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NOTE

Characterization and Cytotoxic Activities of Nonadecanoic Acid Produced by *Streptomyces scabiei* subsp. *chosunensis* M0137 (KCTC 9927)

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The substance 0116p, which exhibits cytotoxicity against human macrophage cell line THP-1, was isolated from a mycelial extract of *Streptomyces scabiei* subsp. *chosunensis* M0137. The cytotoxic substance was purified by Diaion-HP20 adsorption, solvent extraction, Sephadex LH-20 column chromatography, and silica-gel column chromatography. The molecular formula is $C_{19}H_{38}O_2$ (MW 301.10) based on elemental and spectrometric analysis. It was identified as nonadecanoic acid by NMR spectral data. It exhibits cytotoxic activities in various human cancer cell lines, including A549, SK-OV-3, SK-MEL-2 and HCT-15. In addition, 0116p also inhibits IL-12 production in lipopolysaccharide -activated macrophages.

***Key words*:** *Streptomyces scabiei* subsp. *chosunensis* M0137, nonadecanoic acid, anti-tumor activity, inhibitor of IL-12 production

Actinomycetes have been described as the greatest source of antibiotics since Waksman introduced *Streptomyces* into his systemic screening program for new antibiotics in the early 1940s. Actinomycetes have provided about two-thirds (more than 4,000) of the naturally occurring antibiotics that have been discovered, including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, macrolides, β-lactams, and tetracyclins (Nisbet, 1982; Iwai and Takahashi, 1992). In addition to antibiotics, a variety of different approaches using fermentation broths of actinomycetes have been utilized for the development of novel agents for cancer therapies. Until now, cytotoxic substances, such as actinomycin D, mitomycin C, bleomycin and doxorubicin, originating from streptomycetes have been mainly used in cancer therapies (Nakae *et al*., 2000). However, to overcome the secondary effects of these compounds, intensive searches have been aimed at identifying more selective and novel structural agents.

The authors previously isolated over 600 actinomycete strains in soil samples from various locations in Chonnam Province, Korea, that were then screened for the production of various bioactive substances. To develop a new anti-cancer agent, the metabolic products of the isolated streptomycete strains showing cytotoxicity against human macrophage cell line (THP-1) were screened. One streptomycete strain producing potent cytotoxic agents was characterized and identified phenotypically and phylogenetically, and named *Streptomyces scabiei* subsp. *chosunensis* M0137 (KCTC 9927) (Yoo *et al*., 2002). The mycelial extract and culture broth of this strain contained several kinds of cytotoxic substances which were separated by silica-gel column chromatography. The current paper reports on 0116p, isolated from a mycelial extract of *Streptomyces scabiei* subsp. *chosunensis* M0137.

Nonadecanoic acid has already been isolated from several sources, including a fungus (Juzlova *et al*., 1996), marine sponge (Mishra *et al*., 1996), and plant (Hogg and Gillan, 1984; Fukunaga *et al*., 1989), and exhibits inhibitory effects on fibrinolysis and plasmin activity (Kawashiri *et al*., 1986). However, the current study is the first report on the nonadecanoic acid production from the genus *Streptomyces*, along with its biological functions as anti-tumor agent and inhibition of IL-12 production.

A loopful of spores of *Streptomyces scabiei* subsp. *chosunensis* M0137 was inoculated in fermentation medium consisting 2% oatmeal, 1% dried yeast, 1% mannitol, 1%

