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ORIGINAL ARTICLE

Novel *p*-terphenyl glycoside with a rare 2,6-dideoxy-hexopyranose moiety from *Actinomycete* strain SF2911 that inhibits cancer cell migration

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In the course of our search for inhibitors of LPS-induced NO production from microbial strains, an ethyl acetate extract of *Actinomycete* SF2911, isolated from a soil sample collected in Okinawa Prefecture, Japan, showed the inhibitory activity. The active principle was purified and structure determination led to the isolation of one new compound. Since the structure belongs to the terfestatin family, we named it terfestatin D (1). It was found to inhibit cellular migration of breast carcinoma cells as well as NO production. We herein report the isolation, structure elucidation and biological activities of this new compound.
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INTRODUCTION

Nitrogen monoxide (NO) produced by endothelial nitric oxide synthase (eNOS) or inducible NO synthase (iNOS) often plays an important role regulating many physiological functions such as vasodilation and neurotransmission. However, overproduction of NO by iNOS of inflammatory cells such as macrophages often accelerates inflammatory reactions in rheumatoid arthritis, septic shock, multiple sclerosis, diabetes mellitus, amniotic membrane apoptosis and graft rejection.[1] Therefore, suppression of NO production or iNOS expression might be useful for the treatment of these inflammatory disorders. Lipopolysaccharide (LPS) induces iNOS expression that is mainly mediated by nuclear factor-κB (NF-κB). Therefore, screening of LPS-induced NO production inhibitors may provide new NF-κB inhibitors. We previously designed a novel NF-κB inhibitor dehydroxymethylepoxyqinomicin (DHMEQ) from the structure of antibiotic epoxyquinomicin C.[2] It showed potent anti-inflammatory and anticancer activities in animals. We isolated a known glutarimide compound, 9-methylstreptimidone[3] from the culture filtrate of *Streptomyces* and known penicillic acid and dihydropenicillic acid[4] from a fungus *Penicillium* as inhibitors of NF-κB. We also isolated a novel paxilline analog pyrapaxilline from fungus as an NF-κB that inhibited LPS-induced NO production.[5] A novel flavonoid desmal was shown to be an inhibitor of NF-κB.[6] We recently prepared a new epoxide-free analog of DHMEQ called SEMBL.[7] Most of these NF-κB inhibitors were screened firstly as NO production inhibitors. In the present research, we looked for the novel inhibitors of NO production among microbial secondary metabolites. As a result, we found a novel *p*-terphenyl glycoside with a rare 2,6-dideoxygulose moiety named terfestatin D from a *Actinomycete* strain.

RESULTS

Screening and isolation

For the screening of NF-κB and related signaling inhibitors, we looked for compounds that inhibit LPS-induced NO production in mouse monocytic leukemia RAW264.7 cells from microbial culture filtrates. We employed *Escherichia coli*-derived LPS purchased from Sigma-Aldrich. After screening of about 2000 *Streptomyces* culture filtrates, we found that the SF2911 broth inhibited LPS-induced NO production without toxicity. Then, we purify the active compound **1** as described in 'Methods'.

Structure elucidation

Compound **1** was isolated as white amorphous powders. Using high resolution electrospray ionization mass spectroscopy (HRESIMS), a molecular ion was measured at 431.1469 $[M+Na]^+$ (calcd for $C_{24}H_{24}O_6Na$, 431.1465), indicating a molecular formula of $C_{24}H_{24}O_6$ with 13 degree of unsaturation. The ^1H and ^{13}C NMR, and DEPT spectroscopic data revealed seven aromatic methines, seven aromatic quaternary carbons, an oxyquaternary carbon, two oxymethine, an aliphatic methylene, one methyl group and four hydroxyl groups. Careful analysis of these data revealed three aromatic rings and two phenolic protons, which were characteristic of a terphenyl-type sub-structure just like terferol.[8] In addition, a sugar

