J. Antibiot. 61(5): 303–311, 2008

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

SAR Study of a Novel Triene-ansamycin Group Compound, Quinotrierixin, and Related Compounds, as Inhibitors of ER Stress-induced XBP1 Activation

I. Taxonomy, Fermentation, Isolation, Biological Activities and SAR Study

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Received: February 25, 2008 / Accepted: May 19, 2008

Abstract In the course of screening for an inhibitor of ER stress-induced XBP1 activation, we isolated a new member of the triene-ansamycin group compound, quinotrierixin, from a culture broth of *Streptomyces* sp. PAE37. Quinotrierixin inhibited thapsigargin-induced XBP1 activation in HeLa cells with an IC_{50} of 0.067 μM. We found that other triene-ansamycin group compounds such as demethyltrienomycin A and mycotrienin I also inhibited ER stress-induced XBP1 activation. Moreover, we performed SAR study of twelve triene-ansamycin group compounds. The study showed that OH group at C-13 was crucial, and CH_3 group at C-14 would be important for the XBP1 inhibitory activity.

Keywords triene-ansamycin, ER stress, XBP1

Introduction

In tumor microenvironments adverse conditions such as hypoxia, nutrient deprivation and low pH, solid tumor cells are considered to adapt to ER stress by activating X-box binding protein 1 (XBP1) [1, 2]. Indeed, there is now abundant evidence to suggest that XBP1 is a critical transcriptional regulator of this process and required for tumor survival [3～5]. Therefore, we have been screening for an inhibitor of XBP1 activation because the inhibitor of XBP1 activation would be a new type of anticancer drug.

Recently, we have established a screening system for an inhibitor of ER stress-induced XBP1 activation, in which XBP1 activation can be easily detected as luciferase reporter signals in HeLa/XBP1-luc cells [6]. Using this screening system, we have previously reported on the isolation of a novel compound, trierixin (**2**), from a culture broth of *Streptomyces* sp. AC654, as an inhibitor of ER stress-induced XBP1 activation [6]. In continuation of this screening, we found that a culture broth extract of *Streptomyces* sp. PAE37 inhibited ER stress-induced XBP1 activation and isolated a novel triene-ansamycin group compound, quinotrierixin (**1**), as an inhibitor of XBP1. Despite of the structural similarity of **1** to **2**, the XBP1 inhibitory activity of **1** was about 1/5 compared to that of **2** (Table 3). This observation prompted us to perform SAR study to know which functional groups of triene-ansamycin group compounds are required for the inhibitory effects against ER stress-induced XBP1 activation. Because the quinotrierixin-producing strain, *Streptomyces* sp. PAE37, was found to produce several triene-ansamycin group compounds, we further isolated six triene-ansamycin group compounds including three other novel compounds, demethyltrienomycin A (**3**), demethyltrienomycin B (**4**), and demethyltrienomycinol (**5**) from *Streptomyces* sp.

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PAE37, and prepared four derivatives of triene-ansamycin group compounds for SAR study. Furthermore, we also examined whether the XBP1 inhibitory activities of the twelve triene-ansamycin group compounds could correlate with their tumor cell growth inhibitory activities.

Structure elucidation of **1**, **3**, **4**, and **5** will be reported in the accompanying paper [7].

Materials and Methods

General Experimental Procedures

Mass spectra were measured with a JEOL JMS-T100LC mass spectrometer. UV spectra were recorded on a Hitachi U-2800 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained on a JEOL JNM-AL operating at 300 and 75 MHz, respectively. LC/MS system (Waters Corp., USA) with the photo diode array detector (2996) and mass analyzer (micromass ZQ) was used for analysis and preparation.

Taxonomic Studies

The producing strain PAE37 was isolated from a soil sample collected in Sugadaira, Nagano prefecture, Japan. The morphological characteristics of the strain PAE37 were determined on yeast-starch agar and glucose-asparagine agar incubated at 27°C for 6 days.

Preparation of Triene-ansamycin Derivatives

1) Quinotrierixin HQ (**6**)

Quinotrierixin (**1**, 3.0 mg, 4.4 μmol) was dissolved in MeOH (1.0 ml). Five mg of $Na_2S_2O_4$ was added to the solution, and the reaction mixture was stirred for 30 minutes at room temperature. The mixture was filtered, and the filtrate was partitioned with EtOAc/H_2O (each 20 ml). The EtOAc layer was dried over Na_2SO_4 and evaporated to dryness to yield quinotrierixin HQ (**6**) as a colorless powder (2.5 mg, yield 83%).

