PENITRICIN, A NEW CLASS OF ANTIBIOTIC PRODUCED BY *PENICILLIUM ACULEATUM*

IV. BIOSYNTHESIS

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Bioconversion experiments using washed mycelia of *Penicillium aculeatum* NR 5165 and NR 6216 revealed that penitricin (Ro 09-0804, hydroxymethylcyclopropenone) was biosynthesized from *trans*-2-butene-1,4-diol *via* 4-hydroxycrotonaldehyde.

Penitricin, a new cyclopropenone antibiotic, has been isolated from the culture filtrate of *Penicillium aculeatum* Raper and Fennell[1,2,3]. Although a few plants [*Telekia speciosa* (Schreib.) Baumg. and *Lychnophora passerina* Gardm.][4] are known to produce cyclopropenone derivatives, the biosynthetic route for cyclopropenone ring has not yet been clarified. Meanwhile, it has also been observed that all the penitricin-producing strains of *P. aculeatum* coproduced two other metabolites, 4-hydroxycrotonaldehyde (**2**, penitricin B) and *trans*-2-butene-1,4-diol (**1**, penitricin C)[2]. The amounts of **1** and **2** produced varied depending upon the culture conditions and the strains, but there seemed to be roughly an inverse correlation between the amounts of penitricin produced and of **1** and **2**. These findings suggested that **1** and **2** might play an important role in the penitricin biosynthesis. Thus, bioconversion of compounds **1** and **2** has been attempted using washed mycelia of *Penicillium aculeatum* NR 5165 and NR 6216 to confirm the biosynthetic relationship among these compounds. We wish here to report on the biosynthetic pathway of penitricin. In addition, we briefly describe bioconvertibility of *cis*-isomer of butenediol by *P. aculeatum*.

Materials and Methods

Organisms

Penicillium aculeatum NR 5165, a higher producer of penitricin and *P. aculeatum* NR 6216, a spontaneous mutant of NR 5165, were grown and maintained on a malt-extract agar slant containing malt extract (Difco) 1%, Soytone (Difco) 0.1%, yeast extract (Difco) 0.1%, glucose 1% and agar 2%. *Pseudomonas aeruginosa* A_3 was used as the test strain for detection of penitricin.

Preparation of Washed Mycelia

A loopful of conidial mass from the well-sporulating agar slant of NR 5165 was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of PEP medium[1]. The inoculated medium was incubated at 24°C for 4 days on a rotary shaker, shaken at 180 rpm. The resultant culture was filtered through a sterile glass filter (25G2) to collect grown mycelia. The mycelia were washed three times with 200 ml each of sterile saline, and then starved by shaking in 200 ml of 25 mM phosphate buffer (pH 5.7) for 4 hours at 24°C. Subsequently, the mycelia were washed again with sterile saline for use in bioconversion trials.

Assay and Detection of Bioconversion Products

After incubation of the washed mycelia in the solution containing the substrate, the mycelia were

separated by centrifugation for 1 minute at 3,000 rpm at ambient temperature. For detection and quantitative analysis of transformed products, a 50-μl portion of the supernatant was applied to a HPLC column of Shodex Ionpak S801 as described previously[2]. The retention times in minutes for penitricin, **2** and **1** were 16.7, 21.2 and 13.5, respectively. Two TLC systems[2] were also used for detection.

Bioconversion of **1** and Identification of the Product

Washed mycelia (2.5 g, dry cell weight) were suspended in five 500-ml flasks each containing 100 ml of 0.1 M **1**. After 24 hours of incubation, the reaction mixture was filtered to obtain 500 ml of clarified solution. The filtrate was extracted with 1 volume of EtOAc to remove lipophilic impurities. The aqueous layer was applied onto a Sephadex G-10 column (3 liters), which was eluted with H_2O. The fraction active against *P. aeruginosa* A_3 was concentrated to a small volume. Subsequent HPLC separation using Shodex Ionpak S2001 as reported previously and lyophilization at $-25°C$ yielded 40.5 mg of penitricin as a colorless oil: MS *m/z* 85 (M+1), 84 (M^+); IR (KBr) 1830, 1590 cm^{-1}; ^1H NMR (D_2O at 5°C) δ 8.75 (t, 1H, J=1.22 Hz), 4.78 (d, 2H, J=1.22 Hz).

