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

II. ISOLATION AND CHARACTERIZATION

TORU OKUDA, KAZUTERU YOKOSE, TAMOTSU FURUMAI and HIROMI B. MARUYAMA

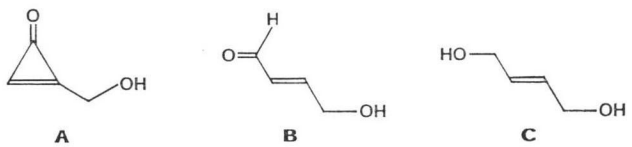
Department of Microbiology and Chemotherapy, Nippon Roche Research Center, 200 Kajiwara, Kamakura 247, Japan

(Received for publication March 29, 1984)

A novel antibiotic, penitricin, Ro 09-0804, has been produced in the culture filtrate of *Penicillium aculeatum* NR 5165. This antibiotic was purified by repeated extraction of culture filtrate with 1-butanol, and passage of the crude extracts through Sephadex G-10, followed by HPLC (Shodex Ionpak S-801). Physico-chemical characterization was made on penitricin, while two open-ringed penitricins B and C co-produced by the producer strain were also identified.

As described in the previous paper[1], a new antibiotic named penitricin was produced by a number of strains belonging to *Penicillium aculeatum*, whereas any other related taxa tested did not produce this antibiotic. Moreover, these penitricin producers co-produce 2 other metabolites, penitricins B and C, which have been identified as 4-hydroxycrotonaldehyde and *trans*-2-butene-1,4-diol, respectively. Their isolation has not been hitherto reported from microorganisms. In this paper, we describe the isolation procedure and the characterization of penitricin. The isolation and identification of 2 other related penitricins B and C are also briefly reported.

Fig. 1. Structure of penitricin (A), penitricins B and C.



Materials and Methods

TLC System

For differentiation of penitricin (Ro 09-0804) from penitricins B and C, a small portion of active broth was spotted on TLC (Merck Kieselgel 60 F_{254}) and developed with EtOAc - MeOH (10: 1). Bioautography against *Pseudomonas aeruginosa* A3, and color reactions with 2,4-dinitrophenyl hydrazine and anisaldehyde-H_2SO_4 were used for detection.

HPLC System

A Waters model pump M-6000A with injector was used in this work. The chromatograph was monitored by a UV detector (Hitachi, Multi-Wave Length UV Monitor 635 M) and a refractive index detector (Shodex RI SE11). All separations were carried out using Shodex Ionpak S801 ($8\phi \times 500$ mm) with a jacket cooled at 5°C or S2001 ($20\phi \times 500$ mm) at ambient temperature. These columns were purchased from Showa Denko Co. They are GPC series columns packed with a microporous cation exchange resin (Na^+ form) for separation of mono-, di-, tri-, or oligosaccharides.

Chemicals

Sephadex G-10 and LH-20 were obtained from Pharmacia Fine Chemicals Co. All other chemicals were reagent grade.

Results and Discussion

Isolation of Penitricin

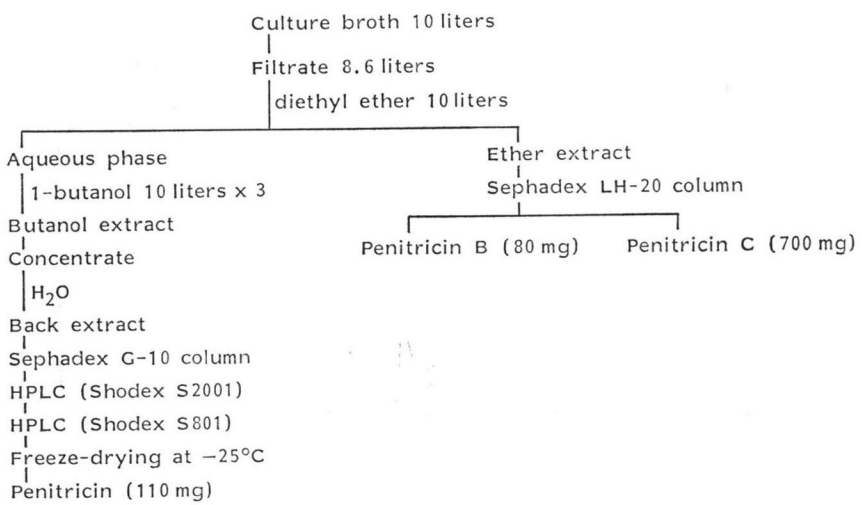
Although penitricin was a water-soluble, neutral compound, it was partially extractable with 1-butanol from culture filtrate. The procedure shown in Fig. 2 was thus established in fairly satisfactory manner in terms of reproducibility and minimizing the degradation.