2) Trierixin Q (**7**)

Trierixin (**2**, 3.2 mg, 4.7 μmol) was dissolved in 2.0 ml of 1.0% methanolic $FeCl_3$ solution and stirred for 1 hour at room temperature. The reaction mixture was partitioned with EtOAc/H_2O (each 20 ml), and the EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo* to yield trierixin Q (**7**) as a red powder (3.1 mg, yield 97%).

3) 13-Keto-mycotrienin I (**8**) and 13-Keto-mycotrienin II(**9**)

Mycotrienin I (**10**, 3.0 mg, 4.7 μmol) was dissolved in 0.5 ml of anhydrous CH_2Cl_2, and 4.0 mg of Dess-Martin periodinane (Sigma-Aldrich, Saint Louis, MO) was added to the solution. After stirring for 3 hours at room temperature, the solution was partitioned with EtOAc (10 ml)/saturated $Na_2S_2O_3$ aq (5.0 ml)+saturated $NaHCO_3$ aq (5.0 ml). The EtOAc layer was dried over Na_2SO_4 and concentrated *in vacuo* to yield 13-keto-mycotrienin I (**8**) as a yellow powder (2.5 mg, yield 84%).

Mycotrienin II (**11**, 3.0 mg, 4.7 μmol) was treated in the same way, and 13-keto-mycotrienin II (**9**) was obtained as a colorless powder (2.6 mg, yield 87%).

Cell Culture

Human epithelial adenocarcinoma cell line HeLa and HeLa/XBP1-luc were cultured in DMEM supplemented with 10% FBS. HeLa/XBP1-luc cells were generated previously [6].

Luciferase Assay

As reported previously [6], HeLa/XBP1-luc cells were seeded in 96-well plates at 2×10^4 cells/well, and then incubated with 0.1 μM of thapsigargin together with or without each triene-ansamycin group compound. After 24 hours of incubation, the cells were lysed in Passive lysis buffer (Promega, Madison, MA), and then luciferase activity was measured using the luciferase assay system (Promega) and a luminometer (Wallac, PerkinElmer, Waltham, MA). IC_{50} values were determined from the dose-response curves of the inhibition of XBP1-luciferase activity when the XBP1-luciferase activity of thapsigargin treatment was defined as 100%.

RT-PCR

As reported previously [6], HeLa cells were seeded in 12-well plates at 5×10^4 cells/well, and then incubated with 0.1 μM of thapsigargin together with or without each triene-ansamycin group compound for 8 hours. Subsequently, total RNA was extracted from HeLa cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). Aliquots 2.0 μg of total RNA were treated with M-MLV reverse transcriptase (Promega) to produce 1st strand cDNA, which was subjected to PCR with KOD Plus polymerase (TaKaRa, Shiga, Japan) using a pair of primers corresponding to nucleotides 505～525 and 609～629 of XBP1 cDNA. The amplified products were separated by electrophoresis on a 6.0% polyacrylamide gel and visualized with ethidium bromide staining.

MTT Assay

HeLa cells were seeded at 2×10^3 cells/well in 96-well plates and cultured overnight. The cells were treated with various concentrations of each triene-ansamycin group compound for 72 hours. Growth was measured by

formazan formation (detected at 570 nm) after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C. IC_{50} values were determined from the dose-response curves of growth inhibition.

Results

Taxonomy of the Producing Strain

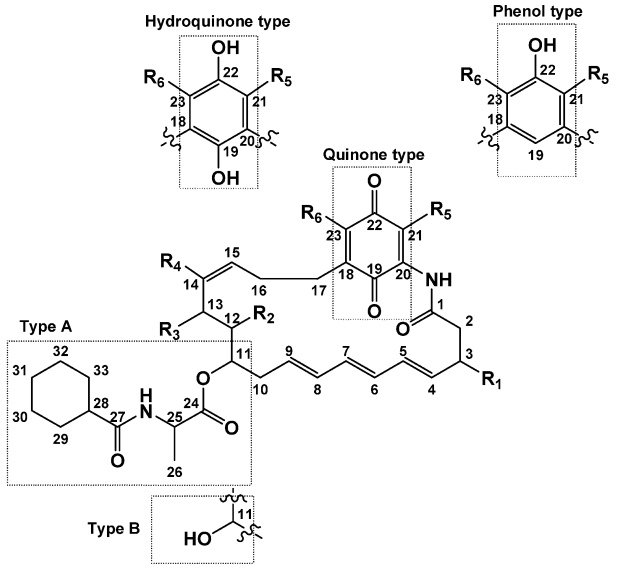
The substrate mycelia of strain PAE37 developed abundantly, were irregularly branched and did not show fragmentation into coccoid forms or bacillary elements. This strain produced dozen of spore chains which were straight, curved or rarely spiraling, on aerial mycelia. LL-diaminopimelic acid was observed in the whole-cell hydrolysates, indicating that the cell wall is type I. On the basis of these morphological and chemotaxonomic characteristics, strain PAE37 was assigned to the genus *Streptomyces*.

