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ORIGINAL ARTICLE

Isomethoxyneihumicin, a new cytotoxic agent produced by marine *Nocardiopsis alba* KM6-1

Takashi Fukuda[1], Misaki Takahashi[1], Kenichiro Nagai[1], Enjuro Harunari[2], Chiaki Imada[2] and Hiroshi Tomoda[1]

A new cytotoxic agent designated isomethoxyneihumicin (1 and 2), a mixture of lactam-lactim tautomers, was isolated along with methoxyneihumicin (3) from the culture broth of the marine *Nocardiopsis alba* KM6-1. The structures of 1 and 2 were elucidated in spectroscopic analyses (1D and 2D NMR data, and ROESY correlations). Isomethoxyneihumicin (15.0 μM) and 3 (15.0 μM) arrested the cell cycle of Jurkat cells at the G2/M phase (66 and 67%) in 12 h. Isomethoxyneihumicin and 3 exhibited cytotoxicity against Jurkat cells with IC_{50} values of 6.98 and 30.5 μM in 20 h, respectively. These results strongly suggest that isomethoxyneihumicin and 3 exhibit cytotoxicity against Jurkat cells by inhibiting the cell cycle at the G2/M phase.
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INTRODUCTION

The challenge to discover new biologically active compounds from various resources including animals, plants and microorganisms is continuing. Microorganisms are expected to become the most important resource for new drug discovery.[1] An extensive number of studies that mainly focused on terrestrial microorganisms as drug discovery resources demonstrated that they produce a number of structurally unique and biologically active compounds including antibiotics, anticancer agents and immunomodulators.[2,3] We recently became interested in poorly studied resources including marine-derived microorganisms. In the course of our search for new bioactive compounds from marine microorganisms, we have identified novel and unique compounds such as seriniquinone[4] (an anticancer agent against melanoma cells) and graphiumins[5,6] (inhibitors of yellow pigment production in methicillin-resistant *Staphylococcus aureus*).

The strain *Nocardiopsis alba* KM6-1 was isolated from sea sediment collected at Chichijima, Ogasawara, Japan in 2013. A new compound designated isomethoxyneihumicin (**1** and **2**) was isolated along with known methoxyneihumicin (**3**)[7] from the culture broth of strain KM6-1 under natural seawater-containing conditions (Figure 1). Isomethoxyneihumicin was found to be a mixture of lactam-lactim tautomers **1** and **2**. In this study, we described the fermentation, isolation, structural elucidation and cytotoxicity of isomethoxyneihumicin.

RESULTS

Collection and identification of strain KM6-1

Strain KM6-1 was isolated from sea sediment collected at Chichijima, Ogasawara, Japan in 2013. This strain was cultured on low carbon Agar medium made with 100% natural sea water for identification. DNA extraction, PCR, sequencing and Basic Local Alignment Search Tool (BLAST) searching followed. The primer set 16S-10F (5′-GTTTGATCCTGGCTCA-3′) and 16S-800R (5′-TACCAGGGTAT CTAATCC-3′) was used to amplify the region under the conditions of 25 cycles at 96 °C for 30 s, 50 °C for 15 s and 60 °C for 4 min. The sequence is available at the National Center for Biotechnology Information (US). On the basis of the BLAST search and its microscopic features, the strain was identified as a *N. alba*.

Fermentation

The strain was inoculated into a 300-ml Erlenmeyer flask containing 50 ml seed medium (1.0% soluble starch, 0.4% yeast extract and 0.2% peptone). The flask was shaken on a rotary shaker at 27 °C for 5 days. The seed culture (1.0 ml) was transferred into a 500-ml Erlenmeyer flask containing 100 ml production medium (1.0% soluble starch, 0.4% yeast extract, 0.2% peptone, 0.1% $CaCO_3$, 0.004% $Fe_2(SO_4)\cdot nH_2O$ and 0.01% KBr in natural sea water). Fermentation was performed at 27 °C for 8 days under shaking conditions (180 r.p.m.).

