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The search for a hair-growth stimulant: new radicicol analogues as WNT-5A expression inhibitors from *Pochonia chlamydosporia var. chlamydosporia*

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ABSTRACT

WNT-5A, a secretory glycoprotein, is related to the proliferation of dermal papilla cells. While searching for an inhibitor of WNT-5A expression, we identified an active compound, radicicol (**1**), and isolated four unique analogues, pochonins G–J (**2**–**5**), from a culture broth of the fungus *Pochonia chlamydosporia* TF-0480. Structural elucidation of **2**–**5** and their biological activities against WNT-5A are described.

It has been pointed out that the stress of modern living has caused an increase in baldness, and the demand for the hair-growth tonic is rising rapidly.[1] The hair shaft is formed by the proliferation and differentiation (keratinization) of follicular keratinocytes. Dermal papillae regulate the proliferation, differentiation, and apoptosis of these follicular keratinocytes, which play a key role in the control of the hair cycle.[2] WNT-5A (wingless-type mouse mammary tumor virus integration site family, member 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.[3,4] To develop a hair-growth stimulant, we have been studying molecules that regulate the proliferation of dermal papilla cells. We recently found that WNT-5A was highly expressed in the dermal papillae of depilated skin. WNT-5A expression inhibitor promotes the proliferation of dermal papilla cells.[5] Using the inhibitory activity against WNT-5A expression as a bioassay guide, we obtained radicicol (**1**)[6] as a potently active compound from a culture broth of the fungus *Pochonia chlamydosporia var. chlamydosporia*. In addition, we were able to isolate four new compounds **2**-**5** (Fig. 1). The present Letter describes the structures of the new compounds and their WNT-5A inhibitory activities.

The inhibition of WNT-5A expression was measured using the QuantiGene assay (a signal amplification nucleic acid probe assay for the direct quantification of cellular mRNA).[7] Cytotoxicity against dermal papilla cells was measured using the Alamar Blue™ assay. Strain TF-0480 was isolated from a soil sample collected in Fujioka City, Tochigi Prefecture, Japan (1994). Based on its morphological characteristics, the strain was identified as *P. chlamydosporia var. chlamydosporia*.[5] The cultured broth[8] was centrifuged, and the supernatant was treated with Diaion HP-20. The resin was washed with water and methanol, and the methanol eluent was extracted with ethyl acetate. The ethyl acetate extract was chromatographically separated, yielding radicicol (**1**) and four new compounds, **2**–**5**, designated as pochonins G–J.[9,10]

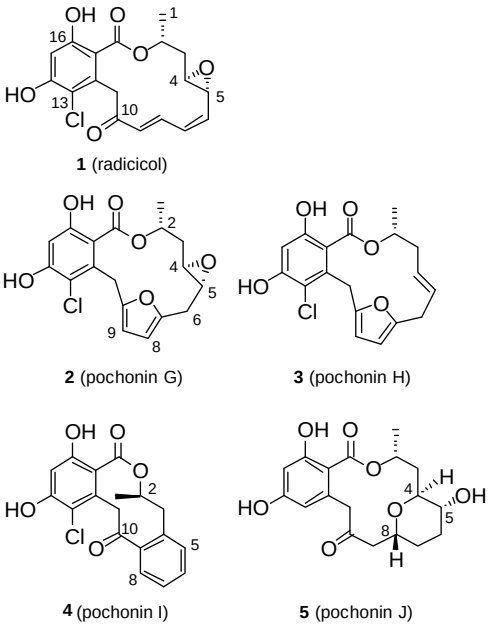

Figure 1. Structure of pochonins G–J (**2**–**5**).