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moiety was also observed. ^{1}H–^{1}H-COSY, HMQC and HMBC spectral analyses also confirmed the presence of two monosubstituted benzene rings, a pentasubstituted benzene ring and a hexopyranose (Table 1, Figure 1). HMBC correlations from H-2/6 to C-1′, from H-2‴/6‴ to C-4′, led to the connection of C-1 to C-1′ and C-1″ to C-4′. Most notably, **1** was found to bear an rare sugar assigned as 2,6-dideoxy-β-xylo-hexopyranoside based upon key COSY (Figure 1, H-1‴/H-2a,b‴/H-3‴/H-4‴/H-5‴/Me-6‴, H-3‴/3‴-OH, H-4‴/4‴-OH), HMBC correlations (Table 1; from H-1‴ to C-2‴ and C-5‴; from H-5‴ to C-1‴), NOESY correlations and *J* couplings (Figure 2). An HMBC correlation from the anomeric proton H-1‴ to C-2′ indicated the sugar moiety was at C-2′. The sugar moiety was connected via a β-linkage on the basis of coupling constant of 9.6 Hz for the anomeric proton. Thus, the structure of **1** was assigned.

Over 100 known *p*-terphenyl derivatives have been reported from fungi and exhibited significant biological activities, for example, potent immune-suppressants, neuroprotective, antithrombotic, anticoagulant, specific 5-lipoxygenase inhibitory and cytotoxic activities.[9] Only 15 have been reported from bacteria, eight of which (terfestatins A–C and echosides A–E) exist as glucosides or glucuronides.[10] Terfestatin A, terphenyl-*β*-glucoside, was identified as a novel inhibitor of auxin signaling isolated from *Streptomyces* sp. F40.[11] Echosides A–E, terphenyl-*β*-glucuronides, were isolated from a geldanamycin-nonproducing mutant strain of *Streptomyces* sp. LZ35, which showed different degrees of inhibitory activity against topoisomerases and moderate antibacterial activity.[12] Terfestatins B and C were discovered from a coal mine fire isolate *Streptomyces* sp. RM-5-8 containing a rare unsaturated sugar moiety, 4-deoxy-α-L-threohex-4-enopyranuronate.[13] There are only three reported unsaturated hexuronic acids among the >3400 known naturally occurring glycosylated bacterial natural products.[14] In this study, we identified a *p*-terphenyl derivatives containing a unique 2,6-dideoxy-β-xylo-hexopyranoside moiety, which has not been reported elsewhere.

Table 1 NMR spectroscopic data of compound 1 (DMSO-d$_6$)[a]

Position	*δ_H (mult, J in Hz)*[b]	*δ_C*[c]	*^{1}H ^{1}H COSY*	*HMBC*
1		137.8		
2/6	7.37 m[d] 2H	130.9	3/5	C-1,1′
3/5	7.44 dd (7.2,7.2) 2H	128.2	4,2/6	C-1,3/5
4	7.37 m[d]	127.3	3/5	C-1,3/5
1′		134.4		
2′		135.2		
3′		148.6		
4′		115.9		
5′		151.8		
6′	6.41 s	107.3		C-1,2′,4′,5′
1″		133.8		
2″/6″	7.37 m[d] 2H	127.4	4″	C-4′
3″/5″	7.46 dd (7.2,7.2) 2H	128.8	4″,2″/6″	C-1″,2″/6″
4″	7.27 m	126.3	3″/5″,2″/6″	C-3″/5″
1‴	4.61 dd (9.6,1.8)	101.8	2‴a,b	C-2′,2‴,5‴
2‴[a]	1.82 ddd (12.0,9.6,3.0)	32.7	1‴,2‴b,3‴	C-1‴
2‴[b]	1.33 dd (12.0,1.8)		1‴,2‴a,3‴	C-1‴,3‴,4‴
3‴	3.70 d (3.0)	67.8	2‴a,b,3‴-OH,4‴	C-4‴, 5‴
4‴	3.01 brs	69.1	3‴,4‴-OH	C-2‴
5‴	3.78 q (6.6)	69.3	6‴	C-1‴,6‴
6‴	1.04 d (6.6)	16.6	5‴	C-4‴, 5‴
3′-OH	8.09 s			C-2′,3′,5′
5′-OH	9.33 s			C-4′,6′
3‴,4‴-OH	4.88 brs[d] 2H			

[a]Chemical shifts (δ) in p.p.m.
[b]600 MHz.
[c]150 MHz.
[d]overlapped signal.