Culture broth of *P. aculeatum* NR 5165 was filtered with aid of Radiolite #900 (Showa Chemical Co.). The filtrate contained penitricin, penitricins B and C. The filtrate was extracted with 1 volume of diethyl ether to separate from penitricins B and C. The aqueous solution was extracted 3 times with 1 volume each of 1-butanol, and the extract was concentrated *in vacuo* at lower than 40°C. The concentrate was then back-extracted with water followed by concentration of aqueous phase to small volume. The concentrate was applied to a Sephadex G-10 column chromatography and eluted with water. Active fractions against *P. aeruginosa* described above were concentrated to a small volume and further purified by the Waters HPLC system (column: Shodex Ionpak S2001, solvent: H_2O, flow rate: 3.0 ml/minute, injection volume max.: 2.0 ml, detection: UV 210 nm and RI). Fractions (retention time: 44 ~ 50 minutes) were combined and evaporated to a small volume, which was rechromatographed on Shodex S801 with jacket cooled at 5°C. The purity of active fractions were checked by TLC or HPLC. After the solution was freeze-dried at −25°C for 48 hours, 110 mg (4% isolated yield) of pure penitricin was obtained from 10 liters of culture broth.

Stability of Penitricin

Penitricin was unstable and easily decomposed especially under alkaline conditions. Fig. 3 shows stability of aqueous solution of penitricin under various pH conditions. Penitricin was unstable above pH 6. The most stable pH range was considered to be between 3 and 5. Under these conditions, half

Fig. 2. Purification procedure.

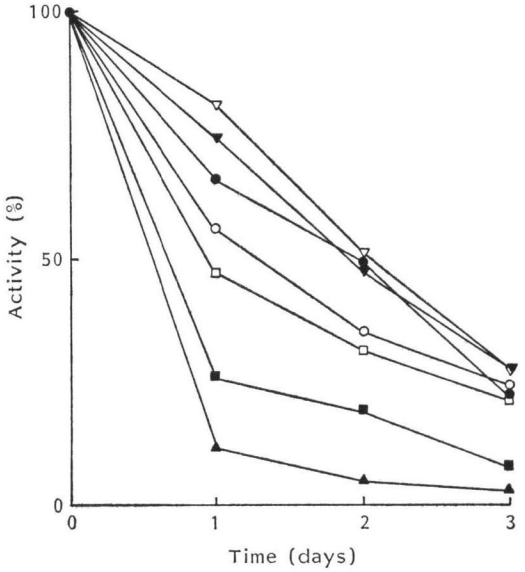


life of penitricin was 2 days at 0°C, half a day at 25°C and about 3.5 hours at 40°C. Decomposition could not be avoided by addition of any protective substances such as buffers or antioxidants. Therefore, the following observation was also made because of the unstable nature of the compound.

When the IR spectrum of penitricin (KBr) was measured on a Hitachi 260-10 spectrometer, the most characteristic absorption bands were observed at 1830 cm^{-1} and 1590 cm^{-1}. After 10 minutes at room temperature, however, absorption peaks at 1720~1700 and 1270 cm^{-1} which were probably derived from decomposed products, appeared. After several hours the intensity of these two peaks increased with a concomitant decrease in peaks at 1830 and 1590 cm^{-1} (Fig. 4).

Fig. 3. pH stability at 0°C, rest of activity (%) after 1~3 days.

○: pH 2.2, ●: pH 3.1, ▽: pH 4.3, ▼: pH 5.1, □: pH 6.1, ■: pH 7.1, ▲: pH 8.0.



