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NOTES

PP2A Inhibitors, Harzianic Acid and Related Compounds Produced by Fungus Strain F-1531

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Serine/threonine phosphatase type 2A (PP2A) is an intracellular protein phosphatase, which catalyzes dephosphorylation of many substrates. We have recently found that specific inhibitors of PP2A augment natural killer cells *in vivo* and inhibit tumor metastasis[1~3]. Thus, a specific inhibitor of PP2A is a candidate for a new immune activator. In the course of our searching for a novel PP2A inhibitor, we have found that the culture broth of Fungus strain F-1531 showed potent inhibitory activity against PP2A. We isolated active materials including two new compounds. These compounds were found to be active only under the chelated condition with zinc ion. In this paper, we describe the fermentation, isolation, physico-chemical properties, and biological activities of harzianic acid-related compounds.

Fungus strain F-1531 was isolated from a soil sample collected in Amagi, Shizuoka prefecture, Japan. Strain F-1531 grown on a agar slant was inoculated into 100 ml of medium containing potato starch 2%, glycerin 1%, soy bean meal 2%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_20$ 0.05% and five glass beads, and cultured at 25°C for 3 days on a rotary shaker (225 rpm). One ml of the seed culture was inoculated into 500-ml flask containing 100 ml of a culture medium containing corn starch 2%, potato starch 1%, beet sugar 1%, Pharmamedia 1%, gluten meal 1%, malt extract 0.5%, $ZnSO_4$ 0.01%, Al_2O_3 0.2%, $CaCO_3$ 0.2% (pH6.0 before sterilization) and cultured at 25°C for 4 days on a rotary shaker (225 rpm).

The fermented broth (10 liters) was filtered and the mycelia were extracted with MeOH. The mycelial extract was concentrated and combined with the broth filtrate and further extracted with BuOH. The organic layer was concentrated under reduced pressure and applied to a silica gel column prepacked with BuOAc : BuOH : MeOH : H_2O = 4 : 4 : 1 : 2. After the column was washed with the same solvents, the active materials were eluted with BuOH : MeOH : H_2O = 4 : 1 : 2. Further purification was carried out by Sephadex LH-20 chromatography using MeOH as an eluent. By repeating this procedure three times, 89.7 mg of **1** was obtained as a yellow powder. Compund **1** (tentatively named **1a**) inhibited PP2A activity at IC_{50} value of 10 μg/ml without effect on other serine/threonine phosphatase type 1 (PP1). On the other hand, compound **1** (tentatively named **1b**) which was purified by reversed phase HPLC (Inertsil ODS-3, GL Science) with 80% MeOH in 20 mM KH_2PO_4 at pH 2 did not show any activity against PP2A. Thus, we examined the structural discrepancy between **1a** and **1b**. All NMR spectra including 2D NMR experiments showed that **1a** and **1b** were essentially the same to harzianic acid,[4] although the peaks of **1a** were broaden. EDS spectra of two compounds revealed the presence of Zn in **1a** and the absence of Zn in **1b**. This was further supported by the mass spectra of two compounds. In the negative mode ESI mass spectrum of **1a**, the base peak was observed at *m*/*z* 794 due to $[2M+Zn]^-$, while **1b** showed the deprotonated molecular ion at *m*/*z* 364 as the base peak. In the ESI-MS/MS spectrum of **1a**, the daughter ion at *m*/*z* 364 was observed from the parent ion at *m*/*z* 794. These results indicated that the active form was composed of **1a** and Zn as 2 : 1 complex. On the other hand, all physico-chemical properties of **1b** are the same as reported harzianic acid[4]. Thus, we concluded that **1** was active only under the chelated conditon with zinc ion.

During the purification process, two new harzianic acid family compounds were isolated. The physico-chemical properties of demethylharzianic acid (**2**) and homoharzianic acid (**3**) were shown in Table 1. The structure determination of **2** was carried out by comparing the spectral data with those of harzianic acid[4]. The molecular formula of **2** was determined to be $C_{18}H_{25}NO_6$ (MW 351) based on the

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Table 1. Physico-chemical properties of **2** and **3**.

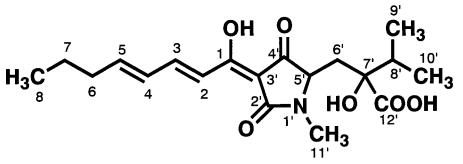
	2	**3**
Appearance	Orange Powder	Orange Powder
Molecular formula	$C_{18}H_{25}NO_6$	$C_{20}H_{29}NO_6$
ESI-MS [*m/z* (M-H)]$^-$	350 $(M-H)^-$	378 $(M-H)^-$
HRESI-MS (*m/z*)		
Calcd:	350.1577 ($C_{18}H_{24}NO_6$)	378.1917 ($C_{20}H_{28}NO_6$)
Found:	350.1586	378.1906
UV λ_{max} nm		
in MeOH:	231, 292, 350	243, 290, 344
0.01N HCl- 90% MeOH:	228, 293, 352	238, 293, 357
0.01N NaOH-90% MeOH:	246, 285, 326	249, 287, 334
Rf value on TLC [a]	0.28	0.28

