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Stresgenin B, an Inhibitor of Heat-induced Heat Shock Protein Gene Expression, Produced by *Streptomyces* sp. AS-9

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Stresgenin B was isolated as an inhibitor of heat-induced heat shock protein (HSP) gene expression from a culture broth of *Streptomyces* sp. AS-9 by silica gel chromatography and HPLC. The molecular formula of the novel compound was determined as $C_{11}H_{13}NO_5$ by high resolution FAB-MS analysis, and the structure was determined by UV, ^1H NMR, ^{13}C NMR, HMQC, HMBC, and NOESY spectra. Stresgenin B inhibited heat-induced luciferase reporter-gene expression directed by the human hsp70B promoter in Chinese hamster ovary (CHO) cells at concentrations lower than the concentrations for inhibition of dexamethasone-induced luciferase reporter-gene expression directed by the mouse mammary tumor virus (MMTV)-LTR promoter. The inhibition of heat-induced reporter gene expression was evident even when cells were exposed to stresgenin B only during heat stress treatment. Moreover, the compound inhibited heat-induced syntheses of hsp72/73, hsp90, and hsp110 and thereby suppressed the induction of thermotolerance. Stresgenin B showed moderate cytotoxic activities against several neoplastic cell lines and also showed antibacterial activities against *Micrococcus luteus*, *Bacillus subtilis* and *Staphylococcus aureus* strains.

Hyperthermia has been extensively studied clinically as a promising modality of cancer therapy[1]. However, there are still some problems to be resolved in the clinical settings, and one of the major problems is the induction of thermotolerance in tumors[2]. Therefore, a material which inhibits the induction of thermotolerance is expected to contribute to improving the effects of fractionated hyperthermia.

Thermotolerance is a transient phenomenon in which cells become heat resistant as a result of prior or continuous exposure to hyperthermia[2,3]. Many experiments have provided pieces of evidence which suggest the importance of hsp70 in induced thermotolerance in mammalian cells[4~10]. A direct correlation between HSP-gene expression and thermotolerance in CHO cells was demonstrated[11], and recent results have clarified that hsp70 is the anti-apoptotic protein which protects cells from cytotoxicity induced by heat[12,13], TNF[13~15], monocytes[16], oxidative stress[17], chemotherapeutic agents[15,18], ceramide[12,13] and radiation[17].

Only a few bioflavonoids including quercetin have been reported to inhibit the heat-induced HSP synthesis through the inhibition of mRNA induction after the heat stress[19,20]. Quercetin also has been reported to prevent cells from acquiring thermotolerance after heat stress[21]. In addition, quercetin potentiated the hyperthermia-induced cytotoxicity in HeLa cells[22] and enhanced the growth-inhibitory effect of hyperthermia on leukemic blast cells by enhancing leukemic cell apoptosis[23]. These findings imply that a selective inhibitor of the heat-induced HSP synthesis might improve hyperthermic cancer therapeutic efficacy.

In the screening using two assays for the human hsp70B

promoter-directed luciferase reporter gene and the MMTV-LTR promoter-directed luciferase reporter gene expressions to find a selective inhibitor of heat-induced HSP gene expression, we isolated a new compound, designated stresgenin B, from the culture broth of *Streptomyces* sp. AS-9 strain. This paper describes taxonomic studies of the producing strain and isolation, physico-chemical properties, structure determination, and biological properties of stresgenin B.

Materials and Methods

Culture Condition of CHO Cells

The CHO cells clones, CHO(pMAM-luc) and CHO (pMAM-HSluc), were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum at 37°C in a CO_2 incubator (5% CO_2 and 95% air) as described previously[24]. The amount of the medium was 200 μl/well for 48-wells plate cultures or 1.5 ml/dish for 35 mm-plastic dish cultures.

Luciferase (Luc) Assay

Luc activity was assayed by using Pika Gene assay kit (Toyo Ink Co.,Tokyo) according to the manufacture's procedure. The luminofluorescence generated by the reaction with luciferine and Luc in a cell lysate was photographed on Polaroid film 612 in Camlight 500 (Analytical Luminescence Laboratory, USA) in the screening assay. For quantitative dose-response assay, luminofluorescence was determined as relative light unit (rlu) by using a luminophotometer (Lumiphotometer TD-4000, Futaba Medical Co., Tokyo).

Heat-induced Expression of the hsp70B Promoter-directed Luc-reporter Gene (HS-Luc expression)

A CHO(pMAM-HSluc) cell clone that stably expresses a human hsp70B promoter-directed Luc-reporter gene depending on heat-shock treatment[24] was cultured in 48-wells plates or in 35 mm-plastic dishes at 37°C for 3 days (100% confluent growth). After the cultures were replaced with fresh medium, cells were assayed for the HS-Luc expression as follows:

1) First screening assay

Cells grown in 48-wells plates were incubated with 5% (v/v) of microbial culture broth samples for 10 minutes at 37°C and then heat-treated for 90 minutes in the presence of added samples in a CO_2 incubator maintained at 42°C. Then, cells were incubated at 37°C for 5 hours to express the Luc-reporter gene. The cells were lyzed in 40 μl of lytic solution (25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid, 10% glycerol, 1% Triton X-100) and 20 μl of the lysate was mixed with 30 μl of luciferine solution (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP) for determination of Luc activity. Samples inhibiting more than 75% of HS-Luc expression were selected.