Isolation

The culture broth (100 ml × 30) was shaken with resin (150 ml, Amberlite XAD7HP, Sigma-Aldrich, St Louis, MO, USA) for 2 h. After filtration by gauze, the fungal body and resin were extracted with acetone (2.0 l) for 2 h. This extract was evaporated to an aqueous solution, which was partitioned between water and EtOAc. The EtOAc fraction was concentrated to yield a crude extract (141 mg). The crude extract was dissolved in a small volume of methanol, applied to an ODS column (6.0 g, 1.5 × 6.0 cm), and eluted stepwise with 30 and

[1]Graduate School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan and [2]Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan
Correspondence: Professor H Tomoda, Graduate School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
E-mail: tomodah@pharm.kitasato-u.ac.jp
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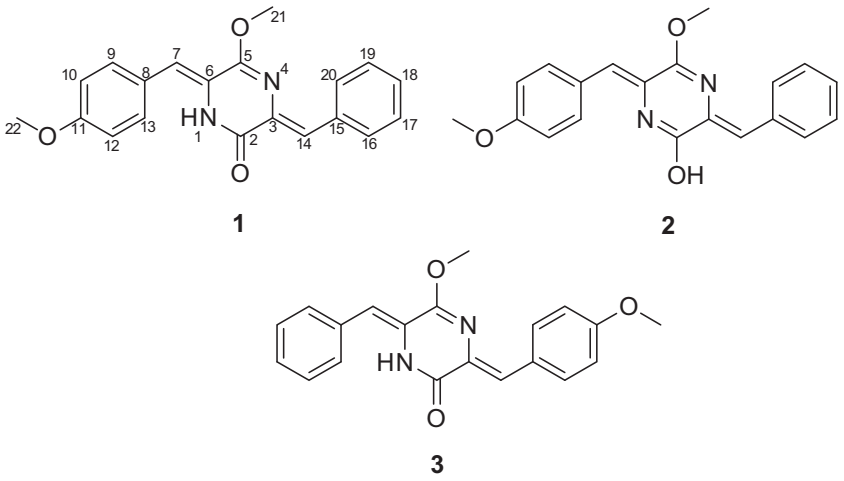

Figure 1 Structures of isomethoxyneihumicin (**1** and **2**) and methoxyneihumicin (**3**).

Table 1 Physicochemical properties of isomethoxyneihumicin (1 and 2)

	1* and *2
Appearance	Fluorescent yellow needles
Molecular formula	$C_{22}H_{18}N_2O_3$
Molecular weight	334
HR ESI-MS *m/z*	$(M+H)^+$
Calcd	335.1395 (for $C_{22}H_{19}N_2O_3$)
Found	335.1355
UV (MeOH)	368, 312, 228
IR ν_{max}^{KBr} cm^{-1}	3432, 2928, 1670, 1638, 1610, 1511

80% aq CH_3CN and CH_3CN (50 ml each). Compounds **1** to **3** were recovered in the 80% aq CH_3CN fraction. This fraction was further purified by HPLC using a reversed-phase C-18 column (10 × 250 mm; PEGASIL ODS SP100, Senshu Scientific, Tokyo, Japan) under the following conditions: solvent, 60% aq CH_3CN; flow rate, 3.0 ml min^{-1}; detection, UV at 210 nm. Isomethoxyneihumicin (**1** and **2**) and **3** were eluted as peaks with respective retention times of 44.9 and 41.3 min. The tautomers **1** and **2** were eluted with the same retention times. These peaks were collected and concentrated to yield 1.7 and 2.5 mg, respectively.

Physicochemical properties of isomethoxyneihumicin

The physicochemical properties of isomethoxyneihumicin are summarized in Table 1. It showed absorption maxima at 368, 312 and 228 nm in the UV spectrum. Absorption at 3432, 1670, 1610 and 1511 cm^{-1} in the IR spectrum suggested the presence of hydroxyl, carbonyl and phenyl groups.