[a] Silica gel 60 F_{254} (Art.5715, Merck) with BuOH-NH_4OH-H_2O-MeOH (4:1:1:0.5)

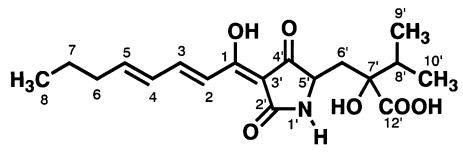
HRESI-MS and ^{13}C NMR information (Table 2). The UV spectrum of **2** was closely resemble to that of **1**. The ^{13}C NMR, DEPT and HMQC spectra of **2** revealed the presence of eighteen carbon signals consisting of three methly, three methylene, six methine and six quaternary carbons indicating the loss of one carbon and two proton atoms compared to **1**. Two side chains in **2** were identical to those of **1** based on the ^1H-^1H COSY and HMBC correlation suggesting that the structural difference between **1** and **2** should occur in five membered rings. In the ^1H NMR spectra, *N*-methyl protons (δ_H 2.94) appeared in **1** was not observed in **2** (Table 2). Any other differences were not observed in all NMR spectra. Thus, the structure of **2** was proposed as shown in Fig. 1.

The molecular formula of **3** was elucidated as $C_{20}H_{29}NO_6$ (MW 379) based on the HRESI-MS and ^{13}C NMR information. The UV spectrum of **3** also showed the similarity to **1** and **2**. The ^1H and ^{13}C NMR spectra of **1** and **3** were also similar to each other except for one additional methylene in **3**, indicating the isopropyl group of **1** was replaced by *sec*-butyl group in **3** (Table 2). This *sec*-butyl group was confirmed by cross peaks from methyl protons (H-11) to one methylene carbon (C-10), and one methine carbon in the HMBC spectrum. The remaining parts of **3** were identical with those of **1**. Thus, the structure of **3** was

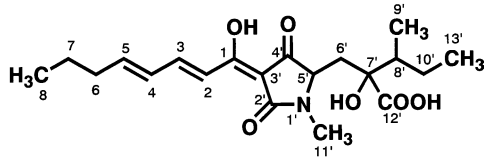
Fig. 1. Structures of harzianic acid-related compounds.



Harzianic acid (1)



Demethylharzianic acid (2)



Homoharzianic acid (3)

Table 2. ^{13}C and ^{1}H NMR assignments of **2** and **3** in chloroform-d_1.

	2		**3**	
Position	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1	175.0		176.3	
2	119.1	7.14 (d, *J*=15.3Hz)	119.1	7.00 (d, *J*=15.3Hz)
3	146.9	7.51 (m)	147.5	7.55 (m)
4	129.9	6.36 (m)	129.6	6.37 (m)
5	149.5	6.36 (m)	149.9	6.37 (m)
6	35.5	2.22 (dt, *J*=6.7, 7.3Hz)	35.5	2.23 (dt, *J*=6.0, 7.3Hz)
7	21.8	1.49 (m)	21.8	1.49 (m)
8	13.7	0.94 (t, *J*=7.3Hz)	13.7	0.94 (t, *J*=7.3Hz)
2′	172.7		173.2	
3′	99.0		98.7	
4′	195.6		197.3	
5′	59.4	4.25 (dd, *J*=10.7, 2.7Hz)	64.0	3.63 (dd, *J*=10.7, 2.7Hz)
6′	38.1	2.04 (dd, *J*=12.0, 10.7Hz)	33.8	1.91 (dd, *J*=14.0, 10.7Hz)
		2.49 (dd, *J*=12.0, 2.7Hz)		2.47 (dd, *J*=14.0, 2.7Hz)
7′	77.2		80.5	
8′	36.0	1.98 (m)	42.7	1.73 (m)
9′	17.1	0.94 (d, *J*=6.7Hz)	12.3	0.97 (d, *J*=8.0Hz)
10′	16.2	1.02 (d, *J*=6.7Hz)	24.2	1.26 (m)
				1.50 (m)
11′			12.2	0.92 (t, *J*=7.3Hz)
12′	181.2		176.7	
13′			26.5	2.96 (s)

Chemical Shifts in ppm from TMS as internal standard.

^{1}H and ^{13}C NMR were measured at 400 MHz and 100 MHz, respectively.

determined as shown in Fig. 1.

These compounds weakly inhibited the growth of human prostate cancer DU-145 cells with IC_{50}s 17 (**1**), 25 (**2**), and 10 (**3**) μg/ml.

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