Ro 22-5417, A NEW CLAVAM ANTIBIOTIC FROM *STREPTOMYCES CLAVULIGERUS*

I. DISCOVERY AND BIOLOGICAL ACTIVITY

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Streptomyces clavuligerus NRRL 3585, a culture which produces a variety of β-lactam antibiotics in the penicillin, cephalosporin and clavam families, was found to produce a new β-lactam antibiotic, Ro 22-5417. The compound, which was neither a substrate for nor inhibitor of several β-lactamases, showed antimicrobial activity in defined minimal medium but little or no inhibitory activity in nutrient-rich medium. The activity was bacteriostatic against *Bacillus* species ATCC 27860 and was antagonized by D- and L-methionine, L-cystathionine, L-homocystine and *O*-acetyl-L-homoserine but not by L-homoserine, L-aspartate, L-cysteine or other common amino acids, vitamins and nucleosides. Our results are consistent with Ro 22-5417 acting as an inhibitor in methionine biosynthesis.

Streptomyces clavuligerus NRRL 3585 has been found to produce a wide variety of β-lactam antibiotics. The organism, first isolated[1] at Eli Lilly and Co., was found[2] to produce penicillin N, 7-(5-amino-5-carboxyvaleramido)-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid and the 7-methoxy analog, cephamycin C; several years later it was also reported[3] to produce deacetoxycephalosporin C. The culture was screened for production of β-lactamase inhibitors at Beecham Pharmaceuticals and found to produce clavulanic acid[4] and its 3-hydroxypropionyl ester[5]. At Glaxo Laboratories the culture was found[6] to produce in addition three clavam-containing antibiotics which showed antifungal activity as opposed to the antibacterial and β-lactamase inhibitory properties of clavulanic acid. A mutant of *S. clavuligerus*, designated IT1, has recently been found[7] to produce several additional antibiotics, *viz.* holomycin and a tunicamycin-related complex of antibiotics.

Methods

Antimetabolite tests were carried out in minimal agar medium[8] as previously described[9].

Bacteriostatic and bactericidal studies with *Bacillus* sp. ATCC 27860 were carried out in shake-flask culture at 36°C in the chemically defined minimal broth of DAVIS and MINGIOLI[8] supplemented with 8 g/liter of D-glucose. The fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of medium on a rotary shaker operating at 250 rpm.

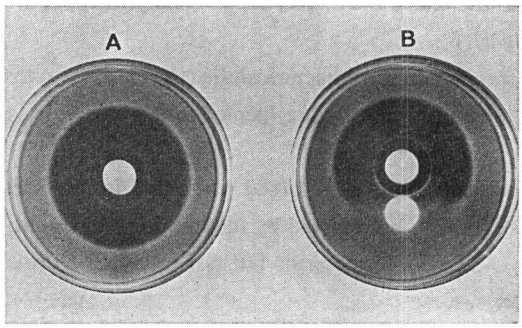
Results and Discussion

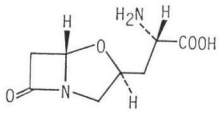
Our interest in *S. clavuligerus* came as a result of antimicrobial screening on chemically-defined minimal agar medium. The organism was initially grown for another project (Dr. N. PALLERONI and Ms. B. PROSSER), and broth of this known organism was included in our screen. Unexpectedly, the broth from *S. clavuligerus* gave an unusually large (55 mm) and visually striking zone of inhibition. The outer zone was antagonized by methionine and methionine containing peptides (Fig. 1). The active

Fig. 1. Inhibition of *Bacillus* sp. ATCC 27860 and reversal by L-methionine.

Photograph A shows the zone of inhibition against *Bacillus* sp. ATCC 27860 in Davis minimal agar medium[8] caused by broth filtrate of *Streptomyces clavuligerus*.

Photograph B shows the reversal of inhibition by L-methionine.





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component **1**, Ro 22-5417, has been isolated and characterized[10], and the absolute stereochemistry established[11].

