EM5519, A NEW BROAD-SPECTRUM ANTIBIOTIC PRODUCED BY *PSEUDOMONAS FLUORESCENS*

Sir:

In the course of searching for antibiotics we have isolated from a strain of *Pseudomonas fluorescens* a novel compound that possesses potent broad-spectrum antibacterial activity. We now wish to report the results of our studies on the taxonomy, isolation, biological and physical properties of this compound, EM5519, and the results of preliminary studies on its mode of action.

Pseudomonas fluorescens SC 12695 was isolated from a water sample taken from the Raritan-Delaware Canal, near Washington Crossing, New Jersey. The organism is a Gram-negative rod that is motile by means of 1 or 2 polar flagella and fluorescens on KING's B medium. It is oxidative, cytochrome oxidase positive, arginine dihydrolase positive and does not grow at 41°C. These characteristics clearly establish the organism as a pseudomonad.

The positive reaction in the arginine dihydrolase test differentiates the EM5519 producer from *P. syringae* and *P. cichorii*, while failure to grow at 41°C separates it from *P. aeruginosa*. A positive gelatin hydrolysis differentiates it from *P. putida*. The failure to form levan from sucrose and a positive denitrification test provide the basis for identification of the organism as *P. fluorescens*.

For production of EM5519 we employed a seed medium composed of glucose 5 g, and yeast-extract 10 g, per liter of distilled water. After inoculation, the culture was incubated on a rotary shaker (280 rpm, 5 cm throw) at 25°C for 24 hours. This growth was used to inoculate the production medium with 1% inoculum. The production medium and the incubation conditions during the production phase were the same as those used for the seed culture. The antibiotic was detected by paper-disc agar-diffusion assay against *Escherichia coli* SC 2927. The fermentation was harvested when maximum antibiotic yields were reached, usually 24 hours after inoculation.

The isolation of EM5519 is accomplished according to the scheme outlined in Fig. 1. EM

Fig. 1. Isolation and purification of EM5519.

Broth supernate (49 liters)

↓ Sorb on Amberlite IRC-72 (Na^+).

Loaded resin

↓ Elute with H_2O - isopropanol - H_3PO_4 (700: 300: 1) and wash eluate with AcOEt.

Aqueous phase

↓ Chromatograph on MCI gel CHP20P with a 0~30% gradient of CH_3CN in pH 2.3 sodium 0.05 M phosphate buffer.

Active eluate

↓ Chromatograph on SP-Sephadex (Na^+) with a NaH_2PO_4 gradient.

Active eluate

↓ Chromatograph on MCI gel CHP20P with a 0~30% gradient of MeOH - CH_3CN (9: 1) in 0.05 M NaH_2PO_4.

Active eluate

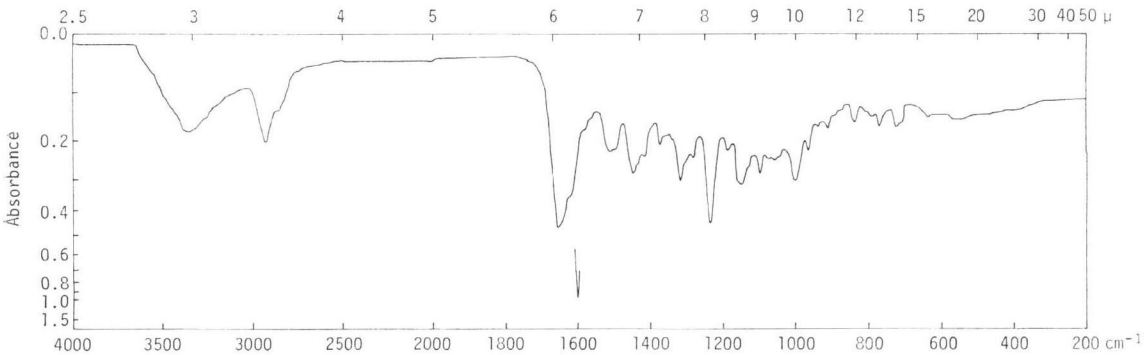
↓ Chromatograph on Sephadex G15 in pH 2.3 sodium 0.05 M phosphate buffer.

Active eluate

↓ Sorb on MCI gel CHP20P and elute with H_2O - CH_3CN - HOAc (90: 10: 1).

EM5519 (18 mg)

Fig. 2. IR spectrum of EM5519.