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Table 1
NMR data (500 MHz) for pochonins G–J (**2**–**5**)

	Pochonin G (**2**)[a]				Pochonin H (**3**)[a]				Pochonin I (**4**)[a]				Pochonin J (**5**)[b]			
Position	δ_C	mult.	δ_H	mult. (*J* in Hz)	δ_C	mult.	δ_H	mult. (*J* in Hz)	δ_C	mult.	δ_H	mult. (*J* in Hz)	δ_C	mult.	δ_H	mult. (*J* in Hz)
1	20.5	q	1.40	d (6.1)	21.1	q	1.38	d (6.1)	18.1	q	1.01	d (6.1)	19.0	q	1.34	d (6.7)
2	72.3	d	5.15	m	71.7	d	5.42	m	73.6	d	5.70	m	72.9	d	5.32	m
3	38.3	t	2.38	ddd (14.0, 8.5, 4.3)	39.9	t	2.39	ddd (13.7, 4.6, 1.8)	37.3	t	3.52	dd (14.6, 4.3)	41.1	t	2.18	dd (15.5, 2.4)
			1.38	ddd (14.0, 9.8, 3.7)			2.25	dt (13.7, 10.7)			2.68	dd (14.6, 2.4)			1.58	ddd (15.5, 7.3, 4.3)
4	56.5	d	2.75	ddd (8.5, 3.7, 1.0)	131.7	d	5.61	ddd (15.2, 10.7, 4.6)	144.5	s			73.0	d	3.42	ddd (10.0, 7.3, 2.4)
5	54.9	d	2.94	ddd (8.5, 3.7, 1.8)	128.3	d	5.40	m	133.6	d	7.37	dd (5.5, 3.7)	83.0	d	3.58	m
6	32.1	t	3.24	dd (15.3. 3.7)	31.4	t	3.18	m	127.9	d	7.43	td (5.5, 3.7)	29.2	t	2.05	m
			2.13	dd (15.3, 8.5)											1.94	m
7	150.4	s			151.9	s			130.0	d	7.44	td (5.5, 3.7)	31.5	t	1.91	m
															1.74	m
8	108.3	d	5.50	br d (2.4)	106.4	d	5.86	d (3.0)	125.2	d	7.62	dd (5.5, 3.7)	77.2	d	4.38	m
9	107.3	d	6.17	br d (2.4)	107.1	d	6.08	d (3.0)	132.2	s			47.9	t	2.65	dd (15.5, 10.0)
															2.40	dd (15.5, 4.0)
10	151.7	s			151.7	s			204.0	s			200.6	s		
11	28.6	t	4.58	d (15.3)	27.9	t	4.58	d (14.3)	47.6	t	4.85	d (17.1)	52.7	t	4.27	d (18.3)
			4.24	d (15.3)			4.17	d (14.3)			4.40	d (17.1)			3.85	d (18.3)
12	137.6	s			138.8	s			138.4	s			139.2	s		
13	114.7	s			114.4	s			116.4	s			113.0	d	5.98	d (2.4)
14	156.4	s			156.2	s			159.6	s			163.2	s		
15	103.6	d	6.52	s	103.2	d	6.47	s	103.6	d	6.56	s	102.9	d	6.14	d (2.4)
16	156.9	s			156.7	s			164.0	s			165.1	s		
17	113.7	s			113.5	s			106.5	s			108.2	s		
18	168.3	s			167.0	s			170.0	s			171.8	s		

[a] In acetone-d_4.
[b] In methanol-d_4.