Ro 22-5417 is produced in a variety of natural and synthetic media. Peak yield is usually obtained at 3 to 4 days. Glycerol, maltose and dextrin serve as good carbon sources for antibiotic production in a soy flour-containing medium, while little or no production occurs with D-glucose, *myo*-inositol, mannitol, sucrose or L-sorbose. The inability of *S. clavuligerus* to use D-glucose for growth[1] and antibiotic production stands in interesting contrast with the microorganism's ability to use maltose and dextrin.

Table 1. *In vitro* antimicrobial activity of Ro 22-5417.

		Minimum inhibitory concentration* (μg/ml)	
		BBL seed agar	Davis minimal agar[8]
Pseudomonas aeruginosa	ATCC 8709	>1,000	>1,000
Proteus vulgaris	ATCC 6380	>1,000	nd
Escherichia coli	ATCC 27856	>1,000	1,000
Klebsiella pneumoniae	ATCC 27858	>1,000	62
Serratia marcescens	ATCC 27857	>1,000	>500
Serratia sp.	ATCC 93	>1,000	500
Acinetobacter calcoaceticus	ATCC 10153	>1,000	>500
Staphylococcus aureus	ATCC 6538 P	>1,000	nd
Micrococcus luteus	ATCC 9341	>1,000	>500
Streptococcus faecium	ATCC 8043	>1,000	nd
Bacillus megaterium	ATCC 8011	>1,000	>500
Bacillus sp.	ATCC 27859	>1,000	nd
Bacillus subtilis	NRRL 558	>1,000	0.25
Bacillus sp.	ATCC 27860	>1,000	0.03
Mycobacterium phlei	ATCC 355	>1,000	>500
Streptomyces cellulosae	ATCC 3313	>1,000	>500
Paecilomyces varioti	ATCC 26820	250	16**
Penicillium digitatum	ATCC 26821	>1,000	31**
Candida albicans	NRRL 477	>1,000	500***
Saccharomyces cerevisiae	ATCC 4226	>1,000	125**

nd=not determined.

* Lowest concentration still showing zone of inhibition by the agar-diffusion well method.

** Biotin and pantothenate added to Davis minimal agar at 1 μg/ml.

*** Biotin added to Davis minimal agar at 1 μg/ml.

In defined minimal medium, Ro 22-5417 showed *in vitro* antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi, but in nutrient-rich medium only weak activity against *Paecilomyces varioti* was observed (Table 1).

Ro 22-5417 did not compete with [^{14}C]benzylpenicillin for any of the cytoplasmic-membrane penicillin-binding proteins of *Escherichia coli* or *Haemophilus influenzae* (Dr. S. MAKOVER, personal communication). Moreover, it was not a substrate or inhibitor of four types of β-lactamase (the inducible penicillinase from *Staphylococcus aureus*, the TEM-1 β-lactamase coded for by plasmid R-1, and the cephalosporinases from *Enterobacter cloacae* and *Proteus vulgaris*).

The activity against *Bacillus* species ATCC 27860 was shown to be bacteriostatic (Fig. 2) and reversible by L-methionine (Fig. 3). Under similar conditions addition of benzylpenicillin led to cell lysis (data not shown).

The inhibition caused by Ro 22-5417 against *Bacillus* species ATCC 27860 was tested in minimal agar medium by a counter diffusion method[9] and was found to be reversed by methionine and several of its biosynthetic precursors (Table 2). Reversal by *O*-acetyl-L-homoserine but not by L-homoserine suggests that Ro 22-5417 inhibits homoserine transacetylase.

The interesting appearance of the zone of inhibition caused by the broth filtrate (Fig. 1A) can now be explained as resulting from the combination of several factors. The inner clear zone of inhibition is due to the bactericidal activity of penicillin N and cephalosporins produced by *S. clavuligerus*. The large outer zone is due to the bacteriostatic action of Ro 22-5417. The inner ring of growth is due to

Fig. 2. Bacteriostatic effect of Ro 22-5417 on *Bacillus* sp. ATCC 27860.

At the time indicated Ro 22-5417 was added at 1 μg/ml (□) and 10 μg/ml (△). A third flask (○) served as the control. Colony counts were determined by the spread-plate method.

