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Structure Determination and Biological Activities of Elaiophylin Produced by *Streptomyces* sp. MCY-846

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A cytotoxic compound, 846I was isolated from the culture of *Streptomyces* sp. strain MCY846 by its cytotoxicity against gastric cancer cell line SNU-1. The IR, UV and NMR spectra of 846I were identical to those of elaiophylin. This compound showed moderate cytotoxicity against several cell lines and exerted strong inhibitory effect on the synthesis of nitric oxide in the lipopolysaccharide stimulated-Raw 264.7 cells.

During the search for novel cytotoxic metabolites from the culture broth of soil microorganisms, *Streptomyces* sp. strain MCY-846 was selected by its cytotoxicity against gastric cancer cell-line, SNU-1. (14) The active principle was isolated by activity guided fractionation of acetone extract of mycelium and further purified by preparative HPLC. The structure of the compound was elucidated by extensive spectroscopic analysis and turned out to be identical to elaiophylin which was first identified from the culture of *Streptomyces melanosporus* (3), then subsequently from other strains of *streptomyces* (1, 2, 5, 9, 19).

Elaiophylin is a macrolide with 16-membered unsaturated lactone ring and has characteristic C2 symmetry structure (5, 13). This compound exhibited anti-helmintic activity (7) as well as activity against gram positive bacteria and protozoa. But there was no previous report either on the cytotoxicity of elaiophylin against tumor cell lines or on the inhibition of nitric oxide synthesis. Here we discuss isolation, spectroscopic characteristics and *in vitro* cytotoxicity of elaiophylin and its effect on the synthesis of nitric oxide in a macrophage cell line, Raw 264.7.

MATERIALS AND METHODS

Fermentation

A slant culture of *Streptomyces* sp. MCY-846 grown on modified Bennett's agar medium consisting of glucose 1%, bacto-peptone 0.2%, beef extract 0.1%, and yeast extract 0.1% (pH 7.2), was inoculated into 500 ml baffled flasks containing 100 ml of seed medium consisting of soluble starch 2%, glucose 1%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.005%, $CaCO_3$ 0.2%, and soybean meal 2.5%. The flasks were cultivated on a rotary shaker for 3 days at 28°C and 200 rpm. A 7.5 liter jar fermentor containing 5 liters of the seed medium was inoculated with 200 ml of the seed culture. The fermentation was carried out for four days at 28°C, 200 rpm and aeration of 200 l/h.

Analytical Methods

The ^1H and ^{13}C NMR spectra were recorded on a Varian UNITY 300 NMR spectrophotometer using standard pulse sequences. Chemical shifts are reported in δ value referenced to $CDCl_3$(δ 7.26) for ^1H and $CDCl_3$(δ 77) for ^{13}C as internal reference and coupling constant (J) are given in Hz. Electrospray Interface (ESI) mass spectral data were obtained on JMS-HS 110A spectrometer. The UV absorption spectrum was measured with a Milton Roy spectronic 3000 array spectrophotometer. The IR absorption spectrum was obtained with KBr disk on a Laser Precision Analytical RFX-65 FT-IR. The melting point was determined on a model of Electrothermal 9100 without correction. Optical rotation was determined on JASCO DIP-181 polarimeter. Preparative HPLC was carried out on a DELTA-PAK C18 (ϕ 19 mm × 300 mm, Waters) and monitored with a UV detector at 253 nm.

Cell Lines and Cell Cultures

Human stomach cancer cell line, SNU-1 and human liver cancer cell line, SNU-354 were obtained from J.-G. Park. Seoul National University, School of Medicine and NIH 3T3 and F25, oncogene ras transformed NIH3T3 were obtained from H. Fujiki, Saitama Cancer Center,

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Japan. They were grown in RPMI-1640 medium supplemented with 10% FBS. Human oral epidermoid cancer cell line KB-3-1 and its vinblastine selected multidrug resistance KB-V1 cells were obtained from M. Gottesman, NIH, U.S.A. Macrophage cell line, Raw 264.7 cells was used to measure nitric oxide production. They were grown in DMEM containing 10% FBS. All cells were incubated at 37°C in humidified atmosphere with 5% CO_2.