Structural elucidation of isomethoxyneihumicin

Isomethoxyneihumicin was a mixture of the tautomers **1** and **2** in an equilibrium of 2:1 in DMSO-d_6 from NMR data. Isomethoxyneihumicin was obtained as fluorescent yellow needles. The molecular formula for **1** was established as $C_{20}H_{18}N_2O_3$ ($[M+H]^+$ *m/z* 335.1355, calcd $[M+H]^+$ 335.1395) on the basis of high-resolution ESI-MS measurements, indicating that **1** contained 12 degrees of unsaturation (Table 1). ^1H and ^{13}C NMR data (in DMSO-d_6) supported the molecular formula (Table 2). The ^{13}C NMR spectrum of **1** showed 20 resolved signals, which were classified into two methyls, 11 sp^2 methines and seven quaternary carbons including one carbonyl carbon (C-2). The ^1H-NMR spectrum of **1** showed two oxygenated methyl signals and 11 olefinic methine signals derived from 9 aromatic protons and one NH-proton signal. The connectivity of all proton and carbon atoms was established by HMQC experiments (Table 2). An analysis of ^1H-^1H COSY data revealed two benzene rings, one monosubstituted and one disubstituted (Figure 2a). An analysis of HMBC spectroscopic data provided further structural information on **1**. The cross peaks from NH-1 (δ 10.03) to C-3 (δ 132.2) and C-5 (δ 155.1) and from H_3-21 (δ 3.98) to C-5 supported the partial structure of the center ring (Figure 2a). The cross peaks from H-7 (δ 6.50) to C-9/13 (δ 131.0) and from H_3-22 (δ 3.78) to C-11 (δ 159.2) supported the *p*-methoxybenzyl part. The cross peaks from H-14 (δ 7.08) to C-16/20 (δ 131.2) supported the benzyl part. Additional cross peaks from H-7 to C-5 and from H-14 to C-2 supported the connectivity of the three partial structures, as shown in Figure 1. The NH proton was confirmed by ^{15}N-gHSQC. A cross peak was also observed from NH-1 (δ 10.03) to N-1 (δ −124.9). Collectively, these data revealed that the planar structure of **1** was a lactam type, as shown in Figure 2a. The conformation of C-7 and C-14 was elucidated by ROESY spectra (Figure 2b). The correlation between NH-1 and H-9/13 and between H-16/20 and H_3-21 supported the 7 *Z*- and 14 *Z*-configurations of the double bonds.

Compound **2** was a lactam-lactim tautomer of **1**. In ^1H-NMR, the isolated signals of **2**, such as H-7 (δ 6.41), H-10/12 (δ 6.86) and H-14 (δ 7.06), were observed as a 1/2 integrated value of the counterpart signals of **1** (Table 2). A clear difference between **1** and **2** was that **2** had one exchangeable signal (δ 10.68), which was identified as a C2-OH proton. In addition, the cross peak from C2-OH to C-3 (δ 132.3) in the HMBC spectrum showed that **2** was formed as a lactim-type ring (Figure 2c).

The structure of compound **3** was identified by comparing data reported previously.[7]

Biological properties

Effects of isomethoxyneihumicin and methoxyneihumicin on the Jurkat cell cycle. The effects of isomethoxyneihumicin (**1** and **2**) and **3** on the cell cycle of Jurkat cells at 3,12 and 20 h were investigated using flow cytometry (Figure 3). The distribution of control cells (without a drug) in the subG1 (2.3%), G1 (46%), S (22%) and G2/M (24%) phases was

almost constant until 20 h. As shown in Figure 3a, when isomethoxyneihumicin (15 μM) was added to Jurkat cells at time 0 h under these conditions, G1 phase cells (46% at 0 h to 13% at 12 h) decreased with a concomitant increase in G2/M phase cells (24% at 0 h to 66% at 12 h) until 12 h. After that, subG1 phase cells (dead cells) markedly increased to a distribution of 60% at 20 h. Similar results were observed for methoxyneihumicin (15 μM), as shown in Figure 3b. These results indicated that the two compounds induced G2/M arrest in Jurkat cells until 12 h, and then cell death at 20 h.

Table 2 NMR spectroscopic data for isomethoxyneihumicin (1 and 2) in DMSO-d6

	1			*2*
Position	δ_C[a]	δ_H[b] *mult (J in Hz)*	*HMBC*	δ_H[b] *mult (J in Hz)*
1-NH		10.03, br.s	3, 5	
2	159.4, C			
3	132.2, C			
5	155.1, C			
6	123.1, C			
7	112.0, CH	6.50, s	5, 9/13	6.41, s
8	125.4, C			
9/13	131.0, CH	7.50, d (8.0)	7, 11, 9/13	7.30[c], m
10/12	114.8, CH	6.96, d (8.0)	8, 11, 10/12	6.86, d (8.0)
11	159.2, C			
14	125.3, CH	7.08, s	2, 16/20	7.06, s
15	134.9, C			
16/20	131.2, CH	8.12, d (7.5)	14, 18, 16/20	8.08, d (7.5)
17/19	128.4, CH	7.40[c], m	15, 17/19	7.40[c], m
18	128.6, CH	7.30[c], m	16/20	7.30[c], m
21	54.7, CH_3	3.98, s	5	3.76, s
22	54.9, CH_3	3.78, s	11	3.78, s
2-OH				10.68, br.s