Fermentation of *Streptomyces* sp. PAE37

A thawed suspension of *Streptomyces* sp. PAE37 was used for inoculation in a 500-ml Erlenmeyer flask containing 100 ml of a seed medium composed of 2.5% D(+)-glucose, 0.2% yeast extract, 1.5% soybean meal and 0.4% $CaCO_3$. The medium was adjusted to pH 7.2 before sterilization. The culture was incubated at 27°C for 2 days on a rotary shaker (150 rpm). Aliquots of this seed culture were added to a 500-ml Erlenmeyer flask containing 100 ml of the same medium. The culture was incubated at 27°C for 3 days on a rotary shaker (150 rpm).

Isolation and Purification of Quinotrierixin (1)

The culture broth (2.0 liters) of *Streptomyces* sp. PAE37 was centrifuged to separate the mycelial cake and the supernatant. The mycelial cake was stirred successively with Me_2CO (1.0 liter) for 1 hour and filtrated. The filtrates was concentrated under reduced pressure to remove Me_2CO and combined with broth supernatant because the XBP1 inhibitory activities of the supernatant and the mycelium



	Structural units	R_1	R_2	R_3	R_4	R_5	R_6	Side chain type
1	Quinone	OCH_3	CH_3	OH	CH_3	H	SCH_3	A
2	Hydroquinone	OCH_3	CH_3	OH	CH_3	SCH_3	H	A
3	Phenol	OCH_3	CH_3	OH	H	H	H	A
4	Phenol	OH	H	OH	CH_3	H	H	A
5	Phenol	OH	H	OH	CH_3	H	H	B
6	Hydroquinone	OCH_3	CH_3	OH	CH_3	H	SCH_3	A
7	Quinone	OCH_3	CH_3	OH	CH_3	SCH_3	H	A
8	Quinone	OCH_3	CH_3	= O	CH_3	H	H	A
9	Hydroquinone	OCH_3	CH_3	= O	CH_3	H	H	A
10	Quinone	OCH_3	CH_3	OH	CH_3	H	H	A
11	Hydroquinone	OCH_3	CH_3	OH	CH_3	H	H	A
12	Phenol	OCH_3	CH_3	OH	CH_3	H	H	A

Fig. 1 Structures of triene-ansamycin group compounds.

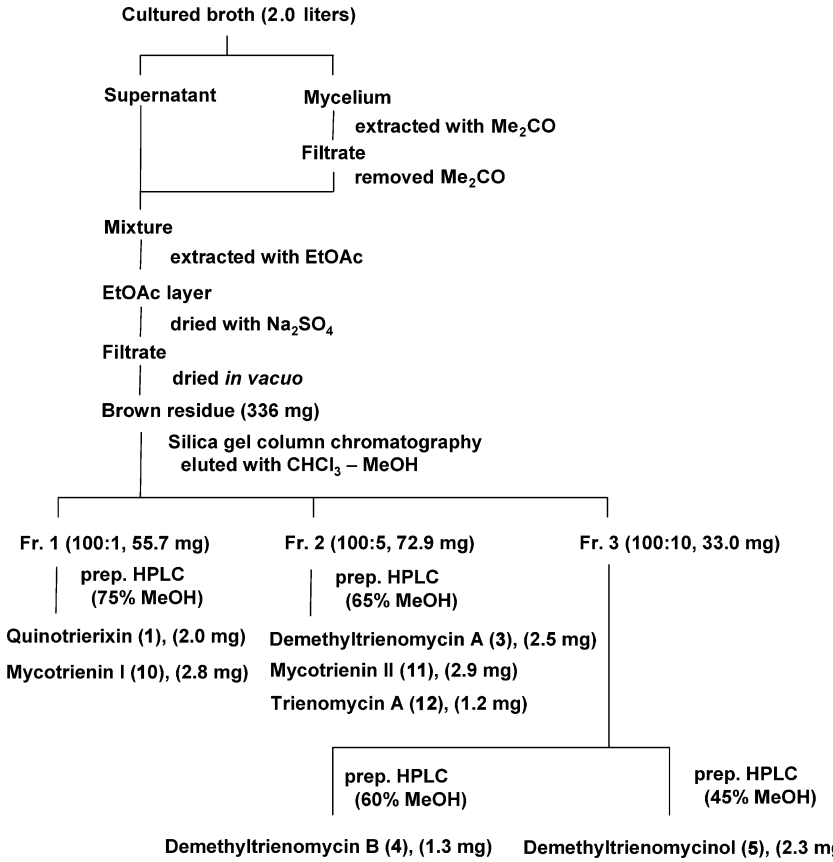


Fig. 2 Isolation procedure of quinotrierixin (**1**) and related compounds from Streptomyces sp. PAE37.