Results and Discussion

Condition for Bioconversion

We have found that PEP medium containing glucose 2%, Polypeptone (Daigo Eiyo Chem.) 1%, $MgSO_4 \cdot 7H_2O$ 0.1% and $CuSO_4 \cdot 5H_2O$ 0.01 mM in 0.05 M phosphate buffer (pH 5.7) is the best medium for production of penitricin[1]. Among these ingredients, glucose and Cu^{2+} ion were indispensible (Table 1). Table 2 shows the effect of presence and absence of copper ion on the bioconversion in relation to penitricin. It was found that the presence of Cu^{2+} in the reaction mixture had no influence, *i.e.* the washed mycelia grown without copper ion in the seed culture were not capable of this conversion, even though the reaction mixture contained copper ion. Therefore, we selected PEP containing $CuSO_4 \cdot 5H_2O$ as the seed culture medium.

Bioconversion of **1** and **2**

Bioconversion of **1** and **2** was examined in various substrate concentrations under the above conditions. The washed mycelia were suspended in solutions of **2** at concentrations ranging 0 to 12.5 mM.

Table 1. Effect of glucose and Cu^{2+} ion.

Medium component	Concentration	Basal medium	Penitricin produced (μg/ml)
Glucose	2%	I	700
Sucrose	2%	I	460
Fructose	2%	I	ND
Maltose	2%	I	ND
$CuSO_4 \cdot 5H_2O$	0.01 mM	II	700
$CuCl_2 \cdot 2H_2O$	0.01 mM	II	520
$Cu(NO_3)_2 \cdot 3H_2O$	0.01 mM	II	350
$NiSO_4 \cdot 6H_2O$	0.01 mM	II	27
$Na_2MoO_4 \cdot 2H_2O$	0.01 mM	II	36
$Pb(CH_3COO)_2 \cdot 3H_2O$	0.01 mM	II	41
$K_2Cr_2O_7$	0.01 mM	II	41

ND: Not detected.

The basal medium I consisted of Polypeptone 1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CuSO_4 \cdot 5H_2O$ 0.01 mM in 0.05 M phosphate buffer (pH 5.7). The basal medium II consisted of glucose 2%, Polypeptone 1%, $MgSO_4 \cdot 7H_2O$ 0.1% in 0.05 M phosphate buffer (pH 5.7).

Table 2. Effect of copper ion on bioconversion of penitricin C.

Washed mycelia grown in	Medium for bioconversion	Inhibition zone vs. A_3 (mm)	Products	
			Penitricin	Penitricin B
PEP	Water	29	+	+
PEP	Medium III	30	+	+
Without Cu	Water	12	−	+
Without Cu	Medium III	14	−	+

PEP medium consisted of glucose 2%, Polypeptone 1%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CuSO_4 \cdot 5H_2O$ 0.01 mM in 0.05 M phosphate buffer (pH 5.7). "Without Cu" means PEP medium not containing $CuSO_4 \cdot 5H_2O$. Medium III consisted of $MgSO_4 \cdot 7H_2O$ 0.1%, $CuSO_4 \cdot 5H_2O$ 0.01 mM in 0.05 M phosphate buffer (pH 5.7).

A_3: inhibition diameter using a paper disk (8 mm) against *P. aeruginosa* A_3. Penitricin C was added to bioconversion medium at concentration of 100 mM. Concentration of the washed mycelia: 42 mg/ml (wet cell weight) incubation condition: 24°C, 180 rpm, 7 hours.

+: Produced (>2 mM), −: not produced (<0.2 mM).

Fig. 1. Bioconversion of penitricin B in various concentrations.
Cell concentration: 42 mg/ml (wet cell weight). Incubation condition: 24°C, 180 rpm, 3 hours.