Assignments made by the interpretation of HSQC and HMBC NMR data.
[a]100 MHz.
[b]400 MHz.
[c]Overlapping.

Cytotoxic activities of isomethoxyneihumicin and methoxyneihumicin in Jurkat cells. The cytotoxicities of isomethoxyneihumicin (**1** and **2**) and **3** against Jurkat cells at 20 h were measured using the MTT assay.[8] Isomethoxyneihumicin and **3** exerted dose-dependent cytotoxic effects with IC_{50} values of 6.98 and 30.5 μM, respectively. The cytotoxic effects of isomethoxyneihumicin and **3** on other cancer cells such as HCT116 (human colon carcinoma) cells, HaCaT (human keratinocyte) cells and CHO (Chinese hamster ovary) cells were investigated; however, no cytotoxicity was observed against these cancer cells at 100 μM until 20 h.

DISCUSSION

Diverse diketopiperazines are produced by fungi and actinomycetes and are known to exhibit various biological activities such as cytotoxic,[9] phytotoxic,[10] antimicrobial[11] and insecticidal activities.[12] For example, a diketopiperazines compound consisting of phenylalanine and isoprenylated dehydrohistidine (named halimide or (-)-phenylahistin) was independently isolated from cultures of marine-derived *Aspergillus* sp. by Fenical *et al.*[13] or of *Aspergillus ustus* by Kanoh *et al.*,[14] respectively. The diketopiperazines inhibited the cell cycle at the G2/M phase by inhibiting tubulin polymerization.[15] Its *tert*-butyl derivative, named plinabulin[16] was entered into a phase III clinical trial for the treatment of advanced metastatic non-small cell lung cancer in 2015.[17] Isomethoxyneihumicin has a diketopiperazine-like core skeleton. There have only been a few samples with this type of skeleton; neihumicin isolated from a culture of *Micromonospora neihuensis* Wu, sp. nov[18] and methoxyneihumicin (**3**) produced by deep-sea-derived *N. alba* SCSIO 03039.[7] These diketopiperazines-related compounds exhibited cytotoxicity against KB (HeLa-derived cells),[19] MCF-7 (human breast adenocarcinoma cells), NCI-H460 (human non-small cell lung cancer cells) and SF-268 (human glioma cells).[7] In the present study, isomethoxyneihumicin was found to arrest Jurkat cells at the G2/M phase without cytotoxic effects until 12 h, and then exhibited cytotoxicity against Jurkat cells. This biological characteristic appeared to be similar to those of halimide[13] and plinabulin.[16] Unfortunately, the compound showed no activity against HCT116 cells, HaCaT cells or CHO cells, at least