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soybean meal, and 0.2% $CaCO_3$. Fermentation was carried out at 30°C for 7 days on a rotary shaker at 180 rpm. The 7-day cultured broth (5 l) was centrifuged at 10,000g for 20 min. The mycelial cake (1.2 kg, wet weight) was suspended in 80% (v/v) aqueous acetone, stored at -20°C for 24 h, and then centrifuged. The acetone was evaporated using a rotary evaporator at 45°C. Diaion-HP 20 (Sigma, USA) (50 g/l) was added to the resulting aqueous solution, which was then stirred for 3 h. The Diaion-HP 20 was separated from the aqueous solution by filtration, and washed with a 20% aqueous methanol. The active substance was eluted from the Diaion-HP20 using an 80% aqueous acetone. The acetone was then removed using a rotary evaporator, and the resulting aqueous solution was extracted with two volumes of *n*-butanol. The butanol was then evaporated using a rotary evaporator at 55°C. Next, 15 ml of acetone was added to dissolve the residue, which was then applied to a column of Sephadex LH-20 (Sigma, USA). The column was eluted with acetone. The active fractions were pooled and dried using a rotary evaporator. The resulting residue was dissolved with 10 ml of dichloromethane, and then applied to a column of silica-gel. The active substance was eluted with hexane:ethylacetate (1:1), and dried using a rotary evaporator. The dried sample was dissolved in 5 ml of methanol, then the temperature was slowly decreased to -20°C. The active substance was recrystallized and washed with cold methanol. The final material was verified as pure by a TLC that produced a R_f value of 0.65 when the same developing mixture was used. The amount of purified substance (0116p) was 115 mg.

Elemental analysis was performed at the Korea Basic Science Institute (KBSI, Seoul, Korea). FT-IR spectra were recorded using a Shimazu FTIR-8101 spectrometer (Shimazu, Japan). The TLC was performed using an aluminum plate coated with silica gel 60 F254 (Merck, Germany), and a hexane:ethylacetate=1:l mixture was used as the developing solution. All mass spectrometric analyses were performed using a JMS-HM110/110A tandom mass spectrophotometer (Jeol, Japan), a four-sector instrument with an E1B1E2B2 configuration. The ion source was operated at 10 KeV accelerating voltage in positive-ion mode with a mass resolution of 1000 (10% valley). The NMR spectra, including homo (^{1}H-^{1}H) and hetero (^{1}H-^{13}C) COSY were obtained on VARIAN UNITY-300 INOVA spectrometers (Varian, USA) using $CDCl_3$ solutions at an ambient temperature (^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz). The chemical shifts are given in ppm using tetramethyl silane as the internal standard.

In vitro cytotoxicity of the substances was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, USA] assay, described by Pieters *et al.* (1988). To measure the *in vitro* anticancer activities, A549 (human non-small cell lung cancer), SK-OV-3 (human ovarian cancer), SK-MEL-2 (human melanoma), and HCT15 (human colon cancer) cell lines were used. A 50% growth inhibitory concentration (GI_{50}) for a particular agent was defined as the drug concentration resulting in a 50% reduction in the cell number relative to the untreated control.

Table 1. Physicochemical properties of 0116p

Appearance	white powder
Molecular formula	$C_{19}H_{38}O_2$
FAB-MASS (positive)	m/z=299.5 $(M^+H)^+$
Elemental analysis	
Found:	C 71.67%, H 11.80%
Melting point	65~67°C
FT-IR (in KBr pellet)	2956, 2919, 1711, 1468, 1433, 1409 and 937 cm^{-1}

Table 2. ^{13}C NMR data of 0116p and authentic nonadecanoic acid*

Carbon position	Chemical shift (ppm)	
	0116p	nonadecanoic acid
1	180.4	180.6
2	34.34	34.23
3	24.95	24.77
4	29.34	29.19
5	29.51	29.36
6	29.70	29.56
7-15	29.92[a]	29.81
16	29.63	29.50
17	32.20	32.05
18	22.97	22.77
19	14.39	14.13

*Accoring to Gunstone *et al.* (1976).
[a]average value of C_7-C_{15}

To examine the effect of 0116p on Interleukin (IL)-12 production, spleen cells were cultured at the concentration of 10^6 cells/ml for approximately 3 h at 37°C. The non-adherent cells were then removed by washing with warm DMEM until visual inspection revealed a lack of lymphocytes (>98% of the cell population). The adherent cells were removed from the plates by incubating for 15 min with ice-cold phosphate-buffered saline and repeated rinsing. The isolated adherent cell population was stimulated with 5 g/ml of LPS in the absence or presence of 0116P over a range of 2 to 100 μg/ml at 2×10^5 cells/well in 96-well culture plates for 48 h. The quantities of IL-12 produced in the culture supernatant were determined by sandwich ELISAs using a monoclonal antibody, as described previously by Kim *et al.* (1997). Standard curves were generated using IL-12 over a range of 0.03 to 4.0 ng/ml. Substance 0116p was obtained as a white solid with a melting point of 65~67°C. All the physicochemical properties including the FT-IR data are summarized in Table 1. The positive FAB-MS spectra suggest that the molecular weight of this fatty acid is 301.10, and the molecular formula would appear to be $C_{19}H_{38}O_2$ when considering the results of the elemental analysis. The