Biological activity

Before purification, SF2911 culture filtrate inhibited LPS-induced NO production without any toxicity in RAW264.7 cells. After purification, however, the effective concentration and cytotoxic concentration of terfestatin D were not much different as shown in Figure 3a. Compounds that inhibit LPS-induced NO production often inhibit cancer cell migration and invasion.[7] Then, we employed highly malignant cancer cell line, human breast carcinoma MDA-MB-231 cells. We evaluated the inhibitory activity of terfestatin D on cellular migration using this cell line by wound healing assay. As shown in Figure 3b, terfestatin D dose-dependently inhibited the migration at 0.1–3 μg ml^{-1} without any toxicity. NF-κB inhibitors often inhibits cellular migration, but terfestatin D did not inhibit the constitutively activated NF-κB (data not shown).

DISCUSSION

Auxin is a growth-promoting hormone in plants. Terfestatin A was isolated as acompound that inhibits expression of the auxin-inducible

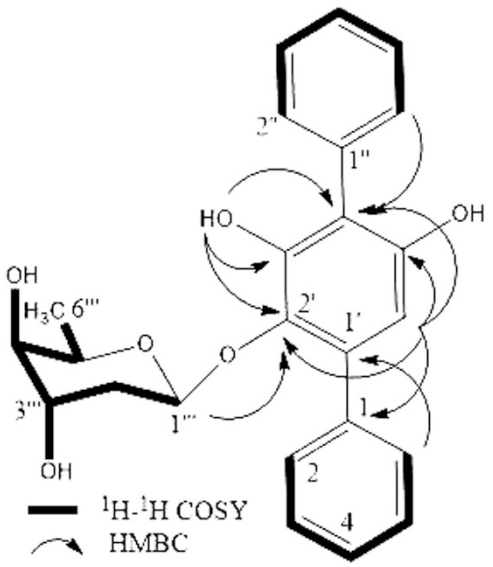

Figure 1 Structure and Key ^{1}H ^{1}H COSY and HMBC correlations of **1.**

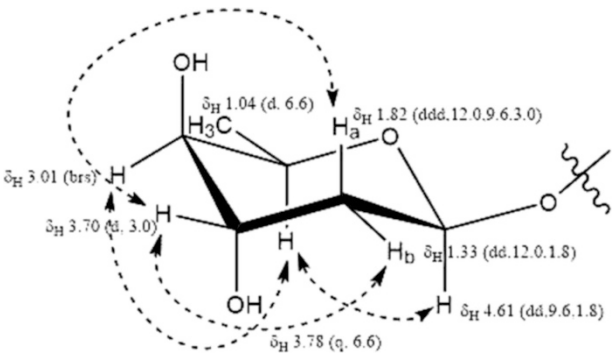

Figure 2 *J*-values and key NOE correlations of the sugar part of **1.**

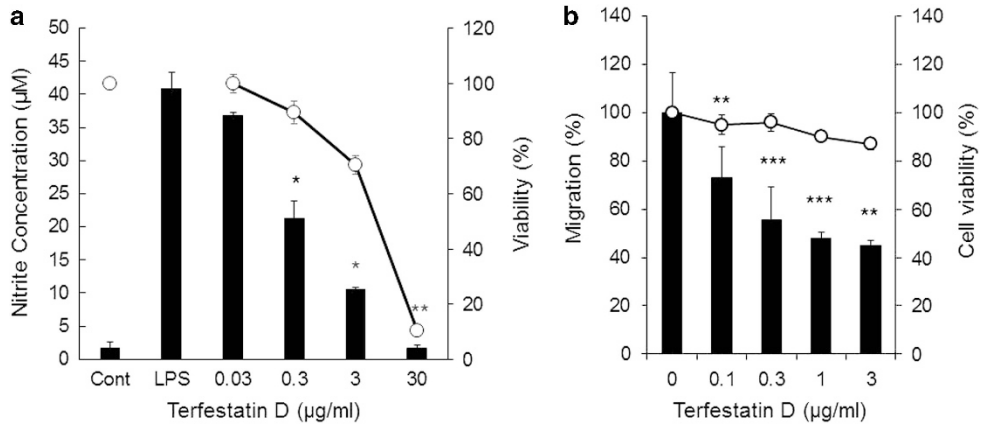

Figure 3 Biological activity of terfestatin D. (**a**) Inhibition of LPS-induced NO production in MDA-MB-231 cells. Bar, NO production; white circle, cellular viability. $*P<0.01$; $**P<0.001$. (**b**) Inhibition of cellular migration by terfestatin D. Bar, migration; white circle, cellular viability. $*P<0.01$; $**P<0.001$.

genes in the plant *Arabidopsis thaliana* action.[15] In the present research, we have isolated a novel related compound as an inhibitor of LPS-induced NO production. It also inhibited migration of breast carcinoma cells. NF-κB inhibitors such as (−)-DHMEQ[16] and SEMBL[7] inhibits cancer cell migration. But terfestatin D did not inhibit NF-κB in MDA-MB-231 cells. Migracin A inhibited cellular migration without inhibiting NF-κB,[16] and it was shown to inhibit IGF-1 expression.[17] Terfestatin D inhibits cellular migration more clearly than LPS-induced NO production. The mechanism for the inhibition of migration remains studied.