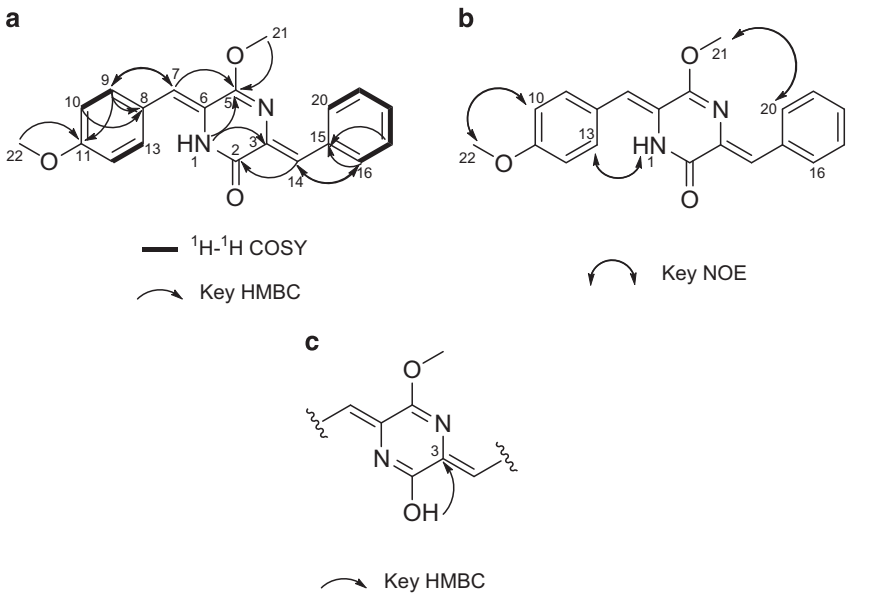

Figure 2 2D NMR data of isomethoxyneihumicin (**1** and **2**). (**a**) ^{1}H-^{1}H COSY and Key HMBC correlations of **1**. (**b**) Key NOESY correlations of **1**. (**c**) Key HMBC correlations of **2**.

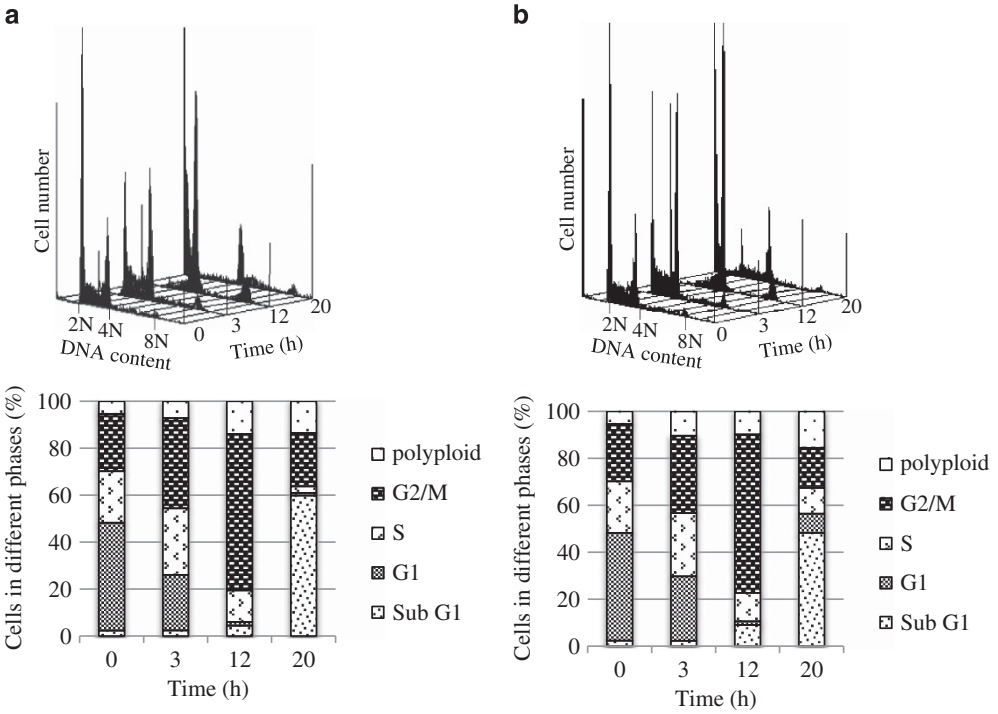

Figure 3 Effects of isomethoxyneihumicin (**1** and **2**) and methoxyneihumicin (**3**) on the cell cycle status of Jurkat cells. (**a**) Jurkat cells (5.0×10^5 cells per ml) were treated with isomethoxyneihumicin (15 μM). The distribution of cells in the cell cycle was analyzed using flow cytometry (upper) at the indicated incubation times (0, 3, 12 and 20 h). The bar graph represents the percentage distribution of Jurkat cells in different phases of the cell cycle at the indicated incubation times (0, 3, 12 and 20 h). (**b**) Jurkat cells (5.0×10^5 cells per ml) were treated with **3** (15 μM). The distribution of cells in the cell cycle was analyzed using flow cytometry (upper) at the indicated incubation times (0, 3, 12 and 20 h). The bar graph represents the percentage distribution of Jurkat cells in different phases of the cell cycle at the indicated incubation times (0, 3, 12 and 20 h).

until 20 h. These results indicate that isomethoxyneihumicin only affects floating cell lines. Further experiments are needed in order to clarify this.