5519 is basic and forms amorphous chloride, acetate, phosphate and tosylate salts. The antibiotic, though labile in alkali, can be extracted as the free base into ethyl acetate from aqueous solution at pH 8.5. EM5519 gives positive RYDON, FOLIN and ninhydrin reactions. It has a UV maximum at 270 nm ($E^{1\%}_{1cm}$ 133) and IR peaks at 3350, 1650 and 1280 cm^{-1} (Fig. 2). EM5519 can be converted to an *N*-acetyl derivative with acetic anhydride - methanol (1: 4), the derivative also exhibiting antimicrobial activity. It has a UV maximum at 271 nm ($E^{1\%}_{1cm}$ 165) and is positive with RYDON and FOLIN reagents. Elemental analysis of *N*-acetyl EM5519 gave the following composition: C 59.29, H 6.72, N 8.85.

Chromatographic and electrophoretic data obtained with EM5519 and the *N*-acetyl derivative are shown in Table 1. The ^1H (Fig. 3) and ^{13}C NMR spectral data and the UV spectrum of EM5519 indicate structural similarities to the saframycin group of antibiotics[1,2]; however, some differences exist. Firstly, hydrolysis experiments reveal the presence of *N*-terminal alanine in EM5519. Secondly, EM5519 has broad spectrum activity while the saframycins are active primarily against Gram-positive organisms.[1]

The broad-spectrum activity *in vitro* is shown in Table 2. The excellent antichlamydial activity is noteworthy. No activity was found when the antibiotic was tested against *Candida albicans*.

Table 1. Chromatographic and electrophoretic mobilities of EM5519 and *N*-acetyl EM5519.

TLC (Merck 60F_{254} silica gel)[a]

Solvent system	Rf	
	EM5519	*N*-Acetyl EM5519
n-BuOH - HOAc - H_2O (3: 1: 1)	0.35	0.40
Acetone - MeOH (5: 1)	0.52	0.75

Electrophoresis[a,b]

Buffer	pH	Mobility	
		EM5519	*N*-Acetyl EM5519
HCO_2H - HOAc - H_2O (1: 3: 36)	1.8	0.79	0.40
0.05 M NaH_2PO_4	4.5	0.68	0.32
Sodium 0.05 M phosphate	7.0	0.55	0.25
Sodium 0.05 M carbonate-bicarbonate	9.2	0.0	0.0

[a] Detection by bioautography against *E. coli* SC 2927.

[b] On Whatman 3 MM paper, 11 volt/cm, 1 hour; mobilities relative to vitamin B_{12} (0) and 1-(2,3 dihydroxypropyl)pyridinium ion (1.0).[4]

Preliminary studies on the mode of action of EM5519 indicate that the compound inhibits

Fig. 3. 400 MHz ^1H NMR spectrum of EM5519 in D_2O.

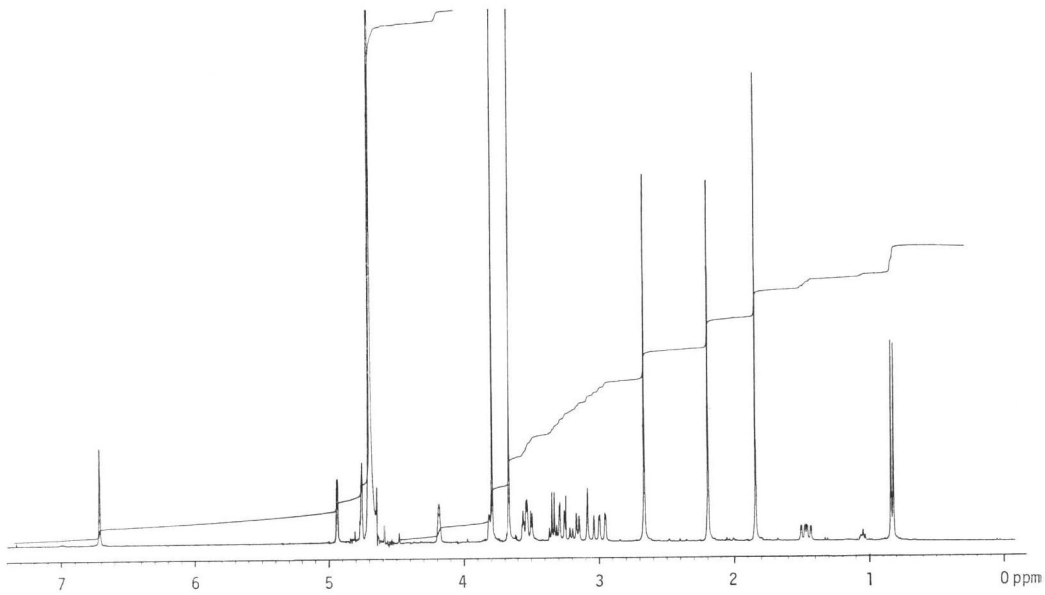
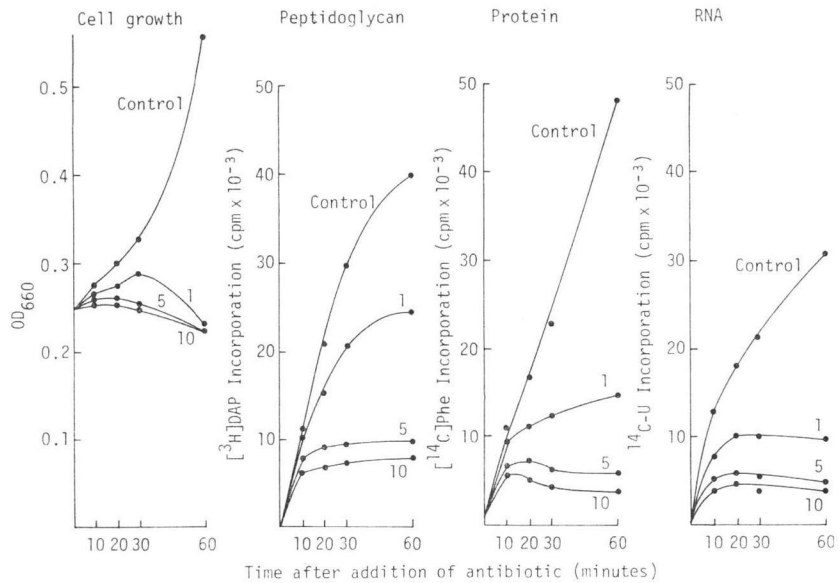