METHODS

General experimental procedures.

Optical rotations were determined on a Jasco P-1020 polarimeter (Tokyo, Japan). The UV data were recorded on a Perkin Elmer Lambda 25 UV/Vis spectrometer. The IR data were recorded using a Nicolet Avatar 330 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). NMR spectra were acquired on a Bruker ASCEND 600 MHz NMR magnet system using TMS as internal standard. HPLC-ESIMS was performed using a 6120 Single Quad LC/MS system (Agilent Technologies, Santa Clara, CA, USA). HR-ESIMS was performed using a Bruker maXis. CD spectra were recorded on a Jasco J-815 CD spectrometer. TLC was performed on Merck TLC plates (silica gel 60 RP-18 $F_{254}S$ and silica gel 60 F254, Darmstadt, Germany), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in EtOH and then heating on a hot plate. HPLC was performed on a Shimadzu LC-20 AT pump equipped with a SPD-20A UV-Vis detector. A YMC-Pack Ph column (4.6 × 250 mm ID 5 μ) and a YMC-Pack Pro C18 column (4.6×250 mm ID 5 μ) were used for analysis and isolation purposes.

Strain and fermentation procedure

Actinomycete strain SF2911 was isolated from a soil collected in Okinawa Prefecture, Japan. This strain was deposited in Meiji Seika Pharma Co, Ltd. A slant culture of SF2911 was used to inoculate in 100- ml Erlenmeyer flasks, containing 15 ml of a seed medium (starch 2.5%, glucose 2.0%, polypepton 0.7%, wheat germ 0.6%, yeast extract 0.45%, soybean meal 0.3%, Lab-Lemco-Powder 0.3%, $CaCO_3$ 0.2%, pH 7.2). The flasks were incubated at 28 °C for 2 days while shaking at 220 r.p.m. A portion of the seed culture was transferred into a 500- ml Erlenmeyer flask containing 80 ml of production medium (malt syrup 1.0%, starch 3.0%, pharmamedia 0.5%, polypepton 0.5%, Bonito Extract 0.4%, TM-solution*, pH 7.5) and incubated at 28 °C for 5 days on a rotary shaker.