were in the same range. This solution was extracted with EtOAc (2.0 liters) twice, and the organic layer was concentrated to give an oily residue (336 mg). The crude oil was subjected to silica gel chromatography (30 g, Silica gel 60, 60~230 μm, Merck) using $CHCl_3$ - MeOH (100 : 1). The active fractions were collected and further purified by preparative ODS HPLC (Sun Fire, 10 μm, 19×250 mm, Waters Corp., USA) with 75% aq MeOH to give pure **1** (2.0 mg). The structure, the isolation procedure, the physico-chemical properties and the ^{13}C-NMR spectral data of **1** were shown in Figs. 1 and 2, Tables 1 and 2, respectively.

Biological Activities of Quinotrierixin (1)

The inhibitory activity of **1** against ER stress-induced XBP1-luciferase activation was first assessed using HeLa/XBP1-luc cells. Treatment of HeLa/XBP1-luc cells with 0.1 μM thapsigargin for 24 hours increased XBP1-luciferase activities, and **1** inhibited thapsigargin-induced activation in a dose-dependent manner with an IC_{50} value of 0.082 μM (Fig. 3). Furthermore, to examine whether **1** also inhibited thapsigargin-induced endogenous XBP1 mRNA splicing in HeLa cells, RT-PCR analysis was performed. As described previously [6], treatment with 0.1 μM thapsigargin for 8 hours induced endogenous XBP1 mRNA splicing. **1** inhibited thapsigargin-induced endogenous XBP1 mRNA splicing in a dose-dependent manner with an IC_{50} value of 0.067 μM (Fig. 4). These results indicated that **1** is an inhibitor of ER stress-induced XBP1 activation.

Isolation and Purification of Six Triene-ansamycin Group Compounds, Including Demethyltrienomycin A (3), Demethyltrienomycin B (4), and Demethyltrienomycinol (5), and Preparation of Four Derivatives of Triene-ansamycin Group Compounds

Recently, we have reported the isolation of trierixin (**2**), a structurally related compound with quinotrierixin (**1**), as an inhibitor of ER stress-induced XBP1 activation [6]. The structural differences between **1** and **2** were the type of a benzenoid moiety (quinone/hydroquinone) and SCH_3 position (C-23/C-21). However, the XBP1 inhibitory activity of **1** was about 1/5 compared to that of **2** (Table 3). Thus, we performed SAR study to know which functional group was required for the inhibitory effects against ER stress-induced XBP1 activation using triene-ansamycin

Table 1 Physico-chemical properties of twelve triene-ansamycin group compounds

	1	**2**	**3**	**4**	**5**	**6**
Appearance	Pale yellow powder	Pale yellow powder	Colorless powder	Colorless powder	Colorless powder	Pale yellow powder
Molecular formula	$C_{37}H_{50}N_2O_8S$	$C_{37}H_{52}N_2O_8S$	$C_{35}H_{48}N_2O_7$	$C_{34}H_{46}N_2O_7$	$C_{24}H_{31}NO_5$	$C_{37}H_{52}N_2O_8S$
Molecular weight	682	684	608	594	413	684
HRESI-MS (*m/z*, Pos.)						
Calcd.	705.3186	—	631.3359	617.3203	436.2100	707.3342
	(as $C_{37}H_{50}N_2NaO_8S$)	—	(as $C_{35}H_{48}N_2NaO_7$)	(as $C_{34}H_{46}N_2NaO_7$)	(as $C_{24}H_{31}NNaO_5$)	(as $C_{37}H_{52}N_2NaO_8S$)
Found.	705.3210	—	631.3360	617.3220	436.2110	707.3331
UV λ_{max}^{MeOH} nm	250, 261, 270, 281, 340	250, 261, 271, 281, 315	213, 250, 259, 270, 282	210, 250, 259, 270, 281	211, 251, 260, 271, 282	251, 261, 271, 281, 316
HPLC (Rt, min)[a]	20.8 (80%MeOH)	15.4 (75%MeOH)	22.0 (65%MeOH)	20.3 (60%MeOH)	19.7 (45%MeOH)	15.5 (75%MeOH)

