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13-BROMOMONOCILLIN I: A NEW WNT-5A EXPRESSION INHIBITOR PRODUCED BY *POCHONIA CHLAMYDOSPORIA* VAR. *CHLAMYDOSPORIA*

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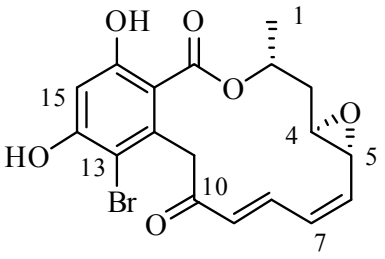
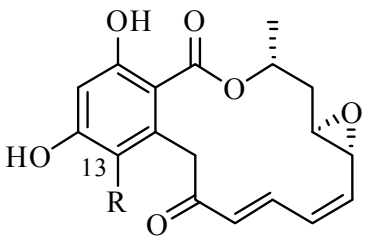
Abstract – WNT-5A is a secretory glycoprotein related to the proliferation of dermal papilla cells. While searching for an inhibitor of WNT-5A expression, a new compound, 13-bromomonocillin I (**1**), was isolated from the fermentation broth of the fungus *Pochonia chlamydosporia*, cultured in NaBr-supplemented medium. 13-Bromomonocillin I (**1**) exhibited WNT-5A expression inhibitory activity with an IC_{50} value of 0.25 μM.

Wingless-type mouse mammary tumor virus integration site family member 5A (WNT-5A) is a secretory glycoprotein that belongs to the WNT family. WNTs are important intercellular signaling molecules that regulate axis formation and organ formation during the fetal stage.[1,2] We have been studying molecules that regulate dermal papilla cell proliferation to develop a hair-growth stimulant. Consequently, we recently found that WNT-5A was highly expressed in dermal papillae of depilated skin. WNT-5A expression inhibitors promote proliferation of dermal papilla cells.[3] Using the inhibitory activity against WNT-5A expression as a bioassay guide, we obtained radicicol (**2**)[4–6] as an active compound. In addition, we isolated 10 new radicicol analogues, pochonins G–P,[7,8] and 10 known radicicol analogues from a culture broth of the fungus TF-0480 that had been identified as *Pochonia chlamydosporia var. chlamydosporia* and reported their structures and WNT-5A expression inhibitory activity.



13-bromomonocillin I (**1**)



R=Cl : radicicol (**2**)
R=H : monocillin I (**3**)

Figure 1. Structure of 13-bromomonocillin I (**1**) and radicicol analogues (**2** and **3**)

We noticed the potent inhibitory activity of radicicol (**2**) against WNT-5A expression (IC_{50} 0.19 μM), which is much stronger than that of monocillin I (**3**) (IC_{50} 1.93 μM), a dechloro derivative of **2**. It is likely that the strong activity of **2** is attributed to the presence of the chlorine atom at C-13. Therefore, we attempted to obtain the monocillin derivatives with C-13 positions that are substituted with other halogens, i.e., fluorine, bromine, and iodine, by supplementing the corresponding sodium halides to the cultivation media. Supplementing NaF and NaI greatly inhibited the growth of the organism, and no fluorine or iodine-derived monocillin was produced.[9,10] However, a new radicicol-type macrolactone 13-bromomonocillin I (**1**) was isolated when the fungus was cultured in a NaBr supplemented medium.[9,11,12] This paper describes the fermentation, isolation, structural elucidation, and WNT-5A expression inhibitory activity of 13-bromomonocillin I (**1**).

Strain TF-0480 was identified as *Pochonia chlamydosporia var. chlamydosporia* based on its morphological characteristics and culture properties.[3] A slant culture of strain TF-0480 was inoculated into 100 mL of a seed medium containing 2.0% glucose, 4.0% mannitol, 2.0% oatmeal, 0.4% yeast extract, 0.014% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4–5H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$ (adjusted to pH 6.0 before sterilization), and was cultured at 26 °C for 3 days on a rotary shaker at 200 rpm. One milliliter of the culture was transferred to a 500 mL Erlenmeyer flask containing 100 mL of a medium containing 12.0% mannitol, 4.0% oatmeal, 0.8% yeast extract, 0.014% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4–5H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$ adjusted to pH 7.0 before sterilization. After incubating the culture at 26 °C for 2 days on a rotary shaker at 200 rpm, NaBr (0.1%) was added and the culture was incubated for an additional 10 days.