***In vitro* Cytotoxicity**

Cells in exponential growth were trypsinized, dispersed in single cell suspension, dispensed in 100 μl volume into 96 well plate and allowed to attach and grow overnight. One hundred μl of medium containing assay sample was added and further incubated for 48 h. Cytotoxicity was measured by the SRB method (18) and the IC_{50} value was calculated using Probits method. In brief, cells were fixed by gently layering 50 μl of cold 50% trichloroacetic acid and incubated at 4°C for 1 h and then washed five times with tap water. Plates were air dried, stained with 0.4% (w/v) sulforhodamine B dissolved in 1% acetic acid and rinsed several times with 1% acetic acid. Plates were air dried and bound dye was solubilized with 10 mM unbuffered Tris base (pH 10.5). Absorbance was read with microtiter plate reader at 570 nm. IC_{50} was the concentration of compound that reduced absorbance to 50% of vehicle-treated controls.

Measurement of Nitric Oxide (NO)

Raw 264.7 cells (1×10^5 cells/well) were incubated with elaiophylin (1, 5, 10, 20, 40 μM) as well as LPS (1 μg/ml) for 18 h at 37°C in 96 well plate in triplicates. Dexamethasone (5, 10, 20, 40 μM) was also included in each experiment as a positive control. After incubation, 200 μl of supernatant in each well was loaded into a new 96 well plate and a few pieces of Zn powder was added to each well. After 20 minutes, 100 μl of supernatant was transferred into another 96 well plate (flat bottom plate) and added 100 μl of Griess reagent (1:1 mixture of 0.1% N-(1-naphthyl)ethylenediamine [Sigma] in H_2O and 1% sulfanilamide in 5% phosphoric acid) into all the wells. Optical density at 550 nm was measured on a microtiter plate reader (Microplate reader, Molecular Devices Co.). Cells treated with LPS only were used as controls for maximum NO_2 level (8).

RESULTS AND DISCUSSION

Strain

The strain MCY-846 was isolated from a soil in Shindoli field, Cheju-island, Korea. Based on the taxonomic properties such as cultural characteristics on ISP media, chemotaxonomic data, utilization of carbon and nitrogen sources, and numerical classification, the isolate, MCY-846, was identified as a *Streptomyces* sp. (14).

Isolation and Purification

Fermentation was performed as described in Materials and Methods. The mycelial cake separated from a cultured broth by continuous centrifugation, was extracted twice with 3 l of acetone. The cell debris was removed by filtration and organic layer was evaporated to dryness. The residue was dispersed in a small volume of water and extracted with methylene chloride. The organic layer was concentrated to a small volume *in vacuo*. The residue was applied to silica cartridge column (P.J. Cobert Associates, Inc.) and was eluted with a linear gradient from chloroform to methanol . Active fractions were collected and separated by MPLC (RP-18, 60A) and further purified by preparative HPLC (Delta Pak C18) eluted with 65% methanol to give colorless powder (25 mg).

Physico-chemical Properties

The physico-chemical properties of compound (846I) are summarized in Table 1. The strong broad IR band at 3429 cm^{-1} indicated the presence of several hydroxyl groups and the absorptions at 1695 cm^{-1} and 1639 cm^{-1} indicated the presence of conjugated ester carbonyl groups. The UV absorption maxima at 253 nm also indicate the presence of a conjugated ester group. The molecular formula of compound 846I was determined to be $C_{54}H_{88}O_{10}$ by HRFABMS. It was soluble in methanol and dimethyl sulfoxide but insoluble in water, acetone, benzene, hexane and ethylacetate. The Rf value was 0.26 when developed in chloroform-methanol=9:1 on silica TLC plate (Merck). It was positive in the color reaction with anisaldehyde-H_2SO_4 and iodine.

