Epopromycins, Novel Cell Wall Synthesis Inhibitors of Plant Protoplast Produced by *Streptomyces* sp. NK04000

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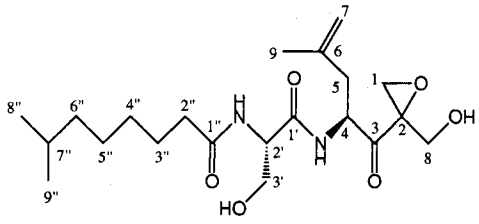
(Received for publication October 28, 1996)

The important difference of plant cells from mammalian cells is the existence of a cell wall which is made of cellulose. It is proposed that an inhibitor of cell wall synthesis might be a safer herbicide. In our screening for novel plant cell wall synthesis inhibitors as herbicides from microbial metabolites, we developed a new screening method using plant (lettuce) protoplasts for measurement of cell wall formation. Using this screening method, *Streptomyces* sp. NK04000 was found to produce the novel cell wall synthesis inhibitors, epopromycins A (**1**) and B (**2**), which are analogues of eponemycin (**3**)[1] (Fig. 1).

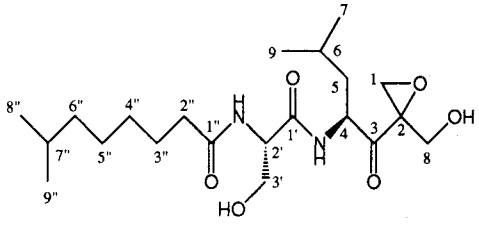
A loopfull of spores of strain NK04000 was inoculated into 100 ml of production medium consisting of glycerin 2%, soy bean meal 2%, soluble starch 2% and NaCl 0.3% (pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days on a rotary shaker (220 rpm). Two milliliters of the above seed culture were transferred to 100 ml of the same medium in a 500-ml Erlenmeyer flask and cultured at 27°C for 4 days on a rotary shaker (220 rpm). Epopromycins were isolated from the culture broth by monitoring inhibition of cell wall synthesis as described in experimental section. The fermentation broth (9.8 liters) was separated into mycelium and filtrate. The filtrate was applied on a column of DIAION HP-20 (Mitsubisi Chemical Ltd.), and after washing the column with water (5 liters) and 50% aqueous acetone (3 liters), the active ingredients were eluted with acetone (4 liters). The active eluate was concentrated *in vacuo* to 300 ml of aqueous solution, which was then extracted three times with EtOAc (3 × 0.5 liters). The extract was evaporated to dryness yielding 0.8 g of crude oil. The mycelial cake was extracted with MeOH (10 liters), the extract was concentrated to aqueous solution, and the resulting solution was extracted three times with EtOAc (3 × 2 liters). These extracts were evaporated to dryness and the crude compound obtained was partitioned between hexane and acetonitrile. The CH_3CN layer was concentrated *in vacuo* to yield 4.2 g of crude oil. Both of the crude oils (5.0 g) were combined and chromatographed on silica gel (Merck, type 60) with a solvent mixture of *n*-hexane and acetone. The active fractions (*n*-hexane - acetone = 1 : 1) were collected and evaporated to dryness and then chromatographed on Sephadex LH-20 with MeOH. The active fraction was collected and concentrated *in vacuo* (0.34 g) and was further purified by preparative HPLC using a ODS-column (Waters μBondasphare 5C18, 19 i.d. × 150 mm) with 70% aqueous MeOH (flow rate 9.6 ml/minute). Two active compounds were collected and concentrated to yield 35 mg of epopromycin A and 17 mg of epopromycin B. At the same time, eponemycin (99 mg) was isolated.

Physico-chemical properties of epopromycin A (**1**) and epopromycin B (**2**) are summarized in Table 1. Epopromycins are soluble in MeOH, MeCN, Me_2CO, EtOAc and $CHCl_3$, and insoluble in water and *n*-hexane. They give positive color reactions to molybdate-phosphoric acid, iodin vapor and Rydon-Smith reagent, but not to ninhydrin, anthrone and ferric chloride tests. The molecular formulas of **1** and **2** were determined by HRFAB-MS to be $C_{21}H_{36}N_2O_6$ and $C_{21}H_{38}N_2O_6$,

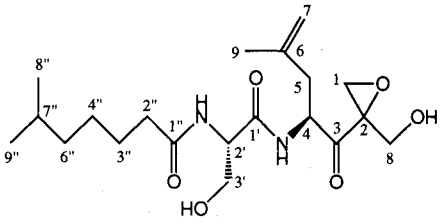
Fig. 1. Structures of epopromycin A (**1**), B (**2**) and eponemycin (**3**).



Epopromycin A (**1**)



Epopromycin B (**2**)



Eponemycin (**3**)[a]

[a] The carbon numberings 6", 7", 8" and 9" are adapted instead of 5", 6", 7" and 8" in the structure of eponemycin for comparative descriptions.

Table 1. Physico-chemical properties of epopromycin A (**1**) and B (**2**).