The molecular formula of pochonin G (**2**), $[\alpha]_D^{20}$ −159 (*c* 1.00, acetone), was established as $C_{18}H_{17}ClO_6$ by HRESI-MS (found *m/z* 363.0641; calc for $C_{18}H_{16}{}^{35}Cl_1O_6$ $[M-H]^-$, 363.0635, Δ +0.6 mmu), which is the same as that of radicicol (**1**) {$[\alpha]_D^{20}$ +195 (*c* 1.00, chloroform), lit.[6b]; $[\alpha]_D^{20}$ +216 (*c* 1.00, chloroform)}. In the ^1H (500 MHz, acetone-d_6) and ^{13}C NMR spectra (125 MHz, acetone-d_6) of **2** (Table 1), several signals from moieties in common with those of **1** were observed: (1) 2-alkyl-3-chloro-4,6-dihydroxybenzoate (C11–C18); (2) a secondary methyl (C-1); (3) an acyloxy methine (C-2); and (4) an epoxide [δ_H 2.75 (1H, ddd, *J* = 8.5, 3.7, 1.8 Hz; H-4); δ_C 56.5 (d; C-4), δ_H 2.94 (1H, ddd, *J* = 8.5, 3.7, 1.8 Hz; H-5); δ_C 54.9 (d; C-5)] adjacent to a methylene [δ_H 3.24 (1H, dd, *J* = 15.3, 3.7 Hz, H-6a), 2.13 (1H, dd, *J* = 15.3, 8.5 Hz, H-6b); δ_C 32.1 (t; C-6)]. The small coupling constant (1.8 Hz) between H-4 and H-5 suggested that the epoxide had an *E*-configuration (in **1**, $J_{H\text{-}4/H\text{-}5}$ = 2.7 Hz). The NMR signals of the dienone group of **1** were absent from the spectrum of **2**; instead, compound **2** exhibited signals assignable to a 2,5-disubstituted furan [δ_H 6.17 (1H, br d, *J* = 2.4 Hz; H-9), 5.50 (1H, br d, *J* = 2.4 Hz; H-8); δ_C 151.7 (s; C-10), 150.4 (s; C-7), 108.3 (d; C-8), 107.3 (d; C-9)]. The proton network from H-1 to H-6 was deduced from the COSY spectrum. The allyl couplings between H-6/H-8 and H-9/H-11 were also determined using the COSY spectrum. The location of the furan was further confirmed by the HMBC spectrum, which showed correlation peaks caused by long-range H/C couplings between H-8/C-6 and H-9/C-11. The HMBC spectrum also indicated that the orientations of the substituents on the benzene ring were the same as those of **1** (Table 1).

The relative stereochemistry of **2** was assigned according to the results of a NOESY experiment (Fig. 2) assisted by MM2 calculation (using Chem 3D). The NOE correlations between H-2/H-5, H-5/H-6β, H-6β/H-8, and H-4/H-6α indicated that the relative configurations of **2** at C-2, -4, and -5 were the same as those of **1**. Pochonin G (**2**) is the first example in the radicicol family to have a furan ring.

The molecular formula of pochonin H (**3**), $[\alpha]_D^{20}$ +229 (*c* 1.00, acetone), was established as $C_{18}H_{17}ClO_5$ by HRESI-MS (found *m/z* 347.0684; calc for $C_{18}H_{16}{}^{35}Cl_1O_5$ $[M-H]^-$, 347.0686, Δ −0.2 mmu). Pochonin H (**3**) contains one oxygen less in comparison with pochonin G (**2**). Actually, the signals caused by epoxide were absent in the ^1H and ^{13}C NMR spectra (acetone-d_6) (Table 1) and were replaced by two olefinic proton signals at δ 5.61 (H-4) and 5.40 (H-5). The coupling constant ($J_{H\text{-}4/H\text{-}5}$ = 15.2 Hz) of these olefinic protons indicated that the new olefin moiety had an *E*-configuration. Except for these, the signals were, overall, similar to those of **2**. The location of the double bond at C-4 was easily deduced from the COSY and HMBC spectra (Table 1). Therefore,