*H_3BO_3 0.5%, $CuSO_4{\cdot}5\ H_2O$ 0.1%, KI 0.25%, $FeCl_3{\cdot}6H_2O$ 1.0%, $MnSO_4{\cdot}5H_2O$ 0.5%, $Na_2MoO_4{\cdot}2H_2O$ 0.2%, $ZnSO_4{\cdot}7H_2O$ 0.5%, $CoCl_2{\cdot}6H_2O$ 0.1%.

Isolation procedures

Total of the cultures (2 l) was extracted with the same volume of 67% aqueous acetone. The extract was filtered and evaporated *in vacuo* to remove the acetone, and then extracted with the same volume of ethyl acetate at pH 3.0, and concentrated *in vacuo* to give a dried brown residue (1.26 g). A part of the extract (997.3 mg) was applied to a Sephadex LH-20 column chromatography (CC) with $CHCl_3$-MeOH (1: 1) to give 6 fractions (Fr.1–Fr.6). Fr.4 (450.0 mg) was further isolated by HPLC with a YMC-Pack Pro C18 column eluted with MeOH-H_2O (5: 95–95: 5 over 30 min, 1.0 ml min^{-1}) to give 5 fractions (Fr.4–1 to Fr.4–5). Fr.4–4 (76.7 mg) was purified by HPLC with a YMC-Pack Ph column eluted with MeOH-H_2O (40: 60 over 20 min, 1.0 ml min^{-1}) to yield **1** (3.8 mg, t_R 9.4 min).

Terfestatin D (**1**). White amorphous powders; $[\alpha]_D^{27}$+29° (*c* 0.68, MeOH); UV (MeOH) λ_{max} (log ε) 264 (4.19) nm; IR (film) ν_{max}: 3446 , 1705 , 1457 , 1367 , 1191 , 758 cm^{-1}; CD (*c* 0.25 μM, MeOH) λ_{max} ($\Delta\varepsilon$) 225 (−1.3), 261 (+3.2); ^1H and ^{13}C NMR see Table 1; LCESIMS *m/z* 431 $[M+Na]^+$; HRESIMS: 431.1469 $[M+Na]^+$ (calcd. for $C_{24}H_{24}O_6Na$, 431.1465).

Cell culture

RAW264.7 cells and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), 200 μg ml^{-1} kanamycin (Sigma, St Louis, MO, USA), 100 units ml^{-1} penicillin G (Sigma), 600 μg ml^{-1} L-glutamine (Sigma), and 2.25 g l^{-1} $NaHCO_3$ under a humidified atmosphere with 5% CO_2 and 95% air at 37 °C.

MTT assay

After the incubation with the chemical for 24 h, 2 (Raw264.7) or 10 (MDA-MB-231) μl of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) solution was added into each plate and incubated for 1–2 h at 37 °C under 5% CO_2. Subsequently, the culture supernatant was replaced with 50 (RAW264.7) or 100 (MDA-MB-231) μl DMSO to dissolve formazan crystal made from succinic dehydrogenase in the mitochondria and its substrate MTT. An absorbance of 570 nm was measured with a microplate reader.

NO production

RAW264.7 cells (3×10^5 cells per ml) were seeded in a 96-well plate (Corning Inc., Corning, NY, USA) with each well receiving 100 μl of the cell suspension. One hour later, the cells were treated with chemicals for 30 min and then stimulated with 10 ng ml^{-1} LPS for 24 h. Then, 50 μl Griess reagent solution was added to each well. The concentration of NO was determined by measuring the absorbance at 570 nm with a microplate reader.

Wound healing assay

MDA-MB-231 cells in 24-well plates were allowed to reach confluence before the surface was uniformly scratched across the center of each well with a pipette tip. The wells were then rinsed twice with serum-free media to remove floating cells and growth media, after which the cells were cultured in serum-free media for 24 h. The initial wounded area and movement of the cells into the scratched area were recorded. Experiments were performed in triplicate in three independent experiments.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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