field ^{13}C chemical shifts for allylic methyls (12-Me: δ 13.1, 16-Me: δ 10.5). These results indicate that **1** is a geometrical isomer of **2** as shown in Figure 1.

Table 1 ^{13}C and ^{1}H NMR data for actinopyrone D (1) and PM050463 (2)

	1 (in $CDCl_3$)		*2* (in CD_3OD)	
No.	δ_C	δ_H (J = *Hz*)	δ_C	δ_H (J = *Hz*)
1	162.1		164.2	
2	100.3		100.1	
3	180.8		183.2	
4	119.9		118.2	
5	153.3		154.4	
6	117.6	6.11 d (12.0)	119.0	6.47 d (15.5)
7	144.8	5.69 dd (12.0, 10.5)	145.1	6.56 dd (15.5, 7.5)
8	34.5	2.89 m	38.8	2.52 m
9	40.3	2.15 2H t (7.0)	41.1	2.24 2H t (7.0)
10	125.3	5.47 m	125.6	5.56 dt (15.5, 7.0)
11	135.6[a]	6.05 d (16.5)	138.5	6.11 d (15.5)
12	135.5[a]		134.7	
13	133.8	5.19 d (10.0)	135.9	5.29 d (9.0)
14	36.8	2.65 m	37.6	2.66 m
15	82.8	3.60 d (9.0)	83.7	3.68 d (8.0)
16	136.5		138.1	
17	123.5	5.46 m	122.7	5.42 m
18	13.1	1.61 3H m	13.1	1.59 3H d (7.0)
2-Me	7.1	1.86 3H s	7.1	1.82 3H s
4-Me	10.4[a]	1.95 3H s	9.6	1.97 3H s
8-Me	20.5	1.06 3H d (6.5)	19.9	1.13 3H d (7.0)
12-Me	13.1	1.73 3H d (1.0)	13.1	1.73 3H d (1.0)
14-Me	17.4	0.78 3H d (6.5)	18.2	0.80 3H d (6.5)
16-Me	10.5[a]	1.61 3H m	11.2	1.58 3H s
1-OMe	55.8	3.93 3H s	56.6	4.06 3H s

[a]Interchangeable.

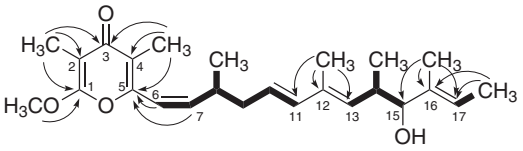

Figure 2 NMR analysis of actinopyrone D (**1**). Bold lines show ^{1}H–^{1}H spin networks and arrows indicate ^{1}H–^{13}C long-range correlations.

Biological activity

Luciferase reporter assay was carried out using human fibrosarcoma HT1080 cells transformed with the luciferase gene under the control of the GRP78 promoter (HT1080 G-L).[8] Treatment of HT1080 G-L cells with 10 mM of 2-deoxyglucose (2DG) for 18 h increased luciferase activity about sevenfold compared with non-treated control. Actinopyrone D (**1**) and PM050463 (**2**) dose-dependently inhibited the luciferase expression by 2DG with IC$_{50}$s of 7.8 and 9.2 nM, respectively (Figure 3). Under the same condition, they did not release lactate dehydrogenase (LDH) from the cells (Figure 3), indicating that these inhibitory effects were not caused by cytotoxicity. Western blot analysis showed that **1** at 100 nM remarkably reduced the expression of GRP78 protein in 2DG-treated HT1080 cells, although **1** did not affect the induction of GRP78 by tunicamycin or A23187 (Figure 4). The effect of **1** on cell viability under ER-stress condition was evaluated using colony formation assay. Actinopyrone D induced cell death in HT1080 cells in the presence of 2DG or under a glucose-deprived condition, whereas no cytotoxicity was observed in HT1080 cells treated with only **1** (Figure 5).

DISCUSSION

Actinopyrone D (**1**) is a new γ-pyrone antibiotic related to PM050463 (**2**) and PM060054 (**3**, Figure 1), which have been isolated from a marine actinomycete *Streptomyces albus* together with their β-D-glucosyl derivatives.[5] Other related metabolites, actinopyrones A (**4**), B (**5**) and C (**6**), have been produced by *Streptomyces pactum*.[9,10] They commonly contain a 2,3-dehydro C$_{17}$ side chain, whereas **1**, **2** and **3** have a 1,2-dehydro side chain. Among them, only **1** contains a *cis* olefin moiety. The activity of **1** and **2** was almost the same and **1** was stable at room temperature, indicating that the geometry of C-6 does not affect their activity.