Structure Determination

The ^{13}C NMR spectra and DEPT spectra revealed the compound have 27 carbon signals containing 6 methyl carbons (6 signals between δ 6 and δ 20), 3 methylene carbons (3 signals at δ 19.36, 33.48 and 35.91), 4 methine carbons (4 signals between δ 38.89 and 41.62)

Table 1. Physico-chemical properties of elaiophylin.

Appearance	white powder
MP	106-109°C
$[\alpha]_D^{25}$ (MeOH)	-86.95 (c 1.18)
UV λ_{Max}^{MeOH} nm (log ε)	253 (4.6)
Mass spectrum	
FAB-MS (m/z)	1047 (M^++Na)
IR υ max (KBr) cm-1	3429, 2972, 2931, 1695, 1639
Molecular formula	$C_{54}H_{88}O_{18}$
Solubility	
Soluble	DMSO, $CHCl_3$, MeOH, EtOH
Insoluble	BuOH, H_2O, Me_2CO, EtOAC, Hexane, Benzene
TLC(Rf)*	
$CHCl_3$-MeOH (9:1)	0.26

*Silica gel TLC plate 60 F254 (Merck).

and 7 carbon atoms attached to alcohol, ether or ester oxygen atoms. The molecular ion peak in the FABMS appeared at m/z 1047(M^++Na) indicating that this compound might be a symmetical molecule. ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY spectra revealed the anomeric carbon signal at δ 93.26 and characteristic hemiketal carbon signal at δ 99.06. Chemical shifts of hemiketal regions were also assigned by comparison with published data for azalomycin (10, 11) and copiamycin (6) which have hemiketal moiety. The singlet at δ 169.95 was assigned to a conjugated ester carbonyl group which is in agreement with the IR absorption maximum at 1695 cm^{-1} (Table 2), UV spectrum (λmax 253 nm) and 4 signals of olefinic carbons at δ 120.97, 131.99, 144.34 and δ 145.04. The ^{1}H NMR spectra showed 6 methyl signals in the upfield region and 4 olefinic proton signals between δ 5.6 and δ 6. The anomeric proton of L-2-deoxyfucose moiety appeared at δ 5.06. From all these findings, the structure of compound 846I was determined to be identical to elaiophylin (Fig. 1).

Biological Activities

In vitro cytotoxicities of elaiophylin against various cell lines are shown in Table 3. Elaiophylin showed moderate cytotoxicity without any differential effect between NIH3T3 and oncogene, ras transformed-NIH3T3 (F25) cells. The compound did not also confer differential cytotoxicity between vinblastine sensitive KB-3-1 and resistant KB-V1 cells.

The minimal inhibitory concentrations (MIC) of

Table 2. ^{13}C and ^{1}H NMR chemical shift data of elaiophylin.

Number	^{13}C[a]		δ H[b]
1	169.95	C	
2	120.97	CH	5.69 (1H, d, J=15.3)
3	145.04	CH	6.98 (1H, dd, J=4.2, 26.7)
4	131.99	CH	6.09 (1H, dd, J=3.9, 25.8)
5	144.34	CH	5.64 (1H, dd, J=5.4, 22.2)
6	41.62	CH	2.54 (1H, m)
7	77.86	CH	4.74 (1H, d, J=10.2)
8	38.89	CH	1.93 (1H, m)
9	70.08	CH	3.90 (1H, m)
10	40.83	CH	1.96 (1H, m)
11	99.06	C	–
12	35.91	CH_2	2. 38 (1H, m), 1.38 (1H, m)
13	70.61	CH	4.12 (1H, m)
14	48.41	CH	1.19 (1H, m)
15	66.56	CH	3.91 (1H, m)
16	19.15	CH_3	1.16 (3H, m)
17	14.93	CH_3	1.11 (3H, d, J=6.0)
18	9.08	CH_3	0.88 (3H, s)
19	7.07	CH_3	1.02 (3H, dd, J=3.3, 17.1)
20	19.36	CH_2	1.44 (1H, m), 1.61 (1H, m)
21	8.75	CH_3	0.83 (3H, t, J=15.3)
1'	93.26	CH	5.06 (1H, brs)
2'	33.48	CH_2	1.79 (2H, m)
3'	66.01	CH	3.93 (1H, brs)
4'	71.44	CH	3.62 (1H, brs)
5'	66.07	CH	3.98 (1H, m)
6'	16.85	CH_3	1.25 (3H, d, J=6.6)