	7	**8**	**9**	**10**	**11**	**12**
Appearance	Pale red powder	Pale yellow powder	Colorless powder	Pale yellow powder	Colorless powder	Colorless powder
Molecular formula	$C_{37}H_{50}N_2O_8S$	$C_{36}H_{46}N_2O_8$	$C_{36}H_{48}N_2O_8$	$C_{36}H_{48}N_2O_8$	$C_{36}H_{50}N_2O_8$	$C_{36}H_{50}N_2O_7$
Molecular weight	682	634	636	636	638	622
HRESI-MS (*m/z*, Pos.)						
Calcd.	705.3186	657.3152	659.3308	—	—	—
	(as $C_{37}H_{50}N_2NaO_8S$)	(as $C_{36}H_{46}N_2NaO_8$)	(as $C_{36}H_{48}N_2NaO_8$)			
Found.	705.3200	657.3142	659.3296	—	—	—
UV λ_{max}^{MeOH} nm	251, 261, 271, 282, 492	251, 261, 271, 282, 389	251, 261, 271, 282, 310	251, 261, 271, 282, 383	251, 261, 271, 282, 310	213, 250, 260, 270, 282
HPLC (Rt, min)[a]	11.4 (80%MeOH)	11.3 (80%MeOH)	18.6 (70%MeOH)	12.8 (80%MeOH)	16.6 (70%MeOH)	17.6 (70%MeOH)

[a] Column, SunFire C_{18} (Waters, 5 mm, 4.6×250 mm); mobile phase, aqMeOH; flow rate, 0.7 ml/minute

Table 2 ^{13}C-NMR assignments of twelve triene-ansamycin group compounds

Carbon No.	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[b]	**6**[a]
C-1	169.4	170.8	168.6	171.0	171.3	169.9
C-2	44.7	43.5	43.3	46.6	46.6	45.0
C-3	78.8	80.0	78.6	72.0	71.7	78.4
C-4	130.8	129.8	129.5	135.3	134.3	129.2
C-5	133.9	135.8	133.4	132.9	132.8	134.1
C-6	129.3	130.3	129.4	131.3	131.0	129.3
C-7	134.1	135.5	134.1	134.4	134.9	134.2
C-8	133.3	134.7	133.4	134.6	134.2	133.7
C-9	129.8	130.4	129.4	130.0	131.6	129.6
C-10	33.0	34.9	33.3	37.1	41.5	33.9
C-11	75.3	76.3	75.6	72.3	69.0	75.0
C-12	38.6	39.8	40.4	39.2	41.6	39.2
C-13	68.5	69.4	66.5	66.9	68.2	68.6
C-14	139.4	139.0	133.4	139.7	139.4	137.5
C-15	123.8	124.8	129.3	125.7	126.6	125.5
C-16	26.1	27.1	34.9	29.8	30.2	27.1
C-17	28.8	33.2	35.7	37.0	37.3	31.9
C-18	142.0	136.9	143.7	144.8	145.1	136.7
C-19	178.7	143.5	111.0	114.0	113.9	141.6
C-20	137.7	127.1	138.1	139.8	139.8	126.9
C-21	115.8	110.9	106.2	107.8	107.8	106.0
C-22	184.1	150.9	157.3	158.6	158.6	150.3
C-23	147.7	116.1	111.9	112.6	112.7	119.0
C-24	172.9	174.1	173.0	173.9	—	172.9
C-25	48.5	49.5	48.5	49.7	—	48.5
C-26	17.7	18.8	17.8	17.2	—	18.0
C-27	176.5	177.4	176.5	179.1	—	176.3
C-28	45.0	46.0	45.0	45.8	—	45.1
C-29	29.4	30.4	29.4	30.4	—	29.4
C-30	25.6	26.6	25.6	26.6	—	25.6
C-31	25.6	26.6	25.7	26.8	—	25.6
C-32	25.6	26.7	25.6	26.6	—	25.6
C-33	29.4	30.5	29.4	30.8	—	29.4
12-CH_3	10.0	10.5	9.6	—	—	9.7
14-CH_3	20.4	21.2	—	18.6	19.1	19.9
3-OCH_3	56.7	57.6	56.8	—	—	56.8
21-SCH_3	—	19.4	—	—	—	—
23-SCH_3	18.0	—	—	—	—	19.4

Chemical shifts in ppm from TMS as internal standard

[a] Recorded at 75 MHz in $CDCl_3$

[b] Recorded at 75 MHz in CD_3OD

Table 2 (Continued)