2) Second screening assay

Cells grown in 48-wells plates were heat-treated at 42°C for 90 minutes, then incubated at 37°C for one hour. After that, samples selected by the first screening were added to the cell culture at 5% (v/v). The cells were incubated at 37°C for 5 hours, lyzed in 40 μl of lytic solution, and 20 μl of the lysate was mixed with luciferine solution (30 μl) for determination of the Luc activity. Samples showing a slight or no inhibition of HS-Luc expression were selected.

3) Dose-response assay

Cells grown in 35 mm-plastic dishes were incubated with various amounts of samples selected by the second screening (less than 5% v/v) at 37°C for 10 minutes, then heat-treated at 42°C for 90 minutes, and further incubated at 37°C for 3.5 hours to express Luc-reporter gene. Cells were lyzed in 160 μl of lytic solution, and 10 μl of the lysate was mixed with luciferine solution (50 μl) for determination of Luc activity.

Dexamethasone-induced MMTV-LTR Promoter-directed Luc-reporter Gene Expression (Dex-Luc expression)

A CHO(pMAM-luc) cell clone stably expresses MMTV-LTR promoter-directed luciferase gene at 3~4 folds elevated levels (induced) depending on dexamethasone treatment[24]. The cells were cultured in 35 mm plastic dishes at 37°C for 3 days (100% confluent growth). After the culture was replaced with fresh medium, cells were incubated with a sample (less than 5% v/v) for 10 minutes at 37°C. Then, dexamethasone (2 μM) was added and cells were incubated at 37°C for 5 hours to express the Luc-reporter gene. After cells were lyzed in 160 μl of lytic solution, 10 μl of the lysate was mixed with luciferine solution (50 μl) for determination of Luc activity. Luc activity of cells incubated without dexamethasone were subtracted from Luc activities of sample-treated and control cells incubated with dexamethasone, and relative Luc activities of sample-treated versus control cells were determined.

Physico-chemical Properties

The mp was measured with a Yanagimoto micro-mp

apparatus and was uncorrected. FAB-MS and HRFAB-MS were obtained on a Jeol JMS-SX102A spectrometer. In the FAB-MS measurement, glycerin was used as a matrix. ^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ as a solvent. The NMR spectra were recorded on a Jeol JNM-GSX400 spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. Chemical shifts were given on the δ scale (ppm). TMS was used as an internal standard at δ 0.0. Coupling constants (*J*-values) are given in Hz. UV and IR spectra were recorded on a Hitachi U-3210 spectrophotometer and a Jasco FT/IR-5300 spectrometer, respectively. Optical rotation was measured with a Jasco DIP-370 digital polarimeter.

Inhibition of HS-Luc Expression Depending on the Co-treatment with Stresgenin B and Heat Shock

Cells grown in 35 mm-plastic dishes were incubated with or without stresgenin B (10.5 μM) at 37°C for 10 minutes, and then heat-treated at 42°C for 90 minutes. The heat-shocked cells without stresgenin B were further incubated at 37°C up to 5 hours in the presence or absence of stresgenin B (10.5 μM). The heat-shocked cells with stresgenin B were either incubated at 37°C up to 5 hours or washed out of stresgenin B twice with 1.5 ml of the fresh growth medium, then incubated in 1.5 ml of the fresh growth medium at 37°C up to 5 hours.

Protein Synthesis

CHO cells grown in a 35 mm-plastic dish (100% confluent growth) and washed with methionine-free warmed growth medium, were incubated for 10 minutes at 37°C with or without stresgenin B. The cells were further incubated for 90 minutes at 42°C (heat shocked) or 37°C (non-heat shocked), and subjected to labeling for 5 hours at 37°C by adding 25 μCi of L-[^{35}S] methionine to the medium. [^{35}S] Methionine-labeled proteins were analyzed by SDS-9% polyacrylamide gel electrophoresis (SDS/PAGE) and autoradiography. The radioactivity of β-actin band (measured with a Bio-Image Analyzer BAS 2000, Fujix) was adjusted to the same level for each lane on SDS/PAGE.

Thermotolerance

The CHO (pMAM-HSluc) cells, cultured overnight to about 35% confluent growth in a 35 mm-plastic dish, were incubated for 10 minutes at 37°C with or without stresgenin B (7.0 μM). The cells were further incubated for 90 minutes at 42°C (heat shocked) or 37°C (non-heat shocked). After washing out stresgenin B twice with 1.5 ml of the fresh growth medium where appropriate, the cells were incubated in 1.5 ml of the warmed fresh growth medium at 37°C for 3.5 hours for HSP synthesis, then subjected to heat challenge at 44°C for up to 150 minutes. The cells were further incubated at 37°C for 24 hours, and viable cell counts were determined with a TCO reagent (Seikagaku-kogyo Co. Ltd.).

Cytotoxic Activity

Several neoplastic cell lines were cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a CO_2 incubator (5% CO_2 and 95% air). After cells were treated with various concentrations of stresgenin B for 48 hours, IC_{50} (μM) values were determined by counting viable cells by microculture tetrazolium assay (MTT assay)[25].

Antimicrobial Activity

Antimicrobial activities (MICs) were determined using the broth dilution method. In these studies, Mueller Hinton broth medium (DIFCO) was used for bacteria, and YNPG broth medium was used for fungi and yeast. Bacteria were incubated for 24 hours at 37°C. Fungi and yeast were incubated for 40 hours at 37°C.