TMS as an internal standard. Chemical shifts were expressed as δ in ppm. [a]in $CDCl_3$ (75 MHz). [b]in $CDCl_3$ (300 MHz). s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet; brs, broad singlet.

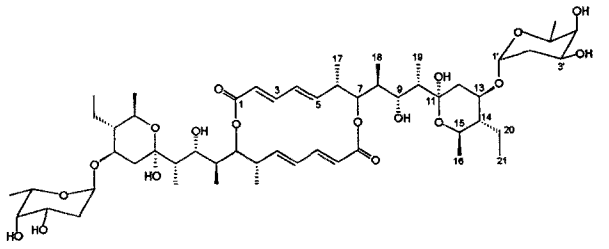

Fig. 1. Structure of elaiophylin.

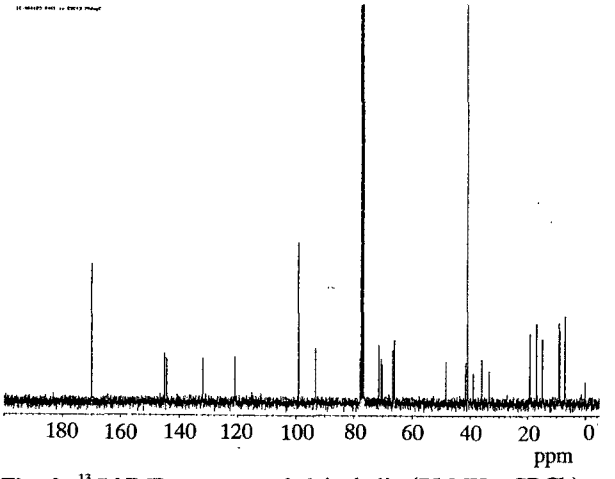

Fig. 2. ^{13}C-NMR spectrum of elaiophylin (75 MHz, $CDCl_3$).

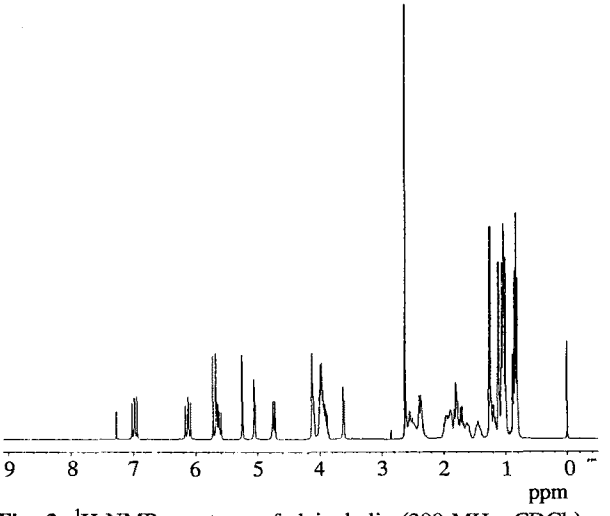

Fig. 3. ^{1}H-NMR spectrum of elaiophylin (300 MHz, $CDCl_3$).

Table 3. *In vitro* test results for elaiophylin against tumor cell lines.

cell lines	IC_{50} (μM)
NIH-3T3	4.00
F25	4.40
SNU-1	0.68
SNU-354	0.39
KB-3-1	0.68
KB-V1	2.58
KB-V1+vinblastine (100 nM)	2.52

Table 4. Antimicrobial activity of elaiophylin *in vitro*.