Carbon No.	**7**[a]	**8**[a]	**9**[b]	**10**[a]	**11**[a]	**12**[a]
C-1	167.3	169.3	171.7	169.7	169.7	168.5
C-2	43.1	43.9	43.5	44.8	43.1	43.5
C-3	78.7	78.1	81.9	79.2	79.6	78.5
C-4	129.8	130.7	131.4	131.3	129.1	130.6
C-5	134.1	134.0	136.8	133.7	134.4	133.5
C-6	129.4	129.8	130.0	129.5	129.5	129.3
C-7	134.5	133.3	135.7	133.7	134.9	134.1
C-8	133.1	133.2	135.0	133.2	133.9	133.4
C-9	129.4	128.5	130.8	129.3	129.6	129.4
C-10	32.9	32.9	33.5	33.0	33.7	33.1
C-11	75.2	73.8	77.9	75.2	75.8	75.5
C-12	40.0	46.9	46.6	39.9	39.0	39.6
C-13	68.0	204.9	210.4	68.0	68.7	68.4
C-14	139.8	136.9	138.2	139.9	137.8	138.6
C-15	127.5	135.6	138.4	122.5	124.3	124.7
C-16	25.6	27.8	31.0	25.6	26.6	29.3
C-17	29.7	29.7	33.0	29.4	31.7	36.2
C-18	136.9	138.1	132.9	137.9	132.7	144.1
C-19	181.0	182.7	142.4	188.2	141.1	110.8
C-20	146.5	144.7	127.1	145.4	125.5	138.4
C-21	136.4	114.8	108.8	114.5	107.5	105.7
C-22	182.5	188.0	151.0	182.5	149.2	157.2
C-23	134.0	133.8	116.1	133.1	115.8	111.9
C-24	173.1	172.2	173.3	172.9	173.3	172.9
C-25	48.6	47.8	50.1	48.5	48.7	48.5
C-26	17.4	18.6	17.2	17.4	17.7	17.8
C-27	176.8	175.4	179.1	176.6	176.9	176.6
C-28	44.9	45.2	45.9	44.9	45.1	45.1
C-29	29.5	29.5	30.6	29.4	29.4	29.5
C-30	25.5	25.6	26.9	25.6	25.6	25.6
C-31	25.6	25.7	26.8	25.5	25.6	25.5
C-32	25.5	25.6	27.0	25.5	25.7	25.7
C-33	29.4	29.5	30.7	29.3	29.4	29.7
12-CH_3	9.8	11.0	13.0	9.6	9.6	9.8
14-CH_3	20.5	21.3	21.6	20.5	20.3	20.3
3-OCH_3	56.7	57.0	56.7	56.6	56.6	56.8
21-SCH_3	17.4	—	—	—	—	—
23-SCH_3	—	—	—	—	—	—

Chemical shifts in ppm from TMS as internal standard

[a] Recorded at 75 MHz in $CDCl_3$

[b] Recorded at 75 MHz in CD_3OD

group compounds. To perform SAR study of triene-ansamycin group compounds, we tried to isolate several triene-ansamycin group compounds from *Streptomyces* sp. PAE37, and to prepare unnatural derivatives of natural triene-ansamycin group compounds.

Since triene-ansamycin group compounds show the characteristic UV spectra (λ_{max} 260, 271 and 281 nm), we searched triene-ansamycin group compounds in the cultured broth of *Streptomyces* sp. PAE37 using PDA-LC system irrespective of their bioactivities. As the result, we found three novel triene-ansamycin group compounds demethyltrienomycin A (**3**), demethyltrienomycin B (**4**),

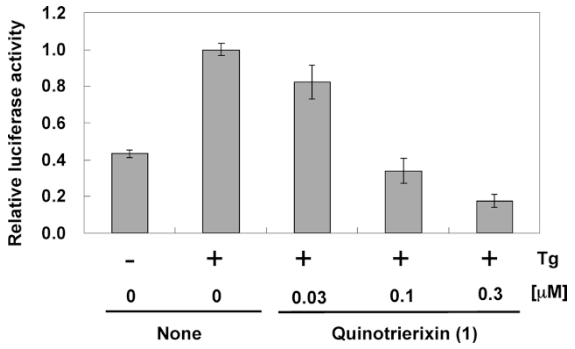


Fig. 3 Quinotrierixin (**1**) inhibited thapsigargin-induced XBP1 activation.

HeLa/XBP1-luc cells were treated with the indicated concentration of quinotrierixin in the presence or absence of 0.1 μM of thapsigargin (Tg). After 24 hours, the cells were lysed and subjected to luciferase assay. Data are the fold of a thapsigargin-treated sample and s.d. of a representative experiment performed in triplicate.

and demethyltrienomycinol (**5**). From 2.0 liters culture, pure **3** (2.5 mg), **4** (1.3 mg), and **5** (2.3 mg) were obtained by using solvent extraction, silica gel column chromatography, and preparative ODS HPLC. Furthermore, in the isolation process of these compounds, mycotrienin I (**10**), mycotrienin II (**11**) [8], and trienomycin A (**12**) [9] were also isolated and identified. The overall purification scheme of these compounds including quinotrierixin (**1**) was summarized in Fig. 2.