Physico-chemical Properties of Penitricin

Penitricin was a colorless oil at room temperature, freely soluble in water, ethanol or methanol, and slightly soluble in acetonitrile. It was practically insoluble in other organic solvents. As already mentioned above, the most characteristic absorption peaks in the IR spectrum were observed at 1830 (carbonyl) and 1590 cm^{-1} (double bond) (Fig. 5). The molecular formula was determined as $C_4H_4O_2$ by a high resolution mass spectrometer (M^+ 84.02034, Calcd 84.0210) and ^{13}C NMR. The other physico-chemical properties are summarized in Table 1.

Fig. 4. Alteration in IR spectrum of penitricin.

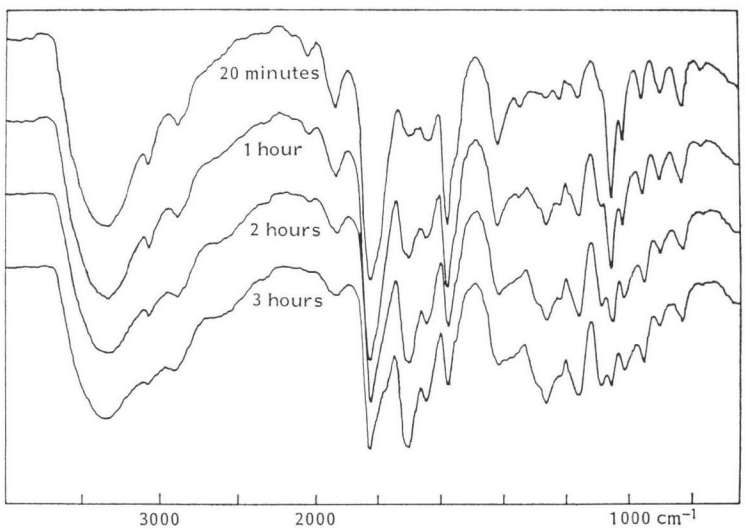


Fig. 5. IR spectrum of penitricin in KBr.

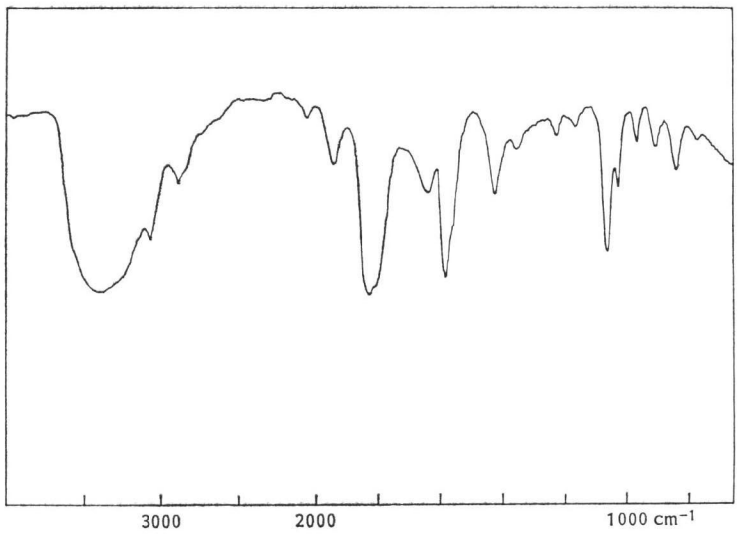


Table 1. Physico-chemical properties of penitricin and penitricin B.

	Penitricin	Penitricin B
EI-MS Calcd for Found	$C_4H_4O_2$: *m/z* 84.02100 *m/z* 84.02034	$C_4H_6O_2$: *m/z* 86.03678 *m/z* 86.03771
UV λ_{max} nm (ε)	241 (sh) in H_2O	283 (114) in MeOH
IR ν_{max} (KBr) cm^{-1}	1830 (C=O), 1590 (C=C)	1690~1670 (C=O)
^1H NMR (100 MHz) δ	in D_2O 8.81 (1H, s), 4.88 (2H, s)	in MeOH-d_4 9.54 (1H, d, J=8 Hz, CHO) 7.12 (1H, dt, J=4, 16 Hz, 3-CH) 6.32 (1H, m, J=8, 16, 2 Hz, 2-CH) 4.40 (2H, dd, J=4, 2 Hz, 4-CH_2)
^{13}C NMR (25 MHz) δ	in D_2O 167.20, 158.90, 146.46, 57.39	in MeOH-d_4 196.2 (d, CHO), 160.0 (d, C-3) 130.9 (d, C-2), 62.0 (t, C-4)
TLC (Merck, Kieselgel 60), Rf		
Acetonitrile - 0.75% AcOH (9: 2)	0.57	0.64
EtOAc - MeOH (10: 1)	0.22	0.50
HPLC*		
Retention time (minutes)	16.7	21.2
Color reaction		
Iodine	+	+
2,4-DNP - H_2SO_4	+	+
$KMnO_4$ - NaOH	+	+
Anisaldehyde - H_2SO_4	−	+
Ninhydrin	−	−
RYDON-SMITH	−	−