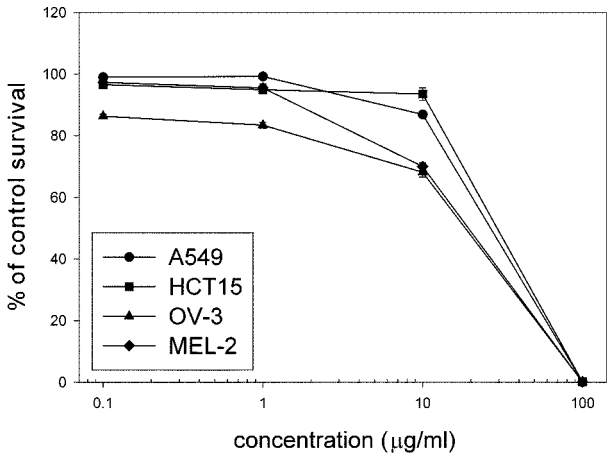

Fig. 1. *In vitro* anti-tumor activities of 0116p (nonadecanoic acid) various in cancer cell lines. A549, human non-small-cell lung cancer; SK-OV-3, human ovarian cancer; SK-MEL-2, human melanoma; and HCT15, human colon cancer cell line.

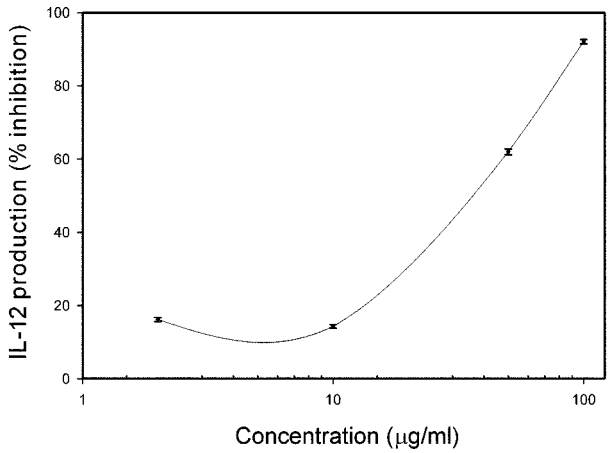

Fig. 2. Inhibitory effect of 0116P on the LPS-induced IL-12 production. Splenic macrophages were stimulated with 5 μg/ml LPS.

analysis of the ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H, and ^{1}H-^{13}C COSY NMR spectra (Table 2) revealed the structure of nonadecanoic acid. From the comparison with the known data (Gunstone *et al*., 1976; Ahn *et al.*, 1995; Burger *et al*., 1999), 0116p was identified as a nonadecanoic acid of molecular formula $C_{19}H_{38}O_2$.

The anticancer activities of 0116p were determined in various cancer cell lines. The GI_{50} values of 0116p for A549, SK-OV-3, SK-MEL-2, and HCT15 were 32.1, 33.57, 33.42, and 22.26 μg/ml, respectively (Fig. 1). *In vitro* anti-tumor activities of 0116p were somewhat weak compared to those of clinical anti-cancer drugs. Lipopolysaccharide (LPS)-activated macrophages produce IL-12 that can induce the differentiation of activated naive CD_4 T cells into effector inflammatory T cells (Kang *et al*., 2000). When the effect of 0116p on IL-12 production from LPS-activated macrophages was observed, 0116p was found to inhibit IL-12 production in lipopolysaccharide (LPS)-activated macrophages (Fig. 2). Inhibition of IL-12 production by 0116p did not result from a general cytotoxic effect since macrophages viability in all cultures remained constant throughout the incubation period in the presence of hypericin concentrations used in the experiment, as demonstrated by trypan blue exclusion test (data not shown). As the IL-12 can induce the differentiation of activated naive CD_4 T cells into effector inflammatory T cells, it is likely that 0116p may have anti-inflammatory effects.

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