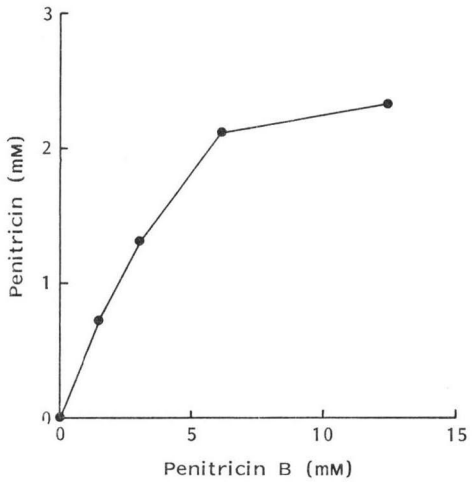


Fig. 2. Time course of penitricin B bioconversion.
●: Penitricin, ▼: penitricin B.
Conditions: See legend in Fig. 1.

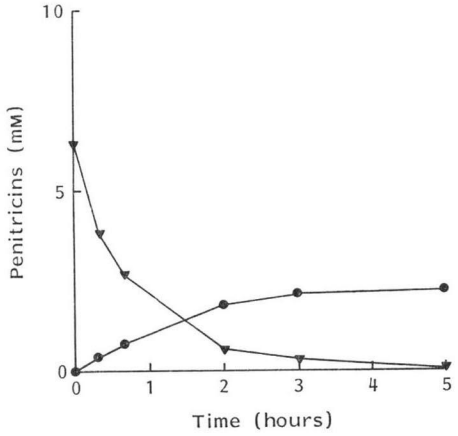
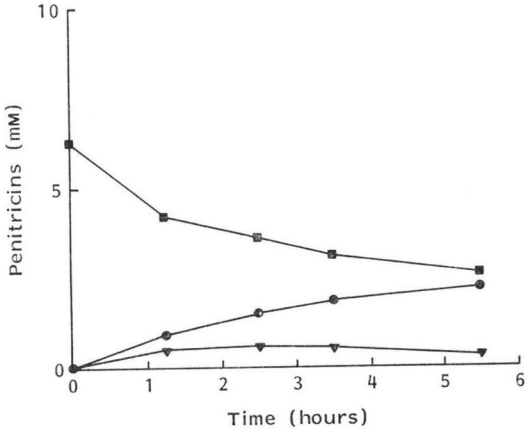


Fig. 3. Time course of penitricin C bioconversion.
●: Penitricin, ▼: penitricin B, ■: penitricin C.
Conditions: See legend in Fig. 1.



The mixtures shaken for 3 hours were analyzed for penitricin concentrations and for **2** remaining. Fig. 1 shows that an approximately linear relationship exists between the concentration of **2** added (up to around 6.25 mM) and the amount of penitricin formed. The time course of bioconversion of **2** at 6.25 mM was then examined (Fig. 2). When the substrate disappeared after 5 hours of incubation, the product penitricin reached its maximum (2.2 mM). However, no *trans*-2-butene-1,4-diol (**1**) was detected by either HPLC or TLC during these experiments. Therefore, neither penitricin nor 4-hydroxycrotonal-

Fig. 4. Difference of time cource between the seed culture with Cu^{2+} and without Cu^{2+}.

a: The seed medium used was PEP medium not containing $CuSO_4 \cdot 5H_2O$, b: The seed medium contained $CuSO_4 \cdot 5H_2O$. Both bioconversion media consisted of penitricin C 120 mM, $CuSO_4 \cdot 5H_2O$ 0.01 mM, $MgSO_4 \cdot 7H_2O$ 0.1% in 0.05 M phosphate buffer (pH 5.7).

Other conditions: See legend in Fig. 1. Symbols: See Fig. 3.