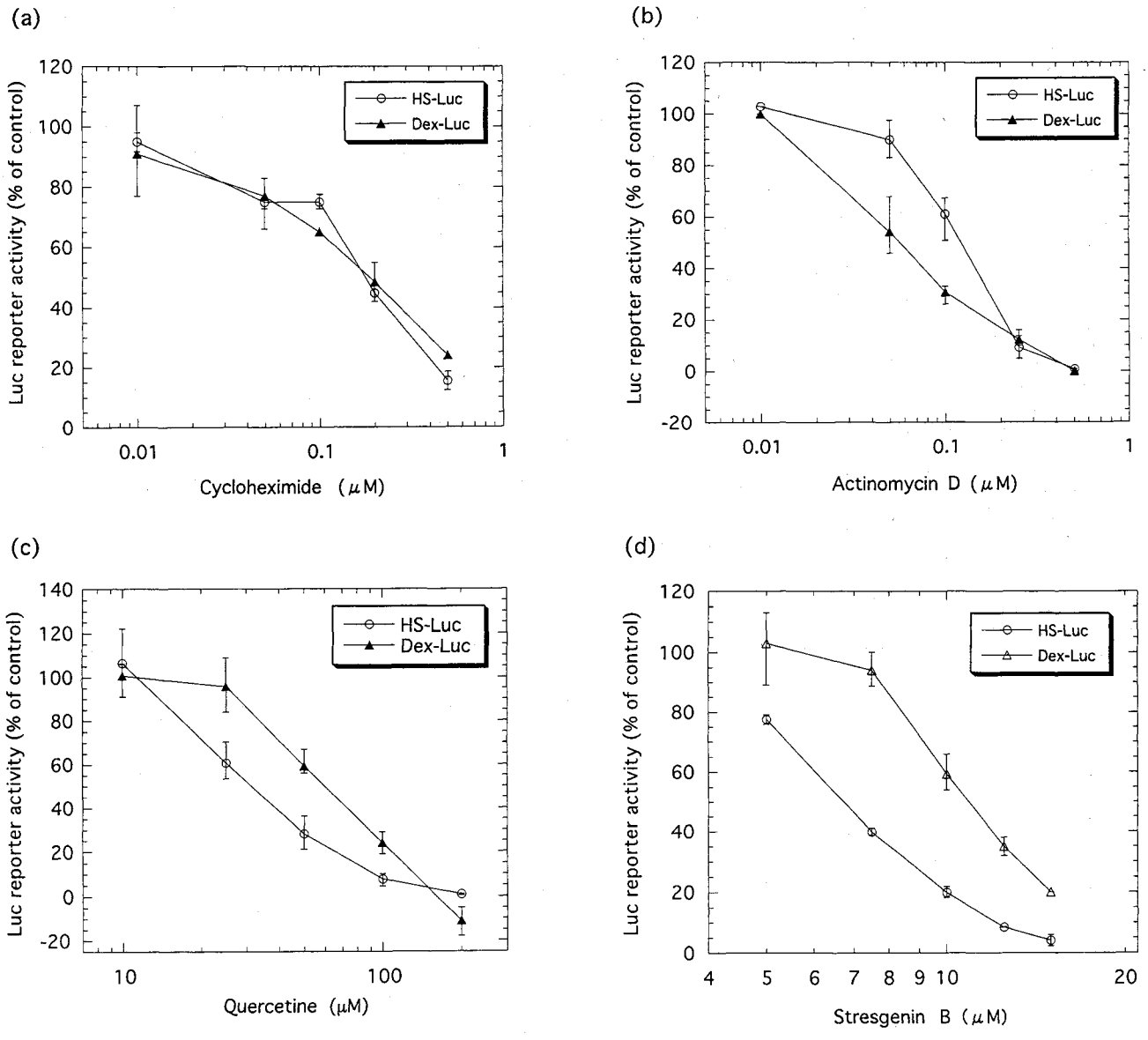
Results

Screening for Inhibitors of HS-Luc Expression

The *Streptomyces* strain AS-9 was selected through three screening steps as a producer of an inhibitor which preferentially suppresses the induction of human hsp70B promoter-directed Luc-reporter expression (HS-Luc expression): In the first screening assay where inhibitors were added to cells from the beginning of heat shock treatment, therefore, inhibitors of promoter activation, mRNA synthesis and/or protein synthesis were detected. For example, both actinomycin D (an inhibitor of RNA synthesis) and cycloheximide (an inhibitor of protein synthesis) inhibited the HS-Luc expression. However, in the second assay in which inhibitors were added after the cells had been incubated for one hour at 37°C after heat shock exposure, cycloheximide exhibited the similar inhibition to that in the first screening assay, but actinomycin D showed much less inhibition of the second assay compared with the first assay. Therefore, protein synthesis inhibitors were excluded by the second assay. The strain AS-9 was selected by these two screening assays.

In the third step, to determine promoter specificity of inhibition, the dose-response effect of the inhibitor was compared between two assays for HS-Luc expression and Dex-Luc expression. The two assays were equally sensitive

Fig. 1. Dose-response inhibition curves of cycloheximide (a), actinomycin D (b), quercetin (c) and stresgenin B (d) for the HS-Luc and the Dex-Luc expressions.



The data are expressed as means of triplicate experiments with all samples assayed in duplicate.

to the inhibition by cycloheximide (Fig. 1-(a)), but the HS-Luc assay was less sensitive to actinomycin D inhibition (Fig. 1-(b)). On the other hand, quercetin, which has been reported to inhibit selectively heat-induced expression of hsp genes[19,20], exhibited a more potent inhibition of the HS-Luc assay compared with the Dex-Luc assay (Fig. 1-(c)). Thus, stresgenin B was isolated from the culture of the strain AS-9 as a preferential inhibitor of the HS-Luc expression (Fig. 1-(d)).