The culture broth was extracted with ethyl acetate and the organic layer was dried over sodium sulfate and concentrated in vacuo to produce a crude extract (7.5 g). The crude extract was purified by silica gel column chromatography (eluent, chloroform:methanol = 98:2), preparative TLC (developing solvent, n-hexane:ethyl acetate = 2:1, twice), and preparative HPLC using two connected columns (YMC-Pack

Table 1. Physico-chemical properties of 13-bromomonocillin I (**1**)

Appearance	colorless oil
MW	409.23
Molecular formula	$C_{18}H_{17}Br_1O_6$
$[\alpha]_D^{20}$	+19.6 (*c* 1.00, acetone)
HR-ESIMS (*m/z*)	
Found	407.0140
Calcd	407.0130 $[M-H]^-$
UV λ_{max} nm (log ε) (MeOH)	218 (4.24), 264 (4.08), and 317 sh (3.64)
IR ν_{max} cm^{-1} (KBr)	3419, 2983, 1709, 1651, 1603, 1581

Pro C_{18} + YMC-Pack Pro C_{18} RS, both 5 μm, 20 × 250 mm) eluted with MeCN:H_2O (+0.25% acetic acid) = 35:65 to produce pure compound **1** (40.8 mg) together with known compounds radicicol (**2**) (1608 mg) and monocillin I (**3**) (28.8 mg). Chlorine of **2** must be originated from the cultivation medium containing oatmeal and yeast extract.

The physico-chemical properties of **1** are summarized in Table 1. The appearance of two molecular ion peaks at m/z 407 and 409 with equal intensities in the low-resolution ESI-mass spectrum (negative ion-mode) indicated that a bromine atom was incorporated in the molecule. The molecular formula of **1** was established as $C_{18}H_{17}Br_1O_6$ by HRESI-MS (found *m/z* 407.0140; calc for $C_{18}H_{16}Br_1O_6$ $[M-H]^-$, 407.0130, Δ +1.0 mmu). The IR spectrum showed the presence of a hydroxyl (3419 cm^{-1}) and a conjugated carbonyl (1709 cm^{-1}) group. The UV spectrum of **1** showed maximal absorptions (λ_{max}) at 218 and 264 nm, similar to that of radicicol (**2**) [λ_{max} 215, 266, 308 (sh) nm].[8] The structure of **1** was elucidated using ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMQC, and HMBC. ^{13}C NMR and DEPT spectra of **1** exhibited 18 carbon signals consisting of one methyl, two methylenes, eight methines, and seven quaternary carbons. The ^{1}H NMR and HMQC spectra of **1** exhibited the signals due to two epoxy protons (δ_H 3.32 and 3.02, δ_C 56.1 and 55.8), four olefinic protons (δ_H 7.42, 6.23, 6.16 and 5.73), and an aromatic proton (δ_H 6.58, δ_C 103.4). The proton network from H_3-1 to H-9 was deduced from the ^{1}H–^{1}H COSY spectrum. The small coupling constant (2.3 Hz) between H-4 and H-5 suggested that the epoxide had an *E*-configuration. The coupling constants ($J_{H\text{-}6/H\text{-}7}$ = 10.7 Hz, $J_{H\text{-}8/H\text{-}9}$ = 16.0 Hz) of the olefinic protons indicated that the two olefins (C6–C7 and C8–C9) had *Z*- and *E*-configurations, respectively. The HMBC correlations between H-15 (δ_H 3.32) and C-13, C-14, C-16, and C-17 (δ_C 106.5, 158.8, 161.0, and 112.1, respectively) indicated the presence of a pentasubstituted aromatic ring. Other critical correlations were as follows: from H_2-11 (δ_H 4.38, 3.94) to C-9 (δ_C 131.2), C-10 (δ_C 196.4), C-13 (δ_C 106.5), and C-17 (δ_C 112.1); from H-9 (δ_H 6.16) to C-11 (δ_C 49.0); from H-8 (δ_H 7.42) to C-10; and from