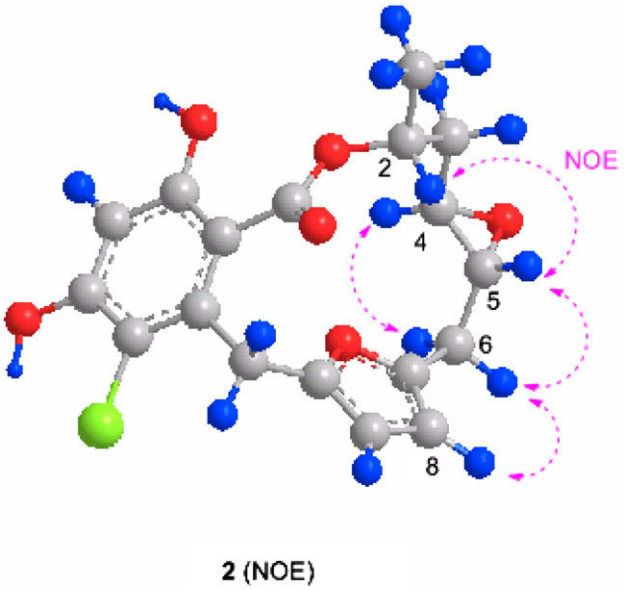

Figure 2. NOEs observed for pochonin G (**2**) in the NOESY spectrum. NOEs are expressed as dotted-line arrow. The conformation was energy-minimized based on MM2 calculation.

pochonin H (**3**) was concluded to be a deoxy derivative of **2**, in which the *E*-configuration of the 4,5-epoxide moiety of **2** was retained as 4,5-olefin in **3**.

The most remarkable feature of the NMR properties of pochonin I (**4**), $[\alpha]_D^{20}$ +128 (*c* 0.72, acetone), $C_{18}H_{15}ClO_5$ by HRESI-MS (found *m/z* 345.0543; calc for $C_{18}H_{14}{}^{35}Cl_1O_5$ $[M-H]^-$, 345.0530, Δ +1.3 mmu), is that it contains a 1,2-disubsituted benzene moiety: The ^{1}H NMR spectrum (acetone-d_6) exhibits aromatic proton signals at δ 7.62 (1H, dd, J = 5.5, 3.7 Hz; H-8), 7.44 (1H, td, J = 5.5, 3.7 Hz; H-7), 7.43 (1H, td, J = 5.5, 3.7 Hz; H-6), and 7.37 (1H, dd, J = 5.5, 3.7 Hz; H-5), and the ^{13}C NMR spectrum shows six aromatic carbon signals at δ 144.5 (s; C-4), 133.6 (d; C-5), 132.2 (s; C-9), 130.0 (d; C-7), 127.9 (d; C-6), and 125.2 (d; C-8). The downfield chemical shift of H-8 (δ7.62) suggested that the benzene ring was conjugated with a carbonyl group. The HMBC signals between H-8/C-10 (δ 204.0), H-5/C-3 (δ 37.3), and H_2-3 (δ 3.52, 2.68)/C-4 and -9 supported the presence of a 1-acyl-2-alkylbenzene group. In the ^{1}H NMR spectrum (acetone-d_6), two methylene protons appeared as an AB-type quartet at δ 4.85 and 4.40 (J_{AB} = 17.1 Hz) (H_2-11), and an ABX-type signal was visible at δ 3.52 and 2.68 (J_{AB} = 14.6 Hz, J_{AX} = 4.3 Hz, J_{BX} = 2.4 Hz) (H_2-3). These properties led us to propose the structure of pochonin I to be **4**.

The molecular formula of pochonin J (**5**), $[\alpha]_D^{20}$ −35.4 (*c* 0.28, methanol), was established as $C_{18}H_{22}O_7$ by HRESI-MS (found *m/z* 349.1306; calc for $C_{18}H_{21}O_7$ $[M-H]^-$, 349.1287, Δ +1.9 mmu). In the ^{1}H and ^{13}C NMR spectra (methanol-d_4) (Table 1), two meta-coupled aromatic protons were present at δ 6.14 (H-15) and 5.98 (H-13) ($J_{H\text{-}13/H\text{-}15}$ = 2.4 Hz), indicating that **5** was a dechloro radicicol analogue. This compound does not exhibit the ^{1}H and ^{13}C NMR signals due to epoxide and olefin moieties but shows three additional oxy-methine signals [δ_H 3.42 (H-4); δ_C 73.0 (C-4), δ_H 3.58 (H-5); δ_C 83.0 (C-5), δ_H 4.38 (H-8); δ_C 77.2 (C-8)] (Table 1), as there is already an analogous signal for position 2. The degree of unsaturation according to the molecular formula implies one more ring, and the HMBC correlation between H-8/C-4 corroborates a tetrahydropyrane ring located between C-4 and C-8.