In addition, we prepared quinotrierixin HQ (**6**), a hydroquinone derivative of **1**, and trierixin Q (**7**), a quinone derivative of **2**, to examine the relationship between benzenoid moiety and the XBP1 inhibitory activity. We also prepared 13-ketomycotrienin I (**8**) and 13-ketomycotrienin II (**9**) to examine the effects of OH group at C-13 on the XBP1 inhibitory activity. The preparation of **6**, **7**, **8**, and **9** is described in Materials and Methods. The structure, the physico-chemical properties and the ^{13}C-NMR spectral data of all triene-ansamycin group compounds were shown in Fig. 1, Tables 1 and 2,

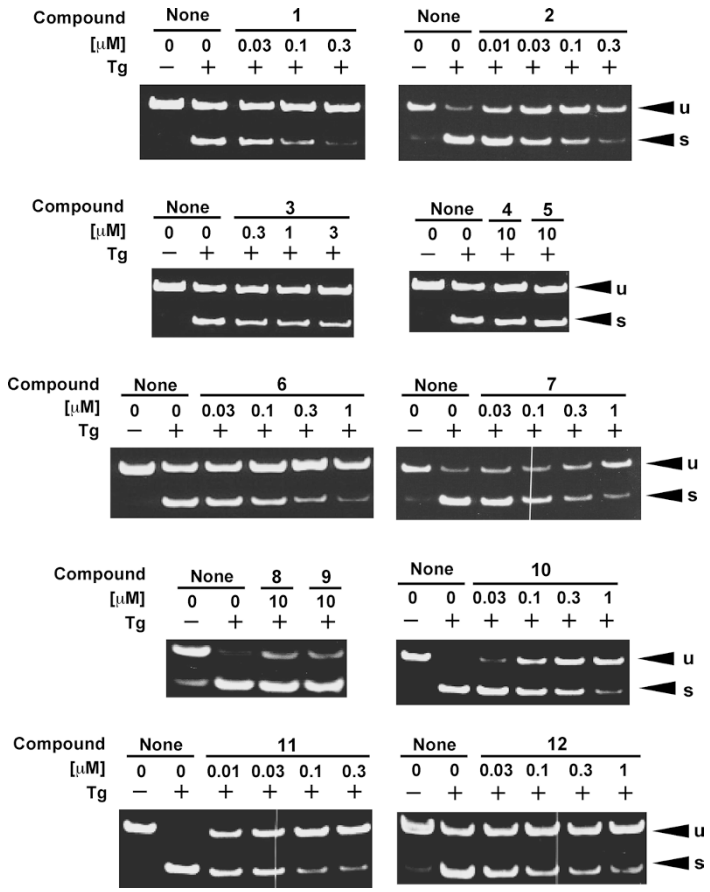


Fig. 4 Triene-ansamycin group compounds inhibited thapsigargin-induced XBP1 mRNA splicing.

HeLa cells were treated with the indicated concentration of triene-ansamycin group compounds in the presence or absence of 0.1 μM of thapsigargin (Tg) for 8 hours. The cells were collected and extracted RNA was subjected to RT-PCR. Spliced- or unspliced-XBP1 mRNA was detected as described in Experimental. [s] and [u] indicate spliced and unspliced XBP1 mRNA, respectively.

respectively.

SAR Study of Twelve Triene-ansamycin Group Compounds as Inhibitors of ER Stress-induced XBP1 Activation

The inhibitory activity of each triene-ansamycin group compound against ER stress-induced XBP1 activation was evaluated by the luciferase assay (Table 3) and RT-PCR (Fig. 4). **10** and **11** inhibited both thapsigargin-induced XBP1-luciferase activation (Table 3) and endogenous XBP1 mRNA splicing (Fig. 4) with IC_{50} of less than 1.0 μM, respectively, whereas **8** and **9** neither inhibited thapsigargin-induced XBP1-luciferase activation nor endogenous XBP1 mRNA splicing even at 10 μM. These results indicated that OH group at C-13 is essential for the XBP1 inhibitory activity. XBP1-luciferase inhibitory activity of **4** was reduced to 1/500 of that of **12**, and **4** was unable to inhibit thapsigargin-induced endogenous XBP1 mRNA splicing up to 10 μM, suggesting that OCH_3 group at C-3 and CH_3 group at C-12 are important for the XBP1 inhibitory activities. The demethylation at C-14 resulted in a reduction of XBP1-luciferase inhibitory activity to 1/20 by comparing **3** and **12**, suggesting that CH_3 group at C-14 is also important for the XBP1 inhibitory activity. Although the XBP1-luciferase inhibitory activity of **7** was 1/20 compared to that of **2**, both **1** and **6** showed almost the same activities, suggesting that the types of a benzenoid moiety (quinone/hydroquinone) are not so significant for their XBP1 inhibitory activities. Furthermore, by comparing **1**, **7**, and **10** or **2**, **6**, and **11**, the following rank orders of XBP1-luciferase inhibitory activities were obtained: **1**>**7**>**10** and **2**>**6**>**11**. The same results were obtained when the XBP1 inhibitory activity was evaluated by thapsigargin-induced endogenous XBP1 mRNA splicing in HeLa cells (Fig. 4). These results suggested that the presence of a SCH_3 group within a benzenoid moiety might be important for showing higher XBP1 inhibitory activity, whereas the position of a SCH_3 group is not so crucial for the XBP1 inhibitory activity.