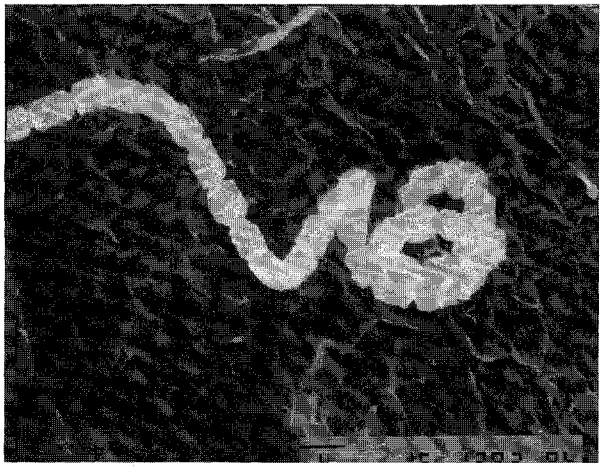
Taxonomy of the Producing Strain

Strain AS-9 was isolated from a soil sample collected in Tokyo, Japan. As morphological characteristics, the aerial mycelium of the cultured strain was well branched and the top of the aerial mycelium was spiral. After growing up, it became divided and formed spiral spore chains. The size of elliptical spore was about 0.7~1.0×0.8~1.3 μm. The surface of the spore was smooth or rugose. No sclerotic granule, sporangium or zoospore was observed (Fig. 2).

The results of cultural characteristics of the strain when grown on six media at 28°C are summarized in Table 1. The color of the surface of colony is indicated according to the symbols described in Color Harmony Manual[26]. The color of aerial mycelium was white to gray on ISP media Nos. 2, 3, 4, 5. Melanoid pigment was observed in tyrosine agar (ISP medium No. 7) but not observed in peptone-yeast extract-iron agar (ISP medium No. 6). The carbon source utilizing pattern according to Pridham-Gottlieb medium[27] is summarized in Table 2. L,L-Diaminopimeric acid was observed as one of the components of the cell wall when the whole-cell hydrolysate of the strain was analyzed by cellulose thin layer chromatography. From the foregoing taxonomic characteristics, the strain AS-9 was found to belong to the *Streptomyces* genus, and named as *Streptomyces* sp. AS-9.

Fig. 2. Scanning electron micrograph of aerial mycelium of *Streptomyces* sp. AS-9 grown at 28°C for 14 days.

Bar represents 1 μm.



Culture and Isolation of Stresgenin B

A stock culture of *Streptomyces* sp. AS-9 was inoculated into a 100-ml Sakaguchi flask containing 20 ml of a medium consisting of soybean meal 1.5%, corn starch MS3600 2.0%, $MgSO_4 \cdot 7H_2O$ 0.2% in tap water (pH 6.8 before sterilization). The flask was incubated at 27°C for 72 hours on a reciprocal shaker (120 rpm). Then, 0.75 ml portions of the culture were transferred to each of ten 500-ml Sakaguchi flasks containing 150 ml of the same medium. After incubation at 27°C for 24 hours on a

Table 1. Cultural characteristics of strain AS-9.

Medium	Growth	Aerial mycerium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar (ISP No.2)	Good	Light brownish gray [3fe,Silver gray]	Brown	Brown
Oatmeal agar (ISP No.3)	Moderate	Grayish yellowish pink [5dc, Pussywillow gray] or Light grayish reddish brown[5fe,Ashes]	Yellowish brown	Light brown
Inorganic salts-starch agar (ISP No.4)	Good	Yellowish gray [2dc,Natural] ~ Medium gray[2fe,Covert gray]	Yellowish brown	Light brown
Glycerol-asparagine agar (ISP No.5)	Good	Yellowish gray[2dc,Natural]	Yellowish brown	Light brown
Peptone-yeast extract-iron agar (ISP No.6)	Bad	None	None	None
Tyrosine agar (ISP No.7)	Good	Yellowish gray [2dc, Natural]	Dark brown	Dark brown

Observation after incubation at 28℃ for 14 to 21 days.

reciprocal shaker (120 rpm), 1.5 g of activated charcoal was added into each flask and the flasks were incubated for a further 72 hours.

The whole culture of ten flasks containing activated charcoal was filtered with the aid of celite 545 (Iwaikagaku, 2% w/v). The carbon combined cake was extracted twice with 400 ml of 50% acetone. The extract was concentrated to about 150 ml by evaporation at 40~45°C, then it was extracted twice with 150 ml of ethyl acetate. The ethyl acetate extract was evaporated to dryness, then dissolved in 2.5 ml of butyl acetate. The 0.5 ml portion was charged on a silica gel column (Wakogel C-200, Wako Pure Chemical; 10×250 mm), and developed with butyl acetate. The active fractions collected form five chromatograms were evaporated to dryness, then dissolved in 2.5 ml of acetonitrile. The 0.5 ml portion was charged on a silica gel column (Keiselgel Si60, Merck; 10×250 mm), and developed with acetonitrile. The active fractions showing UV absorbance at 275 nm collected form five chromatograms were evaporated to dryness, then dissolved in 2.5 ml of water saturated with butanol. The 0.5 ml portion was charged on a Wakogel LP-60C18 column (Wako Pure Chemical, 10×450 mm), and developed with water saturated with butanol. The active fractions showing UV absorbance at 275 nm collected form five chromatograms were evaporated to give 31.6 mg of crude powder. The crude powder was further purified with preparative HPLC using an Inertsil ODS-3 (10 mm i.d, 250 mm length) column and 15% acetonitrile in 0.03 M ammonium acetate buffer (pH 6.0) as a mobile phase (5 ml/minute) detected at UV 275 nm. The pure fraction was collected and lyophilized to give 7.3 mg of stresgenin B as a white powder.