		Epopromycin A (**1**)	Epopromycin B (**2**)
Molecular formula		$C_{21}H_{36}N_2O_6$	$C_{21}H_{38}N_2O_6$
Appearance		Colorless solid	Colorless solid
$[\alpha]_D^{24}$		+26° (*c* 0.1, MeOH)	+34° (*c* 0.1, MeOH)
FAB-MS (*m/z*)		413 $(M+H)^+$, 435 $(M+Na)^+$	415 $(M+H)^+$, 437 $(M+Na)^+$
HRFAB-MS (*m/z*)	Found	413.2666 $(M+H)^+$	415.2793 $(M+H)^+$
	Calcd	413.2652 for $C_{21}H_{37}N_2O_6$	415.2808 for $C_{21}H_{39}N_2O_6$
UV λ_{max}^{MeOH}		End absorption	End absorption
IR ν_{max} (KCl) cm^{-1}		3301, 2928, 1722, 1644, 1536, 1050	3292, 2956, 1718, 1645, 1540, 1048
Rf value[a]		0.3	0.3

[a] Silica gel TLC (Kieselgel 60F 0.25 mm, Merck) was used with developing solvent *n*-hexane - acetone (1 : 1).

Table 2. ^1H and ^{13}C NMR chemical shifts of epopromycins A (**1**), B (**2**) and eponemycin (**3**).

Carbon No.	Epopromycin A (**1**)[a]		Epopromycin B (**2**)[a]		Eponemycin (**3**)[b]	
	^{13}C NMR[c]	^1H NMR[d]	^{13}C NMR[c]	^1H NMR[d]	^{13}C NMR	^1H NMR
1	49.4	3.12 (d, 4.8), 3.33 (d, 4.8)	49.4	3.10 (d, 5.1), 3.31 (d, 5.1)	49.3	3.12 (d, 5.0), 3.35 (d, 5.0)
2	62.4		62.2		62.5	
3	207.2		207.9		207.5	
4	51.1	4.63 (m)	51.7	4.52 (m)	51.3	4.61 (ddd, 6.6, 10.3, 3.6)
5	38.3	2.09 (dd,10.3, 14.3), 2.60 (dd, 3.3, 14.3)	38.7	1.28 (m)	38.1	2.08 (dd, 10.3, 14.1), 2.59(dd, 3.6, 14.1)
6	139.9		25.3	1.67 (m)	140.0	
7	115.0	4.78 (s), 4.86 (s)	23.3	0.95 (d, 3.7)	114.6	4.79 (br, s), 4.83 (br, s)
8	61.4	3.72 (d, 12.5), 4.22 (d, 12.5)	61.5	3.72 (dd, 5.9, 12.5), 4.22 (dd, 5.9, 12.5)	61.3	3.75 (d, 12.6), 4.21 (d, 12.6)
9	21.6	1.75 (s)	21.1	0.94 (d, 3.7)	21.6	1.75 (s)
4-NH		7.26 (d, 6.6)		7.20 (d, 7.3)		7.03 (d, 6.6)
1′	171.4		171.6		171.2	
2′	53.3	4.51 (ddd, 7.3, 6.2, 4.0)	53.3	4.52 (ddd, 7.0, 6.1, 3.5)	53.7	4.48 (ddd,7.3, 5.5, 3.5)
3′	62.7	3.59 (dd, 6.2, 11.4), 3.95 (dd, 4.0, 11.4)	62.6	3.59 (dd, 6.1, 11.0), 4.01 (dd, 3.5, 11.0)	62.7	3.58 (dd, 5.5, 11.4), 4.02 (dd, 3.5, 11.4)
2′-NH		6.64 (d, 7.3)		6.54 (d, 7.0)		6.48 (d, 7.3)
1″	173.9		174.0		174.0	
2″	36.5	2.21 (t, 7.7)	36.5	2.22 (t, 7.2)	36.4	2.21 (t, 7.7)
3″	25.6	1.60 (m)	25.6	1.60 (m)	25.7	1.60 (m)
4″	27.1	1.28 (m)	27.1	1.28 (m)	26.9	1.28 (m)
5″	29.5	1.28 (m)	29.5	1.28 (m)	—[e]	—
6″	38.8	1.15 (m)	38.8	1.15 (m)	38.6[e]	1.16 (m)
7″	27.9	1.51 (m)	27.9	1.50 (m)	27.7[e]	1.52 (m)
8″	22.6	0.86 (d, 6.6)	22.6	0.86 (d, 7.0)	22.5[e]	0.86 (d, 6.6)
9″	22.6	0.86 (d, 6.6)	22.6	0.86 (d, 7.0)	22.5[e]	0.86 (d, 6.6)

[a] Measured in $CDCl_3$; δ ppm from TMS as an internal standard.
[b] Data were quoted from ref. 1 ($CDCl_3$, ^{13}C: 100 MHz, ^1H: 400 MHz).
[c] 100 MHz in $CDCl_3$.
[d] 400 MHz in $CDCl_3$.
[e] The carbon numberings 6″, 7″, 8″ and 9″ are adapted instead of 5″, 6″, 7″ and 8″ in the structure of eponemycin for comparative descriptions.