Strains (ATCC #)		MIC (μg/ml)
Staphylococcus aureus	6538P	6.25
Staphylococcus aureus	25923	50
Staphylococcus epidermidis	12228	50
Micrococcus luteus	9341	3.125
Enterococcus casseliflavus	14432	12.5
Enterococcus faecalis	29212	> 50
Bacillus subtilis	6633	3.125
Escherichia coli	25922	> 50
Citrobacter freundii	8090	> 50
Entrobacter aerogenes	13048	> 50
Serritia marcescens	14756	> 50
Proteus vulgaris	13315	> 50
Candida albicans	10231	> 50
Candida tropicalis	13803	> 50
Cryptococcus neoformans	7224[a]	> 50
Saccharomyces cerevisiae	9763	> 50
Aspergillus niger	9642	> 25
Aspergillus flavus	9643	> 25
Penicillium citrinum	9849	> 25
Alternaria mali	44899	> 25
Fusarium oxysporum f. sp. cucumerinum	9761[b]	> 25
Aureobasidium pullulans var. melanogenum	9348	25

[a]KCTC number. [b]IFO number.

elaiophylin against bacteria, yeast, and fungi were determined by the agar dilution method (4). The results are given in Table 4. 8461 possessed an antibacterial activity against Gram positive bacteria but very weak activity was observed against Gram negative bacteria, yeast, and fungi.

Interestingly, the compound significantly inhibited production of prostaglandin E2 (PGE2) (data not shown) and of nitric oxide (NO) in a macrophage cell line, Raw 264.7 cells stimulated with lipopolysaccharide. Prostaglandin, such as PGE2 contributes in cell transformation, tumor growth and metastasis (15). PGE2 also regulates NO production in macrophage (16). These facts imply that inhibition of PGE2 and NO production might have good correlation with cytotoxic effect to cancer

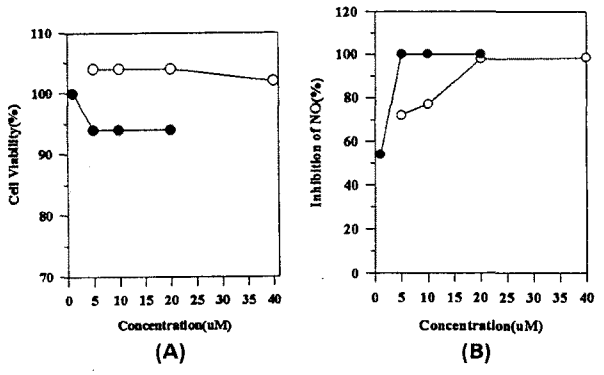

Fig. 4. Effect of elaiophylin on the viability (A) and inhibition of NO production (B) in Raw 264.7 cells.
The addition of LPS (1 μg/ml) did induce 62 μM NO production in the cell. —○—, dexamethasone; —●—, elaiophylin.

cells as well as antiinflammatory effect. As shown in Fig 4. elaiophylin exhibited more potent inhibition than dexamethasone in the production of NO at its non-toxic concentration to Raw 264.7 cells. This result indicate that elaiophylin has at least considerable potential as an inhibitor of NO production

Nitric oxide is an important second messenger which is involved in the pathophysiological mechanism of variety of disease states (12). Particularly NO production is accompanied in a inflammatory response, i.e. macrophage cell line Raw 264.7 cells produce significant amount of NO upon stimulation with lipopolysaccharide. Therefore, modulation of NO production in macrophage would be an important target on the development of antiinflammatory agent. The remarkable effect of elaiophylin on the production of NO in the Raw 264.7 cells suggest that the compound be further evaluated its effect on the regulation of nitric oxide synthase in the Raw 264.7 cells (17).

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