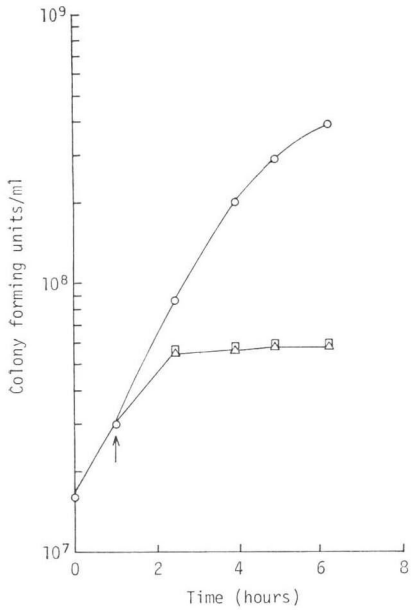

Fig. 3. Bacteriostatic effect of Ro 22-5417 on growth of *Bacillus* sp. ATCC 27860 and reversal by L-methionine.

At the time indicated Ro 22-5417 was added at 1 μg/ml (△) to two flasks while a third flask served as a control (○). L-Methionine was added at 200 μg/ml (□) after four hours. The optical density of diluted samples was monitored at 600 nm with 1 cm light-path.

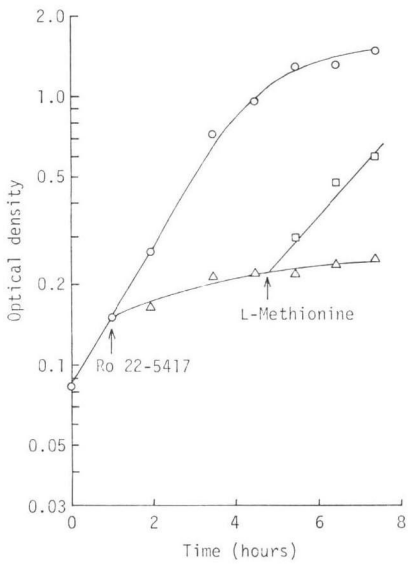

Table 2. Results of reversal study.

The following compounds antagonized the activity of Ro 22-5417 when tested by a counter-diffusion method[9]:	
L-Methionine	L-Homocystine
D-Methionine	*O*-Acetyl-L-homoserine
L-Cystathionine	L-Glutamine (slightly)

The following compounds did not antagonize the activity of Ro 22-5417:			
L-Homoserine	D-Glutamic acid	Calcium pantothenate	Dithiothreitol
L-Aspartic acid	4-Hydroxy-L-proline	Folic acid	D,L-Lipoic acid
L-Lysine	L-Proline	Nicotinic acid	Choline
L-Cysteine	L-Glutamine	Nicotinamide	
L-Threonine	L-Histidine	*p*-Aminobenzoic acid	Adenosine
L-Asparagine	L-Phenylalanine	Pyridoxal	Cytidine
L-Isoleucine	L-Tryptophan	Pyridoxamine	Guanosine
L-Valine	L-Tyrosine	Pyridoxine	Thymine
L-Leucine	Glycine	Riboflavin	Uridine
L-Alanine	L-Serine	Thiamine	Adenosine-3′,5′-cyclic-monophosphate
D-Alanine	D,L-Diaminopimelic acid	Biotin	
L-Arginine	*N*-Acetyl-D-glucosamine	Vitamin B_{12}	Arabinose-5′-phosphate
L-Glutamic acid		Glutathione	

reversal of Ro 22-5417 inhibition by methionine and related compounds present in the broth filtrate. This can be evidenced by the fact that a paper disc containing a combination of 5 μg Ro 22-5417, 4 μg L-methionine, and 2 μg benzylpenicillin produces a zone of inhibition very similar to that in Fig. 1A against *Bacillus* sp. ATCC 27860 on Davis minimal agar.

After completion of our work and its preliminary communication[12], the discovery, isolation, and structure determination of a closely related compound, (−)-3-(2-hydroxyethyl)-1-aza-4-oxabicyclo-[3.2.0]heptan-7-one was reported[13]. The compound, produced by *Streptomyces antibioticus*, shows biological properties very similar to those of Ro 22-5417.

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