Isomethoxyneihumicin is a mixture of lactam-lactim tautomers **1** and **2** at a ratio of 2:1 in DMSO-d_6. Although methoxyneihumicin (**3**) was previously reported to form a single structure, we found that it is also a mixture of lactam-lactim tautomers at a ratio of 5:1 in DMSO-d_6 (data not shown).

EXPERIMENTAL PROCEDURES

General experimental procedures

ESI-MS spectrometry was conducted on a JMS-T100LP spectrometer (JEOL, Tokyo, Japan). UV and IR spectra were measured with a U-2800 spectrophotometer (HITACHI, Tokyo, Japan) and FT/IR-460 plus spectrometer (JASCO, Tokyo, Japan), respectively. The various NMR spectra were measured with UNITY 400 (Agilent Technologies, Santa Clara, CA, USA). Reversed-phase HPLC separation was performed using a Senshu Pak PEGASIL ODS SP100 column (10×250 mm) at a flow rate of 3.0 ml min^{-1} using the SHIMADZU LS20AT pump and SHIMADZU LS20AS UV detector (SHIMADZU, Kyoto, Japan). Absorbance was read with Power Wave 340 (Bio Tek Instruments, Winooski, VT, USA).

Materials

Soluble starch and $Fe_2(SO_4)\cdot nH_2O$ were purchased from Wako Pure Chemical Industries. (Osaka, Japan), yeast extract and peptone from Becton Dickinson (Sparks, MD, USA), and $CaCO_3$ and KBr from Kanto Chemical (Tokyo, Japan). Natural sea water was purchased from Shozikido (Shizuoka, Japan). RPMI 1640, thiazolyl blue tetrazolium bromide (MTT), propidium iodide, ribonuclease A and IGEPAL CA-630 were purchased from Sigma-Aldrich. Trisodium citrate dihydrate was purchased from Wako Pure Chemical Industries. Penicillin (1.0×10^4 units ml^{-1}) and streptomycin (1.0×10^4 mg ml^{-1}) solution was obtained from GIBCO (Grand Island, NY, USA). Fetal bovine serum was from BioWest (Riverside, MO, USA).

Cell culture and cell cycle analysis of Jurkat cells

Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units per ml penicillin and 100 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Jurkat cells (5.0×10^5 cells in 200 μl) prepared in a 96-well microplate were treated with samples (0–100 μM) at 37 °C for 20 h. Cells were then suspended in 200 μl of 0.1% sodium citrate solution containing 50 μg ml^{-1} propidium iodide, 20 μg ml^{-1} ribonuclease A and 0.3% IGEPALCA-630 (Krishan's solution). The cell cycle status was assessed in an analysis of the DNA content using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA). Ten thousand cells were analyzed, and the distribution of cells in different phases (subG1, G1, S and G2/M) was calculated using the program ModiFit LT ver. 2.0 (Verity Software House, Topsham, ME, USA) according to the manufacturer's protocol.

MTT analysis

The cytotoxic activity of compounds against Jurkat cells was evaluated by the MTT assay, as described previously.[8] In brief, Jurkat cells (5.0×10^5 cells in 100 μl) in a 96-well microplate were treated with samples (0–100 μM) at 37 °C for 20 h. After being incubated, cells were treated with 10 μl MTT solution (5.5 mg ml^{-1} in phosphate-buffered saline), and were then incubated at 37 °C for 4 h. A 90-μl aliquot of the lysis solution (40% *N*, *N*-dimethylformamide, 2.0% CH_3COOH, 20% SDS and 0.03 M HCl) was added to each well, and the plates were incubated for 2 h. The absorbance at 550 nm of each well was read with Power Wave 340 (Bio Tek Instruments).

The inhibition of cell growth was defined as (absorbance-sample/absorbance-control)$\times100$. The IC_{50} value was defined as a sample concentration that causes 50% inhibition of cell growth. In almost the same manner, the cytotoxic activity of compounds against other cells (HCT116 cells, HaCaT cells and CHO cells) was evaluated by the MTT assay.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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