Table 3 Biological activities of twelve triene-ansamycin group compounds

Compound	XBP1-luciferase inhibition	Cell growth inhibition
1	0.082	0.055
2	0.016	0.010
3	0.40	0.86
4	>10	>10
5	>10	>10
6	0.078	0.20
7	0.32	0.10
8	>10	>10
9	>10	>10
10	0.95	0.60
11	0.10	0.090
12	0.019	0.029

IC_{50} value (μM)

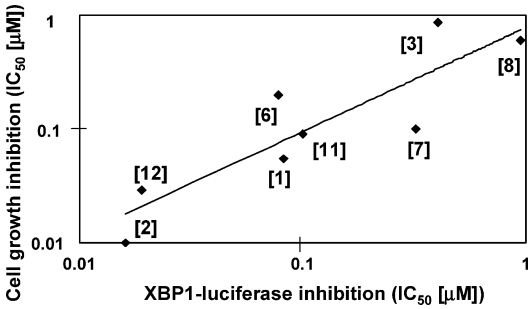


Fig. 5 Correlation between inhibitory activities of triene-ansamycin group compounds against XBP1 activation and against tumor cell growth.

The parenthetic numbers indicate the number of compounds.

Effects of Twelve Triene-ansamycin Group Compounds on Inhibition of Tumor Cell Growth

Previously, we showed that **2**, **11** and **12** inhibited not only ER stress-induced XBP1 activation but also tumor cell growth with the subequal IC_{50} value, respectively [6]. Therefore, we next examined the effects of each triene-ansamycin group compound against the cell growth of HeLa cells by MTT assay. As shown in Table 3, most compounds except **4**, **5**, **8** and **9**, also inhibited cell growth with IC_{50} value less than 1.0 μM. Interestingly, there was high correlation between inhibitory effects of triene-ansamycin group compounds against XBP1 activation and those against tumor cell growth with the correlation coefficient at 0.71 ($t_{(6)}$=3.51, p<0.05) (Fig. 5).

Discussion

Several biological activities of triene-ansamycin group compounds have been reported. Furthermore, the partial structures of triene-ansamycin group compounds that are crucial for their biological activities have also been reported. For example, it has been reported that the acyl chain (cyclohexanecarbonylalanyl moiety) attached at C-11 was crucial for cytotoxicity of mycotrienin I (**10**) and mycotrienin II (**11**) against mouse leukemia cells [10]. In addition, trienomycin A (**12**), which has the acyl chain

linked at C-11, has more potent inhibitory activity against NO production in microglia cells than trienomycin G, which has the acyl chain linked at C-13 [11]. On the other hand, it was reported that OH group at C-19 and the double bonds at C-4, C-6, and C-8 were crucial for the inhibitory activity of mycotrienin II (**11**) against osteoclastic bone resorption [12]. Furthermore, it has been reported that OH group at C-13 of trienomycin A (**12**) was important for its cytocidal activity against HeLa S_3 cells [13], which is consistent with our findings that both **8** and **9** showed only weak their inhibitory activities against cell growth of HeLa cells (Table 3).

There were many reports that triene-ansamycin group compounds showed antitumor effects *in vitro* and *in vivo* [6, 14∼16], however, underlying mechanisms for the inhibition of tumor growth by these compounds are unclear. In this study, we revealed highly correlation between inhibitory activities of triene-ansamycin group compounds against XBP1 activation and tumor cell growth. These results raised the possibility that the antitumor activities of triene-ansamycin group compounds would be due to the inhibitory effects against XBP1 activation. These findings suggest that this group of compounds may have promise of clinical utility against tumors. The *in vitro* and *in vivo* anti-tumor activity should be explored further in additional to the pharmacokinetics. Also it would be of interest to explore other tumor cells to see if they have alternative pathways for cellular survival under stress.

Acknowledgements We thank Ms. N. Kinoshita (Institute of Microbial Chemistry, Tokyo) for the experiment of taxonomic studies. This study was partly supported by grants from the New Energy and Industrial Technology Development Organization (NEDO) and a grant from Nateglinide Memorial Toyoshima Research and Education Fund of Keio University.

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