Table 2. Carbon utilization of strain AS-9.

Carbon source	growth
L-Arabinose	+
D-Xylose	±
D- Glucose	+
D- Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	+
Raffinose	+
D- Mannitol	+

+; positive, ±;slightly positive

Physico-chemical Properties and Structure of Stresgenin B

The physico-chemical properties of stresgenin B are summarized in Table 3. It shows positive color reactions with ferric chloride and Rydon-Smith reagents. It is negative to ninhydrin and 2,4-dinitrophenylhydrazine reagents. Its molecular formula was determined as $C_{11}H_{13}NO_5$ by high-resolution FAB-MS analysis using NBA as a matrix. The UV spectrum in MeOH solution shows a maximum at 275 nm, indicating the presence of a dienone moiety. The ^1H NMR, ^{13}C NMR and HMQC spectra suggest that there are one *C*-methyl, one *O*-methyl, two oxymethine carbons, four olefinic carbons, and two carbonyl carbons as well as one quarternary replaced carbon in the molecule.

The plane structure of stresgenin B was determined by the HMBC spectrum as shown in Fig. 3. The ^1H and ^{13}C NMR assignments are shown in Table 4. The relative

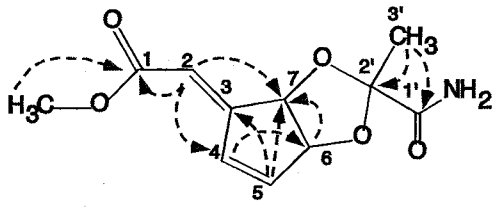
Table 3. Physico-chemical properties of stresgenin B.

Appearance	White powder
Solubility	Soluble in MeOH, $CHCl_3$, Acetone
MP(℃)	183-184
$[\alpha]_D^{25}$ (*c* 0.157,MeOH)	−12.6
Molecular formula	$C_{11}H_{13}NO_5$
HRFAB-MS(m/z)	
$(M+H)^+$	$C_{11}H_{14}NO_5$
Calcd:	240.0872
Found	240.0875
UV $\lambda_{max}^{MeOH}(\varepsilon)$	275 (25,800)
IR $\nu_{max}^{KBr}(cm^{-1})$	3429, 1716, 1593, 1439, 1197, 1064, 551

Table 4. ^{1}H and ^{13}C NMR chemical shifts of stresgenin B.

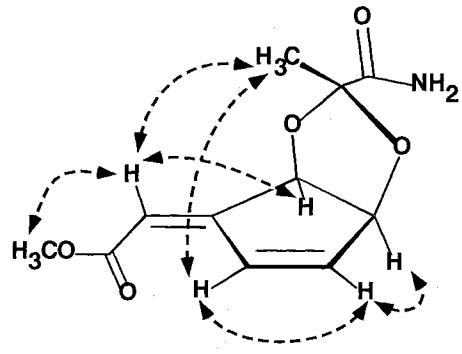
Carbon No.	^{13}C Chemical shifts (ppm)	^{1}H chemical shifts (ppm)
C-1	164.5	
C-2	136.2	7.43 br
C-3	130.0	
C-4	124.4	6.43 ddd(J=9.7,1.8,1.8Hz)
C-5	129.1	6.56 d(J=9.7Hz)
C-6	80.9	4.42 m
C-7	79.2	4.46 m
C-1'	172.1	
C-2'	109.8	
C-3'	22.9	1.66 s
O-Me	52.2	3.79 s

Fig. 3. The plane structure of stresgenin B.



Arrows indicate HMBC correlation.

Fig. 4. Relative stereochemistry of stresgenin B.



Arrows indicate NOESY correlation.

configuration of the molecule was also determined by the NOESY spectrum as shown in Fig. 4.

Preferential Inhibition by Stresgenin B of HS-Luc Expression Relative to Dex-Luc Expression

As shown in Fig. 1-(d), the HS-Luc expression was more sensitive to stresgenin B inhibition than was the Dex-Luc expression. The 50% inhibition concentration (IC_{50}) of 7.0 μM for HS-Luc expression was 4.9 times lower than that of quercetin (34 μM; Fig. 1-(c)).

Inhibition of HS-Luc Expression Depending on the Co-treatment with Stresgenin B and Heat Shock

As shown in Fig. 5, heat-shocked cells without stresgenin B (○) were induced for Luc activity during the recovery incubation up to 5 hours at 37°C. However, the Luc induction was strongly inhibited when cells were heat-shocked with stresgenin B (10.5 μM) only during the duration of heat shock (●) and during the total period including the recovery incubation (□). On the other hand, the Luc induction was only slightly affected by stresgenin B when it was added only during the recovery period (△).