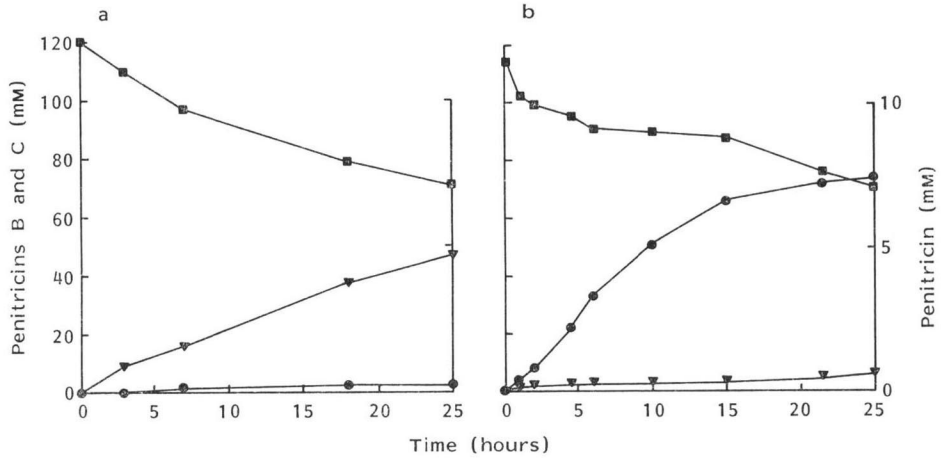
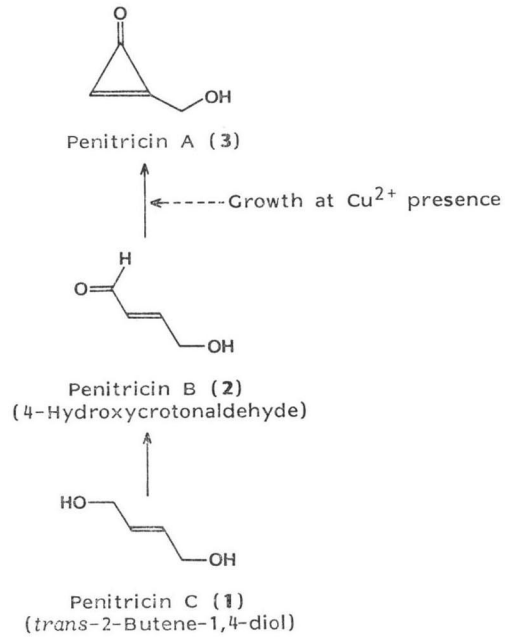


Fig. 5. Biosynthetic pathway of penitricin.



dehyde (**2**) could be a biosynthetic precursor for **1**.

Fig. 3 shows the time course of bioconversion from 6.25 mM compound **1** to penitricin. Penitricin production was observed within 2 hours of incubation and reached 2.2 mM after 5.5 hours. As already described above, the product bioconverted from **1** was isolated and identified as penitricin. Moreover, a small amount (0.6 mM) of **2** was also detected during the 5.5-hour incubation, indicating that penitricin might be biosynthesized from penitricin C (**1**) *via* penitricin B (**2**). In order to confirm this assumption, **1** was bioconverted using the washed mycelia grown in a seed culture without copper ion. When the washed mycelia thus obtained was suspended in 120 mM **1** and incubated for 10 hours (Fig. 4a), **1** was bioconverted into **2** more significantly than seen in Fig. 3. As for penitricin, a much lesser amount was produced as presented in Fig. 4a. Fig. 4b shows the effects of using washed mycelia grown in PEP medium containing copper ion. The results reported in Fig. 4a and Fig. 4b not only support the copper ion requirement for penitricin biosynthesis but also define the biosynthetic pathway of **2**.

Meantime, we obtained a spontaneous mutant (strain NR 6216) from NR 5165 by single colony selection, which accumulated a large amount of **1** and **2** with only trace amounts of penitricin. Strain NR 6216 was cultured in PEP medium for 4 days. Thereafter, when the washed mycelia of strain

NR 5165 was added to the culture filtrate of strain NR 6216 and incubated at 24°C, **1** and **2** in the culture filtrate disappeared within 1 hour.

On the basis of these findings, we propose the biosynthetic pathway for penitricin as shown in Fig. 5.

Bioconversion of *cis*-2-Butene-1,4-diol

When 0.1 M *cis*-isomer of butenediol was used instead of **1** as a precursor, a small amount (48 μg/ml) of penitricin was detected after 2 hours of incubation with the washed mycelia. Therefore, strain NR 5165 was also capable of using *cis*-2-butene-1,4-diol as a penitricin precursor.

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