The actinopyrone-group antibiotics show various activities including antimicrobial activity, cytotoxic activity, vasodilating activity and EGF signaling inhibitory activity.[5,9] Their structural features are related to those of the piericidin-family antibiotics,[11] which consist of a similar or the same side chain and a substituted pyridine ring. Among them, piericidin A has been reported to inhibit the transcriptional expression of GRP78 induced by ER stress.[12] Piericidins are known to be mitochondrial NADH-ubiquinone

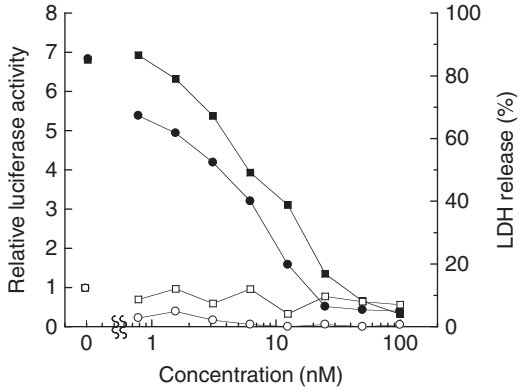

Figure 3 Effects of actinopyrones D (**1**) and PM050463 (**2**) on the luciferase expression and lactate dehydrogenase (LDH) release in HT1080 G-L cells. HT1080 G-L cells were treated with **1** (●, ○) or **2** (■, □) in the presence of 2-deoxyglucose (10 mM) for 18 h at 37 °C. The relative luciferase activity (●, ■) compared with non-treated control was measured with a luminometer. The LDH release (○, □) was calculated as free LDH/total LDH using Wako LDH-Cytotoxic test.

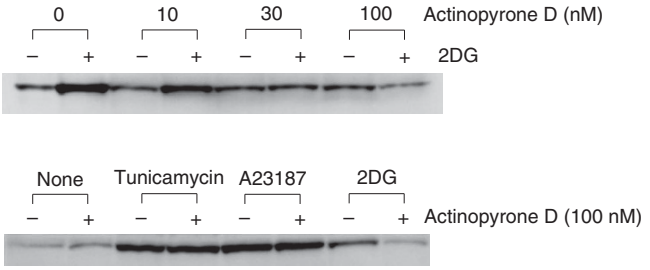

Figure 4 Western blot analysis of GRP78 expression. HT1080 cells were treated with actinopyrone D (**1**) in the presence or absence of endoplasmic reticulum-stress inducers for 18 h. The cell lysate was analyzed by SDS-polyacrylamide gel followed by western blotting. Tunicamycin, A23187 and 2-deoxyglucose (2DG) indicate 6.25 μg ml^{-1} of tunicamycin, 4 μM of A23187 and 10 mM of 2DG, respectively.

oxidoreductase inhibitors.[13] Similar action might cause the above activities of actinopyrones except antibacterial activity. Further studies on the biological activities of actinopyrone D are in progress.

EXPERIMENTAL PROCEDURE

Microorganism

Strain RAG92 was isolated from a soil sample collected at Noguchi, Nikko-shi, Tochigi, Japan. Genomic DNA was extracted by the standard method.[14] The 16S rRNA gene fragment was amplified by PCR using primers with additional *Xba*I and *Hin*dIII sites (5′-GCTCTAGAAGAGTTTGATCCTGGCTCAG-3′ and 5′-ACCAAGCTTAAGGAGGTGATCCAGCCGCA-3′).[15] After heating at 98 °C for 1 min, PCR was performed for 30 cycles (98 °C 10 s, 57 °C 30 s, 68 °C 2 min) using KOD Plus ver. 2 DNA polymerase (Toyobo, Osaka, Japan). The PCR product was linked with *Xba*I/*Hin*dIII-digested pGEM-7Z (Promega, Madison, WI, USA) by using T4 DNA ligase. DNA sequencing was carried out with a PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequence was compared with the bacterial sequence data stored in NCBI (National Center for Biotechnology Information) database by using BLAST algorithm.[16] The sequence revealed high-sequence identity with *Streptomyces spiralis* NBRC 14215 (99.1%), *Streptomyces albus* subsp. *albus* JCM 10204 (98.7%) and *Streptomyces fumigatiscleroticus* NBRC 12999 (98.7%). Accordingly, strain RAG92 was identified as a member of the genus *Streptomyces* and named *Streptomyces* sp. RAG92. The 16S rRNA gene sequence of *Streptomyces* sp. RAG92 reported here has been deposited in the GenBank, DDBJ and EMBL databases under accession number AB915873.

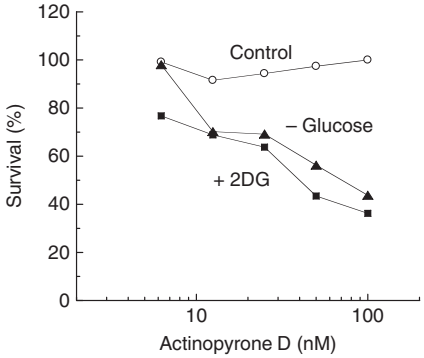

Figure 5 Selective cytotoxicity of actinopyrone D (**1**) in the presence of an endoplasmic reticulum (ER)-stress inducer. HT1080 cells were treated with **1** for 18 h in the presence or absence of an ER-stress inducer. The cells were plated and incubated for 7 days to form colonies. The cell viability was calculated as percent of colony number in control culture. +2DG, 2-deoxyglucose (10 mM), −glucose, glucose-deprived condition.