Inhibition of Heat-induced HSP Synthesis by Stresgenin B

As shown in Fig. 6, heat-shocked cells (lane 2) were induced for syntheses of hsp72/73 which were immunoprecipitated with the monoclonal antibodies against human hsp70 (hsp72) or Chinese hamster cell hsc70 (hsp73) as

Fig. 5. Inhibition of HS-Luc expression depending on the co-treatment with stresgenin B and heat shock.

○: control, △: stresgenin B was added only during the recovery incubation, □: stresgenin B was added during the heat shock and the recovery incubation, ●: stresgenin B was added only during the heat shock treatment.

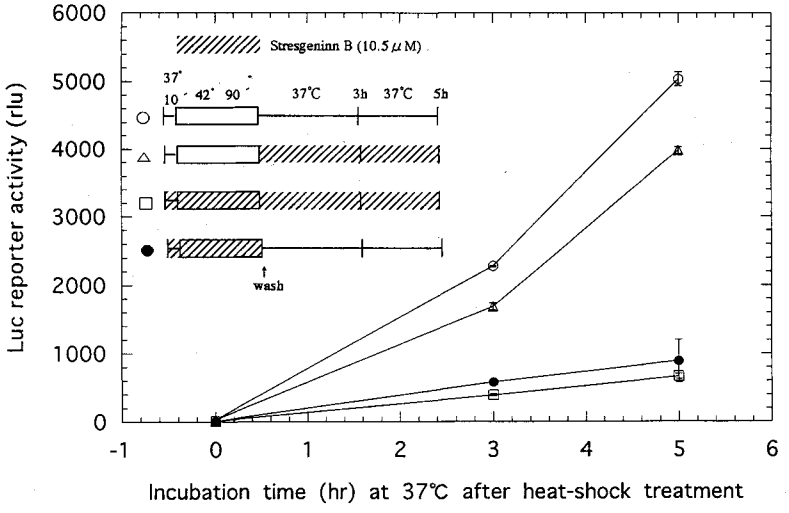
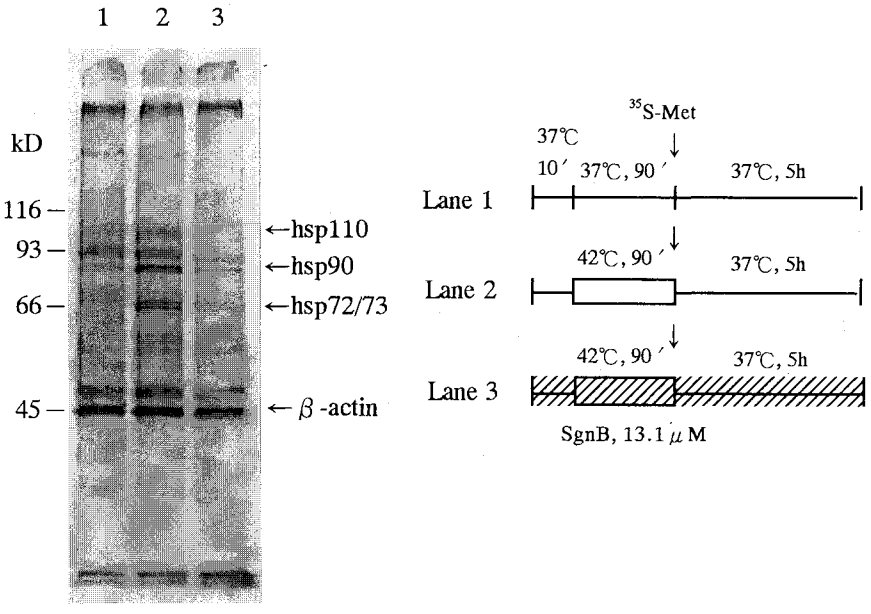


Fig. 6. Inhibition of heat-induced HSP synthesis by stresgenin B (Sgn B).

Lane 1: control, Lane 2: heat-shocked, Lane 3: heat-shocked with stresgenin B.



described previously[11], and two proteins whose molecular weight are corresponding to hsp90 or hsp110. When cells were exposed to stresgenin B during heat stress and subsequent [^{35}S]-methionine labeling, HSP synthesis was greatly inhibited compared with β-actin synthesis (Lane 3).

Suppression of Thermotolerance Expression by Stresgenin B

As shown in Fig. 7, non-heat-shocked cells (○) decreased the number of viable cells as increasing the time

Table 5. Cytotoxicity of stresgenin B.

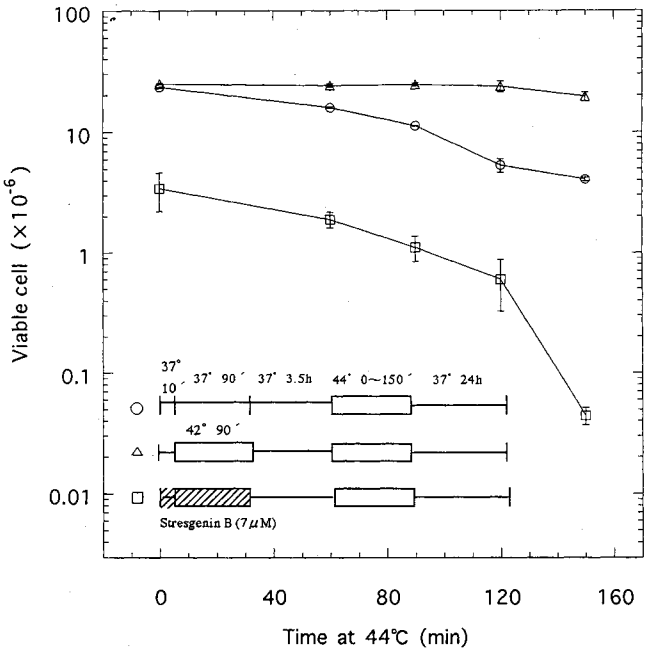
Cell lines	IC_{50}(μM)
K562	2.6
PC6	6.3
HT1080	13.7
HT29	5.9
MCF7	19.5
MKN28	5.6

Table 6. Antimicrobial activities of stresgenin B.