2,4-DNP: 2,4-Dinitrophenylhydrazine.

* Shodex Ionpak S801, solvent: H_2O, flow rate: 1.5 ml/minute, detection: RI and UV_{210nm}.

Based on these observations together with NMR spectral data, this antibiotic was considered to be hydroxymethylcyclopropenone (Fig. 1 **A**). The details of structure determination and structural confirmation by chemical synthesis are reported in the accompanying paper[2]. Naturally occurring cyclopropenones have been isolated from *Compositae*[3], but this is the first report of cyclopropenones of microbial origin.

Isolation and Identification of Penitricins B and C

Penitricins B and C were found to be simultaneously produced in the culture filtrate of *P. aculeatum* NR 5165. The culture filtrate of NR 5165 was extracted with diethyl ether and the extract was then chromatographed on Sephadex LH-20 column in methanol to separate penitricins B and C from each other, as shown in Fig. 2. Both compounds were purified as oily materials. Both compounds showed some activity against *P. aeruginosa*.

Penitricin B was a colorless oil, freely soluble in water, methanol and diethyl ether. The IR spectrum showed a characteristic peak at 1690~1670 cm^{-1} (unsaturated carbonyl). The molecular formula was determined as $C_4H_6O_2$ by a high resolution mass spectrometer. On the basis of these characteristics together with ^1H and ^{13}C NMR spectra (Table 1), penitricin B was identified as 4-hydroxycrotonaldehyde (Fig. 1 **B**).

Penitricin C was also a colorless oil, and soluble in water or methanol. The IR spectrum showed a broad peak at 3400~3300 cm^{-1} (OH). The di-trimethylsilyl derivative of penitricin C had the molecular formula $C_{10}H_{24}O_2Si_2$ (EI-MS spectrum *m/z* 232.13184 (M^+), Calcd *m/z* 232.13152). Penitricin C was identified as *trans*-2-butene-1,4-diol (Fig. 1 **C**) from consideration of ^1H and ^{13}C NMR spectra and direct comparison with an authentic material purchased from Tokyo Kasei Co. The *trans* configuration was determined by gas chromatography in comparison with the authentic materials.

Although penitricins B and C have been reported as synthetic materials[4], this is the first report of these materials as natural products. Moreover, they were both produced by all of the penitricin producers. Together with their structural similarlity, this finding indicated that they may be biosynthetically related to penitricin, and these studies will be reported elsewhere.

Acknowledgment

We thank Mr. T. KAMIYAMA and Mr. S. OSHIMA for their technical assistance. Thanks are also due to Mr. N. NAKAYAMA for his NMR and MS operations.

References

1) OKUDA, T.; Y. YONEYAMA, A. FUJIWARA & T. FURUMAI: Penitricin, a new class of antibiotic produced by *Penicillium aculeatum*. I. Taxonomy of the producer strain and fermentation. J. Antibiotics 37: 712~717, 1984
2) OKUDA, T.; N. SHIMMA & T. FURUMAI: Penitricin, a new class of antibiotic produced by *Penicillium aculeatum*. III. Structural confirmation by chemical synthesis and biological activity. J. Antibiotics 37: 723~727, 1984
3) BOHLMANN, F.; J. JAKUPOVIC, L. MUELLER & A. SCHUSTER: Naturally occurring cyclopropenone derivatives. Angew. Chem., Int. Ed. Engl. 20: 292~293, 1981
4) GWINNER, M. W.: Nouveaux hydroxyènals substitués en position 4, e procédé pour leur préparation. Fr. Pat. 1,584,471, Feb. 19, 1968