Table 2. NMR data (500 MHz, acetone-d_6) for 13-bromomonocillin I (**1**) and radicicol (**2**)

	13-bromomonocillin I (**1**)				radicicol (**2**) [8]			
position	δ_C	mult.	δ_H	(J in Hz)	δ_C	mult.	δ_H	(J in Hz)
1	18.5	q	1.53	d (6.9)	18.5	q	1.55	d (6.6)
2	71.7	d	5.40	ddq (3.8, 3.3, 6.9)	71.9	d	5.43	ddq (3.8, 3.3, 6.6)
3	37.1	t	2.41	dt (15.3, 3.8)	37.0	t	2.43	ddd (15.1, 3.3, 2.7)
			1.77	ddd (15.3, 8.4, 3.3)			1.78	ddd (15.1, 8.5, 3.8)
4	56.1	d	3.02	ddd (8.4, 3.8, 2.3)	56.1	d	3.04	dt (8.5, 2.7)
5	55.8	d	3.32	br s	55.8	d	3.19	br s
6	135.6	d	5.73	dd (10.7, 3.8)	135.9	d	5.75	dd (10.7, 3.6)
7	130.3	d	6.23	dd (10.7, 9.2)	130.4	d	6.24	dd (10.7, 9.6)
8	138.7	d	7.42	dd (16.0, 9.2)	139.0	d	7.50	dd (15.9, 9.6)
9	131.2	d	6.16	d (16.0)	131.2	d	6.12	d (15.9)
10	196.4	s			199.1	s		
11	49.0	t	4.38	d (16.0)	46.5	t	4.44	d (16.2)
			3.94	d (16.0)			3.90	d (16.2)
12	137.8	s			136.4	s		
13	106.5	s			115.6	s		
14	158.8	s			157.9	s		
15	103.4	d	6.58	s	103.8	d	6.57	s
16	161.0	s			160.6	s		
17	112.1	s			111.0	s		
18	168.7	s			168.8	s		

H-2 (δ_H 5.40) to C-18 (δ_C 168.7). These data suggested that compound **1** has a 14-membered macrolactone the same as **2**. The ^{13}C NMR spectrum of **1** was similar to that of **2**, with the exception of the signal of the quaternary carbon at C-13 (δ_C 106.5) in the former (Table 2). The higher shift of C-13 of **1** than that of the C-13 (δ_C 115.6) of **2** indicated that the bromine atom was attached to C-13 instead of the chlorine atom of **2**.[13] These data demonstrated that compound **1,** named 13-bromomonocillin I, was a new analogue of **2** with the replacement of the chlorine atom in **2** by a bromine atom (Figure 1).

The relative stereochemistry of **1** was assigned according to the results of the NOESY experiment (Figure 2). The NOE correlations between H_3-1/H-11a and H-11b, H_3-1/H-4, H-3a and H-3b/H-5, and H-5/H-8 indicated that the relative configurations of **1** at C-2, C-4, and C-5 were the same as those of **2**.

The WNT-5A expression inhibitory activity and cytotoxicity against dermal papilla cells of compounds **1**–**3** are shown in Table 3. The WNT-5A expression inhibitory activity of 13-bromomonocillin I (**1**) is stronger than that of monocillin I (**3**) and as potent as that of radicicol (**2**). The cytotoxicity of **1** against dermal papilla cells is more than five times weaker than that of **3**. These data indicate that a halogen atom at C-13 in the radicicol-type 14-membered macrolactone contributes to enhancement of WNT-5A expression inhibitory activity and the reduction of cytotoxicity, and that chlorine and bromine atoms at this position had similar effects. We have already found that active compounds with WNT-5A