Test organisms	MIC(μ g/ml)
Staphylococcus aureus FDA209P	12.5
Bacillus subtilis ATCC6633	3.1
Micrococcus luteus ATCC9341	3.1
Escherichia coli K-12	>50
Klebsiella pneumoniae ATCC10031	>50
Pseudomonas aeruginosa IFO12582	>50
Candida albicans ATCC10231	>50
Aspergillus fumigatus IFM41088	>50

Fig. 7. Suppression of thermotolerance expression by stresgenin B after heat-shock treatment of CHO cells.

○: control, △: heat-shocked, □: heat-shocked with stresgenin B.



of heat challenge at 44°C. However, conditioning heat-shocked cells (△) by exposure to 42°C for 90 minutes and subsequent incubation for 3.5 hours at 37°C, did not decrease the number of viable cells in the heat challenge for 150 minutes, indicating thermotolerance induction by the conditioning heat-shock. On the other hand, cells exposed to stresgenin B (7.0 μM) (□) only during the heat stress (42°C, 90 minutes) significantly decreased the number of viable cells as increasing the time of heat challenge at 44°C. This clearly shows that stresgenin B suppressed thermotolerance induction by the conditioning heat-shock treatment. The heat-shock treatment alone showed no significant growth inhibition (as seen at time 0 in Fig. 7), and the exposure to stresgenin B (7.0 μM) alone during 48 hours incubation at 37°C showed only a moderate inhibition of cell growth (61% of control; data not shown). However, combined treatment with heat-shock and stresgenin B reduced viable cell number to 16% of control (see at time 0 in Fig. 7). These results indicated that 42°C hyperthermia enhanced the cell-killing activity of stresgenin B.

Cytotoxicity and Antimicrobial Activity

As shown in Table 5, stresgenin B showed moderate cytotoxic activities against various neoplastic cell lines. Stresgenin B also showed antibacterial activities, but it showed no inhibitory activity against fungi and yeasts (Table 6).

Discussion

We have isolated stresgenin B from the culture broth of *Streptomyces* sp. AS-9 as an inhibitor of heat-induced HSP gene expression. The inhibitor was determined to be a novel low molecular weight compound with a unique chemical structure. Stresgenin B exhibited a preferential inhibition of the HS-Luc expression compared with the Dex-Luc expression, and also inhibited rather selectively heat-induced syntheses of hsp72/73, hsp90 and hsp110 in CHO cells (Fig. 6).

Stresgenin B, actinomycin D, and cycloheximide inhibited the HS-Luc expression in the first screening assay in which cells were heat-shocked in the presence of the

inhibitor and subsequently incubated at 37°C for Luc expression. However, in the second screening assay where cells were exposed to the inhibitor after one hour of the recovery incubation at 37°C following heat-shock treatment, cycloheximide showed the same inhibition as that of the first screening assay, but actinomycin D showed much less inhibition of the second assay (data not shown). Stresgenin B exhibited only a slight inhibition of Luc expression in the second assay (data not shown). Actually, stresgenin B showed strong inhibition of Luc induction even when it was present only during heat shock period, whereas it slightly inhibited when present only during the recovery incubation (Fig. 5). These results suggest that stresgenin B inhibits heat-induction of the hsp70B promoter activity and does not significantly affect the post-transcriptional process for Luc expression.

Exposure of cells to stresgenin B only during the heat stress (42°C, 90 minutes) inhibited the thermotolerance acquisition of CHO cells (Fig. 7), as expected by its inhibition of heat induced hsp70 synthesis[4,11]. Furthermore, combined treatment of cells with mild heat shock (42°C, 90 minutes) and exposure to stresgenin B induced a synergistic cell-killing (Fig. 7). These activities of stresgenin B propose the usefulness of this compound combined with hyperthermia as a modality of cancer therapy.

Although the mechanism of inhibition of heat-induced HSP gene expression has not yet been identified, stresgenin B has similar biological activities to those of quercetin[19~23]. Stresgenin B, however, has some advantages: 1) at least 4.9 times more potent than quercetin for inhibition of heat-induced HSP promoter activation and 2) more water-soluble than quercetin.