Table 2. Activity *in vitro* of EM5519.

Organism	MIC (μg/ml)[a]	Organism	MIC (μg/ml)[a]
S. aureus SC1276	0.05	*P. mirabilis* SC3855	>50
S. aureus SC2399	0.1	*P. rettgeri* SC8479	1.6
S. aureus SC2400	0.1	*P. vulgaris* SC9416	1.6
S. aureus SC10165	0.1	*S. typhosa* SC1195	0.05
S. faecalis SC9011	3.1	*S. sonnei* SC8449	0.05
S. agalactiae SC9287	<0.05	*E. cloacae* SC8236	0.4
M. luteus SC2495	<0.05	*E. aerogenes* SC10078	3.1
E. coli SC8294	0.4	*C. freundii* SC9518	0.1
E. coli SC10857	0.05	*S. marcescens* SC9783	1.6
E. coli SC10896	0.05	*P. aeruginosa* SC9545	25
E. coli SC10909	<0.03	*P. aeruginosa* SC8329	6.3
K. aerogenes SC10440	0.1	*A. calcoaceticus* SC8333	1.6
K. pneumoniae SC9527	0.05	*Chlamydia trachomatis*[b]	≤0.03

[a] Antibacterial activity measured by agar dilution assay: (10^4 CFU).
[b] Cultured in McCoy cells.

Fig. 4. Mode of action of EM5519.



primarily RNA synthesis (Fig. 4). Effects on DNA synthesis could not be quantitated accurately due to poor incorporation of [^{14}C]thymidine into TCA-precipitable material.

From the data given, we believe that EM5519 is a new compound from a pseudomonad with potent antibiotic activity.

When our studies were being brought to conclusion, we became aware of a patent,[8] granted to MUNAKATA *et al.*, of Yoshitomi Pharmaceutical Industries, Ltd., concerning their novel antibiotics, Y-16482 α and β. TLC and ^{1}H NMR spectroscopic comparisons of EM5519 with these compounds, kindly supplied by Yoshitomi, proved the identity of EM5519 with Y-16482 α.

Acknowledgement

The authors thank Drs. C. C. KAO and S. K. TANAKA for the *in vitro* analysis and Mr. P. PRINCIPE for providing fermentation broths.

EDWARD MEYERS
RAYMOND COOPER
WILLIAM H. TREJO
NAFSIKA GEORGOPAPADAKOU
RICHARD B. SYKES

The Squibb Institute
for Medical Research
P.O. Box 4000
Princeton, New Jersey 08540,
U.S.A.

(Received November 2, 1982)

References

1) ARAI, T.; K. TAKAHASHI & A. KUBO: New antibiotics, saframycins A, B, C, D and E. J. Antibiotics 30: 1015~1018, 1977
2) ARAI, T.; K. TAKAHASHI, K. ISHIGURO & K. YAZAWA: Increased production of saframycin A and isolation of saframycin S. J. Antibiotics 33: 951~960, 1980
3) T. MUNAKATA, *et al.* (Yoshitomi Pharm. Ind.): PCT Int. Appl. WO 82/00146, Jan. 21, 1982
4) GRUSZECKI, W. & Z. LEDOCHOWSKI: Research on tumor inhibiting compounds. XXXII. Heterocyclic quaternary amines derived from 3-chloro-1,2-propanediol. Rocznicki Chem. 40: 1313~1314, 1966