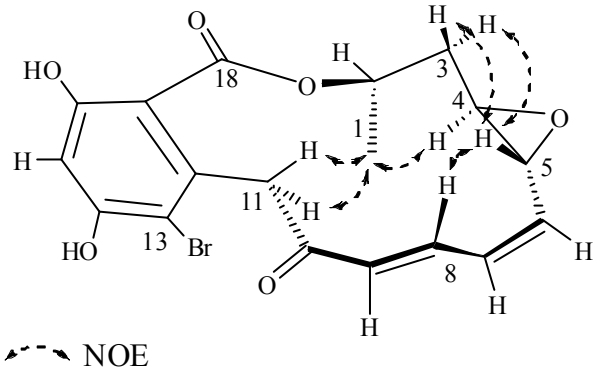


Figure 2. Key NOESY correlations for 13-bromomonocillin I (**1**)

Table 3. WNT-5A expression inhibitory activities of 13-bromomonocillin I (**1**), radicicol (**2**), and monocillin I (**3**)

compound	IC_{50} (μM) WNT-5A	TC_{50} (μM)[a]
13-bromomonocillin I (**1**)	0.25	>15.3
radicicol (**2**)	0.19	15.3
monocillin I (**3**)	1.93	2.90

[a] Half maximal toxic concentration against dermal papilla cells

expression inhibitory activity also exert dermal papilla cell proliferation (will be reported in another article), and the new WNT-5A expression inhibitor, 13-bromomonocillin I (**1**), is expected as a potent hair-growth stimulant.

EXPERIMENTAL

General Experimental Procedures

NMR spectra were recorded at 500 MHz using a JEOL ECA 500 spectrometer (JEOL Ltd.). Chemical shifts were reported in parts per million (ppm), and the solvent peaks were utilized as an internal standard. The coupling constants (*J*) were given in hertz (Hz). Electrospray ionization (ESI) mass spectra were obtained using a Micromass Platform LC mass spectrometer (Micromass Ltd.). Optical rotations were measured using an AUTOPOL V digital polarimeter (Rudolph Research Analytical). UV spectra were recorded using a V-520 UV/Vis spectrophotometer (JASCO). IR spectra were measured using a Spectrum One FT-IR spectrometer (Perkin Elmer).

Microorganism

Strain TF-0480 was isolated from a soil sample collected in Fujioka City, Tochigi Prefecture, Japan. The strain was deposited at the National Institute of Advanced Industrial Science and Technology in Japan as FERM BP-8332.

Biological assay

Inhibition of WNT-5A expression: quantification of mRNA using the QuantiGene method

Human dermal papilla cells were cultured in MEM (Invitrogen) containing 12% fetal bovine serum (FBS). The dermal papilla cells in the fifth subculture generation were sowed in a 96-well plate at a density of 1 × 10^4 cells/well and cultured overnight. The medium was then replaced with one that did or did not contain the test compounds and the culture was continued for an additional 24 h. After 24 h, the amount of WNT-5A mRNA was measured using a QuantiGene High Volume Kit (Bayer Medical) by the branched DNA (bDNA) signal amplification method.[14] In accordance with the manufacturer's protocol, the cells were lysed with a lysis mixture and the lysis solution was added to the capture plate. Next, a set of probes specific to WNT-5A[3] was added, and the reaction proceeded at 53 °C for 20 h. After the plate was washed using 0.1 × SSC (3.0 M NaCl and 0.3 M sodium citrate) containing 0.03% lauryl sulfate, an amplification probe containing bDNA was added and reacted at 46 °C for 1 h. After the plate was washed again, a probe labeled with alkaline phosphatase was added and reacted at 46 °C for 1 h. After the plate was washed for last time, the Lumi-Phos Plus substrate was added, and the reaction proceeded at 46 °C for 30 min. Luminescence was measured using a WALLAC 1420 ARVOsx (Perkin Elmer).

Dermal papilla cell cytotoxicity

Dermal papilla cells were sowed in a 96-well plate to yield a density of 5 × 10^3 cells/well and cultured for 16 h in MEM containing 12% FBS. The medium was then replaced with either a medium to which no compounds had been added or a medium that contained the test compounds, and the culture continued for an additional 24 h. The medium was then replaced with a medium containing 10% Alamar Blue™ (Wako Pure Chemical Industries), and the culture was continued for an additional 4 h. Finally, the fluorescence intensity (Ex 544 nm, Em 590 nm) was measured using a WALLAC 1420 ARVOsx.

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