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References

1) ABE, M. & M. HIRAOKA: Hyperthermia in combination with radiation in the treatment of cancers. *In* Stress proteins in Biology and Medicine. *Eds.*, MORIMOTO, R. I.; A. TISSIERES & C. GEORGOPOULOS, pp. 117~129, Cold Spring Harbor Laboratory Press, New York, 1990
2) GERNER, E. W. & M. J. SCHNEIDER: Induced thermal resistance in HeLa cells. Nature 256: 500~502, 1975
3) HENLE, K. J. & L. A. DETHLEFSEN: Heat fractionation and thermotolerance: a review. Cancer Res. 38: 1843~1851, 1978
4) LI, G. C. & Z. WERB: Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc. Natl. Acad. Sci. USA 79: 3218~3222, 1982
5) LASZLO, A. & G. C. LI: Heat-resistant variants of Chinese hamster fibroblasts altered in expression of heat shock protein. Proc. Natl. Acad. Sci. USA 82: 8029~8033, 1985
6) ANDERSON, R. L.; I. V. KERSEN, P. E. KRAFT & G. M. HAHN: Biochemical analysis of heat-resistant mouse tumor cell strains: a new member of the HSP70 family. Mol. Cell. Biol. 9: 3509~3516, 1989
7) RIABOWOL, K. T.; L. A. MIZZEN & W. J. WELCH: Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. Science 242: 433~436, 1988
8) JOHNSTON, R. N. & B. L. KUCEY: Competitive inhibition of hsp70 gene expression causes thermosensitivity. Science 242: 1551~1554, 1988
9) ANGELIDIS, C. E.; I. LAZARIDIS & G. N. PAGOULATOS: Constitutive expression of heat-shock protein 70 in mammalian cells confers thermoresistance. Eur. J. Biochem. 199: 35~39, 1991
10) LI, G. C.; L. LI, Y. K. LIU, J. Y. L. CHEN & W. M. F. LEE: Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. Proc. Natl. Acad. Sci. USA 88: 1681~1685, 1991
11) AKAGAWA, H.; A. ISHII & S. MIZUNO: Suppression of thermotolerance development through cycloheximide-induced negative control of stress protein gene expression. J. Biochem. 123: 226~232, 1998
12) MOSSER, D. D.; A. W. CARON, L. BOURGET, C. DNEIS-LAROSE & B. MASSIE: Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol. Cell. Biol. 17: 5317~5327, 1997
13) BUZZARD, K. A.; A. J. GIACCIA, M. KILLENDER & R. L. ANDERSON: Heat shock protein 72 modulates pathways of stress-induced apoptosis. J. Biol. Chem. 273: 17147~17153, 1998
14) JAATTELA, M.; D. WISSING, P. A. BAUER & G. C. LI: Major heat shock protein hsp70 protects tumor cells from tumor necrosis factor cytotoxicity. EMBO J. 11: 3507~3512, 1992
15) JAATTELA, M.; D. WISSING, K. KOKHOLM, T. KALLUNKI & M. EGEBLAD: Hsp 70 exerts its anti-apoptotic function downstream of caspase-3-like proteases. EMBO J. 17: 6124~6134, 1998
16) JAATTELA, M. & D. WISSING: Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. J. Exp. Med. 177: 231~236, 1993
17) SIMON, M. M.; A. REIKERSTORFER, A. SCHWARZ, C. KRONE, T. A. LUGER, M. JAATTELA & T. SCHWARZ: Heat shock protein 70 overexpression affects the response to ultraviolet light in murine fibroblats. J. Clin. Invest. 95: 926~933, 1995
18) KARLSEDER, J.; D. WISSING, G. HOLZER, L. OREL, G. SLIUTS, H. AUER, M. JAATTELA & M. M. SIMON: HSP70 overexpression mediates the escape of a doxorubicin-induced G2 cell cycle arrest. B. B. R. Comm. 220: 153~159, 1996
19) HOSOKAWA, N.; K. HIRAYOSHI, A. NAKAI, Y. HOSOKAWA, N. MARUI, M. YOSHIDA, T. SAKAI, H. NISHINO, A. AOIKE,

K. KAWAI & K. NAGATA: Flavonoids inhibit the expression of heat shock proteins. Cell Struct. Funct. 15: 393~401, 1990

20) HOSOKAWA, N.; K. HIRAYOSHI, H. KUDO, H. TAKECHI, A. AOIKE, K. KAWAI & K. NAGATA: Inhibition of the activation of heat shock factor *in vivo* and *in vitro* by flavonoids. Mol. Cell. Biol. 12: 3490~3498, 1992

21) KOISHI, M.; N. HOSOKAWA, M. SATO, A. NAKAI, K. HIRAYOSHI, M. HIRAOKA, M. ABE & K. NAGATA: Quercetin, an inhibitor of heat shock protein synthesis, inhibits the acquisition of thermotolerance in a human colon carcinoma cell line. Jpn. J. Cancer Res. 83: 1216~1222, 1992

22) KIM, J. HO; S. H. KIM, A. A. ALFIERI & C. W. YOUNG: Quercetin, an inhibitor of lactate transport and a hyperthermic sensitizer of HeLa cells. Cancer Res. 44: 102~106, 1984

23) LAROCCA, L. M.; F. O. RANELLETTI, N. MAGGIANO, S. RUTELLA, E. O. LA BARBERA, C. RUMI, F. SERRA, M. T. VOSO, M. PIANTELLI, L. TEOFILI & G. LEONE: Differential sensitivity of leukemic and normal hematopoietic progenitors to killing effect of hyperthermia and quercetin used in combination: role of heat-shock protein-70. Int. J. Cancer 73: 75~83, 1997

24) MIZUNO, S.; A. ISHII, Y. MURAKAMI & H. AKAGAWA: Stress dose-dependent suppression of heat shock protein gene expression by inhibiting protein synthesis during heat shock treatment. Cell Struct. Funct. 22: 7~13, 1997

25) MOSMANN, T.: Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assay. J. Immunol. Methods 65: 55~63, 1983

26) TAYLER, H. D.; L. KNOCHE & W. C, GRANVULLE (*Ed.*): Color Harmony Manual, Container Corporation of America, Chicago, 1948

27) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107~114, 1948