respectively. The IR and NMR spectra of two compounds are similar to those of eponemycin (charts not shown). The ^1H NMR spectrum (Table 2) revealed the presence of three methyls (δ 0.86, d×2 and 1.75, s), one exomethylene (δ 4.78, s and 4.86, s), one epoxide methylene (δ 3.12, d and 3.33, d) and two amides (δ 6.64, d and 7.26, d). These spectral properties suggested epopromycins are analogues of eponemycin. The ^1H and ^{13}C NMR spectra of **1** are similar to those of eponemycin. The signal of C-5″ (δ 29.5) of **1** and the absence of a corresponding signal of **3** revealed that there is additional one methylene group in the acyl moiety of **1**. The FAB-MS fragment ions of **1** compared with that of eponemycin also showed the existence of one additional methylene group in the acyl moiety. Therefore, it was demonstrated that epopromycin A (**1**) has the partial structure of *N*-7-methyl-octanoyl moiety instead of *N*-6-methyl-heptanoyl moiety of eponemycin (Fig. 1). Comparative

analyses of ^{1}H NMR, ^{1}H-^{1}H and ^{13}C-^{1}H COSY spectra of **1** and **2** revealed the reduction of exomethylene moiety of **1** in the molecule of **2**, that is the disappearance of exomethylene protons, the appearance of methyl C-7 (δ 0.95, d) signal and the upfield shift of a methyl C-9 (from δ 1.75, s in **1** to 0.94, d in **2**). Therefore, epopromycin B (**2**) is a dihydro derivative of epopromycin A (**1**) (Fig. 1). SUGAWARA *et al.* reported that the optical rotation of authentic eponemycin was $+32\pm2°$[1]. The optical rotations of epopromycin A and B were $+26°$ and $+34°$, respectively (Table 1). It can therefore be presumed that the stereochemistry of epopromycins is consistent with eponemycin.

Epopromycin A and B inhibited the cell wall synthesis of lettuce protoplast at $0.1\sim0.2\,\mu g/ml$ and $0.2\sim0.4\,\mu g/ml$, respectively, while eponemycin did not show any inhibition at these dosages. Epopromycins showed herbicidal activities at $2000\,\mu g/ml$ in the greenhouse pot test, but their herbicidal selectivities between weeds and crops were not sufficient for further field works. Epopromycin A and B did not show any antimicrobial activities at $100\,\mu g/ml$ against Gram-positive bacteria, Gram-negative bacteria and fungi. Epopromycins showed potent cytotoxicity against B16 melanoma (IC_{50}; A = 0.003, B = 0.003 $\mu g/ml$) *in vitro*.

Experimental

IR spectrum was recorded on a Perkin Elmer model 1600 FT-IR. FAB-MS spectrum was obtained with a JEOL JMS-AX505HA mass spectrometer. NMR spectra were recorded on a JEOL GX-400 NMR spectrometer with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz.

Isolation of lettuce protoplasts, determination of cell wall synthesis and cell viability were performed as follows. Lettuce seeds (*Lactuca sativa*), sterilized in 1.0% NaOCl for 10 minutes and washed three times with sterile water, were grown at 25°C for 4 days with 16 hours daylength (3000 lux). The young leaves of lettuce were cut into pieces and incubated in an enzyme solution consisting of Cellulase Onozuka RS 0.5% (W/V, Yakult Honsha Co., Ltd.), Pectolyase Y23 0.02% (Seishin Pharmaceutical Co., Ltd.) and 0.5 M mannitol (pH 5.8) at 25°C for 16 hours without shaking. After the reaction mixture was filtered through gauze, protoplasts were collected by centrifugation (700 rpm, 2minutes) and washed three times with 0.5 M mannitol (pH 5.8). These protoplast were suspended at 2×10^{4} cells/ml in Murashige and SKOOG's medium (partially modified, NH_4NO_3 80 mg/liter, naphthaleneacetic acid 0.2 mg/liter, benzyladenine 0.2 mg/liter, sucrose 10 mg/liter and agarose (SIGMA type II) 1.0 mg/liter) in plastic petri-dishes. After adding 40 μl of fermentation broth for screening or sample solution (50% aqueous EtOH) the petri-dishes were sealed with film and incubated at 25°C for 5 days in darkness. Inhibition of cell wall synthesis was detected by staining cell walls of protoplasts with Calcofluor White[2] (SIGMA) 0.1% (final concentration) and observing their fluorescence with fluorescence microscopy under UV light. Cell viability was determined by staining them with fluorescein diacetate[3] (SIGMA) 0.01% (final concentration) and observing them with fluorescence microscopy under UV light. The tested broth which inhibits cell wall formation without killing protoplasts was selected as a positive sample.

Acknowledgment

The authors wish to express their thanks to Ms. K. ISHIZAKA for her technical assistance.

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