The twist-boat conformation of the tetrahydropyran ring (C4–C8) in **5** was deduced from the ^{1}H–^{1}H coupling constant ($J_{H\text{-}4/H\text{-}5}$ = 10.0 Hz; anti) and NOESY correlation (H-4/H-7a).

The relative stereochemistry of **5** was deduced from the ^{1}H–^{1}H coupling constants and NOESY data (Fig. 3). The $J_{H\text{-}3a/H\text{-}4}$ and $J_{H\text{-}3b/H\text{-}4}$ values were relatively large and small (7.3 and 2.4 Hz, respectively), indicating that the relationships of H-3a/H-4 and H-3b/H-4 were anti and gauche, respectively. The NOE correlations between H-1/H-4, H-4/H-9a, and H-3b/H-5 indicated that the relative configurations of **5** were α-methyl (C-2), α-H (C-4), α-OH (C-5), and β-H (C-8).

The WNT-5A expression inhibitory activities and cytotoxicities against dermal papilla cells of pochonins G–J (**2**–**5**) and radicicol (**1**) are summarized in Table 2.

Radicicol (**1**) showed the strongest WNT-5A inhibitory activity of the tested samples, although it also showed high cytotoxicity. The inhibitory activity of pochonin G (**2**) was ten-fold weaker than that of **1**. It is noteworthy that compound **2** showed no cytotoxicity at concentrations above 100 μM. Pochonins H (**3**), I (**4**), and J (**5**) are practically inactive, which implies that the 4,5-epoxide moiety present in **1** and **2** may be necessary for radicicol-type compounds that are designed to inhibit WNT-5A expression.

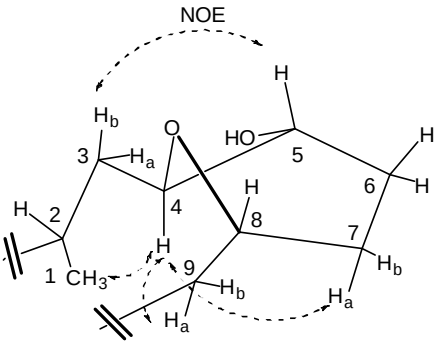

Figure 3. Relative stereochemistry of the C1–C9 part of pochonin J (**5**). NOEs are expressed as dotted-line arrow.

Table 2
WNT-5A expression inhibitory activities of radicicol (**1**) and pochonins G–J (**2**–**5**)

	IC_{50} (μM)	TC_{50} (μM)[a]
Compound	WNT-5A	
Radicicol (**1**)	0.19	15.4
Pochonin G (**2**)	8.15	>100
Pochonin H (**3**)	>100	>100
Pochonin I (**4**)	89.4	>100
Pochonin J (**5**)	>100	>100

[a] Half maximal toxic concentration against dermal papilla cells.

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8. The fermentation of TF-0480 was carried out at 26 °C for 6 days under an aeration rate of 1.0 v/v/minute and agitation at 300 rpm in 1-ton jar fermentors containing 600 l of production medium consisting of 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% $MnSO_4{\cdot}4$–$5H_2O$, and 0.0005% $CuSO_4{\cdot}5H_2O$, adjusted to pH 6.0 before sterilization.
9. A series of radicicol analogues have been isolated from the *Pochonia* species, and they have been named pochonins A–F.[11] The present new analogues, therefore, were named pochonins G–K as the successors of pochonin F.
10. Pochonins G (**2**), H (**3**), I (**4**) and J (**5**) were yielded as colorless oils (**2**; 67.2 mg, **3**; 13.4 mg, **4**; 9.9 mg, **5**; 4.3 mg).
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