Actinopyrone D (1)

Colorless oil; $[\alpha]_D^{23}$ −120° (*c* 0.026, MeOH); FAB-MS (*m/z*) found: 401.2694 $(M+H)^+$, calcd. for $C_{25}H_{37}O_4$: 401.2692; UV (MeOH) λmax nm (ε) 227 (35 100), 272 (12 800); IR ν_{max} (KBr) 3390, 1660 cm^{-1}; ^{13}C and ^{1}H NMR ($CDCl_3$) see Table 1.

Spectroscopic measurements

UV and IR spectra were measured on a UV-1700 spectrometer (Shimadzu, Kyoto, Japan) and a Spectrum 100 FT-IR spectrometer (PerkinElmer, Waltham, MA, USA) in the attenuated total reflection mode. Mass spectra were obtained on a JMS-SX102A spectrometer (JEOL, Akishima, Tokyo, Japan) in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. NMR spectra were obtained on a JNM-LA400 spectrometer (JEOL) with ^{1}H NMR at 400 MHz and with ^{13}C NMR at 100 MHz. Chemical shifts are given in p.p.m. relative to $CDCl_3$ (δ_H 7.24 and δ_C 77.0) or CD_3OD (δ_H 3.30 and δ_C 49.0).

Cells and cell culture

HT1080 G-L cells were established by transfection of HT1080 human fibrosarcoma cells with the plasmid pGRP70pro160-Luc containing a firefly luciferase gene under the control of the GRP78 promoter.[8] The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose at 37 °C.

Reporter assay

HT1080 G-L cells were plated into 96-well plates at a density of 2×10^3 cells/well. After incubation for 6 h at 37 °C, the cells were treated with samples in the presence or absence of 2DG (10 mM) for 18 h at 37 °C. After the medium was removed, 100 μl of Cell Culture Lysis Reagent (Promega) were added to each well. Each 50 μl portion of the cell lysate was transferred into a white 96-well plate. Luciferase activity was measured with a TR717 microplate luminometer (Applied Biosystems) using Luciferse Assay Reagent (Promega).

LDH release assay

HT1080 G-L cells were plated into 96-well plates at a density of 2×10^3 cells/well. After incubation for 6 h at 37 °C, the cells were cultured with various concentrations of samples for 18 h at 37 °C. The conditioned medium was transferred into another 96-well plate, and the residual cells were dissolved in 100 μl of 0.5% Triton X-100. LDH-Cytotoxic Test (Wako, Osaka, Japan) was used to measure LDH concentrations in the conditioned medium and cell lysate. The rate of free LDH was calculated according to the below equation by

using the LDH concentration in the conditioned medium as the amount of free LDH and the LDH concentration in the cell lysate as the amount of intracellular LDH. Rate of free LDH (%) = (amount of free LDH)/(amount of free LDH + amount of intracellular LDH) × 100.

Western blotting

HT1080 cells were cultured in 9-cm Petri dishes at a density of 2×10^5 cells/dish for 24 h at 37 °C. After incubation with or without samples under ER stress for 18 h at 37 °C, the cells were collected using a cell scraper and lysed in a SDS sample buffer consisting of 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl (pH 6.8). Protein concentrations of the lysates were measured with Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Aliquots containing 45 μg of protein were resolved on 7.5% SDS-polyacrylamide gel and transferred by electroblotting to a polyvinylidene difluoride membrane. The membrane was pretreated with PBS-T (Tween 20 0.05%, NaCl 0.8%, KCl 0.02%, $Na_2HPO_4 \cdot 12H_2O$ 0.29%, KH_2PO_4 0.02%) containing 10% skim milk for 1 h and incubated with 2 μg ml^{-1} of mouse monoclonal anti-KDEL (Enzo Life Sciences, Farmingdale, NY, USA) in PBS-T for 1 h. The membrane was washed with PBS-T and incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin antibody (1:2000) (GE Healthcare, Piscataway, NJ, USA) for 1 h. After washing with PBS-T, the protein expression of GRP78 was detected with an ECL detection system (GE Healthcare) using an ImageQuant LAS 4000mini System (GE Healthcare).

Colony formation assay

HT1080 cells were plated in 9-cm Petri dishes at a density of 2×10^5 cells/dish. The cells were treated with various concentrations of samples in the presence or absence of 2DG (10 mM) or under a glucose-deprived condition for 18 h. Trypsinized cells were plated at 200 cells/dish and incubated for 7 days to form colonies. After the colonies were stained with 0.01% crystal violet, the cell viability was calculated as percent of colony number in control culture.

ACKNOWLEDGEMENTS

We thank F Hasegawa, Tokyo University of Science, for assistance with mass spectrometry.

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