SQ 26,180, A NOVEL MONOBACTAM. I

TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

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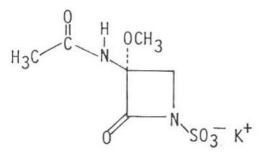
Strains of *Chromobacterium violaceum*, isolated from various environments, were found to produce a novel monocyclic β-lactam antibiotic. The antibiotic, SQ 26,180 was weakly active against Gram-positive and Gram-negative bacteria with the exception of mutants hypersensitive to β-lactam antibiotics. The compound was highly stable to β-lactamases and acted as an inhibitor of the P99 enzyme from *Enterobacter cloacae*. SQ 26,180 showed affinity for penicillin-binding proteins 1a, 4 and 5/6 of *Escherichia coli* and inhibited R61 DD-carboxypeptidase.

The production of monocyclic β-lactam antibiotics (monobactams) by bacteria has been recently reported[1,2,3]. Over the past few years, we have screened approximately 1 million microorganisms for the production of β-lactam antibiotics. In order to test a large variety of eubacteria, many soil, plant, and water samples from numerous ecosystems were examined. Enrichment procedures and specific media were used to enhance the efficiency of isolation of representative bacteria from the various samples. Isolates were screened in an extremely sensitive and selective screening system for detecting β-lactam antibiotics using a strain of *Bacillus licheniformis* SC 9262, hypersensitive to β-lactam containing molecules.

The structure of SQ 26,180, a simple methoxylated monobactam, is shown in Fig. 1. This paper describes the taxonomy of *Chromobacterium violaceum* SC 11,378 and the fermentation and biological properties of SQ 26,180. A preliminary account of this work has appeared[2]. Isolation, structure determination, synthesis and physicochemical properties of SQ 26,180 are described in the accompanying paper[4].

Fig. 1. Structure of SQ 26,180.



Taxonomy

Chromobacterium violaceum SC 11,378 was isolated from a soil sample collected in the New Jersey Pine Barrens. Although *Chromobacterium* strains were isolated from many samples, indicating they are relatively widespread in nature, strains producing SQ 26,180 were isolated from only eight locations (Table 1). Furthermore, nine *Chromobacterium violaceum* strains, one *Chromobacterium lividum* and one *Chromobacterium marismortui* strain obtained from the American Type Culture Collection were all nonproducers. The producing strains of *Chromobacterium violaceum* showed the following characteristics.

Morphology

The organism was an aerobic Gram-negative rod, motile by means of a single polar flagellum with an occasional single smaller lateral flagellum. Rods often showed barring, bipolar staining and lipid inclusions.

On nutrient agar violet colonies predominated with occasional nonpigmented clones appearing; pigment production was enhanced on media rich in tryptophan or yeast extract. The violet pigment was soluble in ethanol but insoluble in water or chloroform. In nutrient broth the culture produced a violet ring on the wall of the tube but no confluent pellicle.

Table 1. Collection sites of *Chromobacterium violaceum* strains producing SQ 26,180.

Sample	Collection site
Cedar forest soil	New Jersey Pine Barrens
Swamp water	New Jersey Pine Barrens
Creek water	New Jersey Pine Barrens
Blue spruce needle compost pile	Waterbury, CT
Small unidentified plant	Barnegat Bay, NJ
Sulfur-iron bog sediment	Great Swamp Refuge, NJ
Decaying root mass of swamp plant	Mercer County Park, NJ
Forest soil	West Windsor, NJ
Oak leaf litter	Hacklebarney State Park, NJ

Physiological and Biochemical Characteristics

The organism was mesophilic, with good growth between 15°C and 37°C; no growth occurred at 4°C or above 37°C.

Casein was hydrolyzed strongly and esculin hydrolysis was negative. Glucose, fructose and trehalose were fermented, whereas L-arabinose was not utilized either fermentatively or oxidatively[5]. HCN was produced.

The above key characters provided the basis for identification of this organism as *Chromobacterium violaceum* and its differentiation from *Chromobacterium lividum*[6].

Chromobacterium violaceum SC 11,378 produced an extracellular β-lactamase which was purified to homogeneity as follows. Centrifuged broth was first treated with ammonium sulfate; β-lactamase activity was observed in the precipitate obtained from 55~85% saturation. The enzyme was then fractionated on Sephadex G-75 (50 mM phosphate buffer, pH 7.0, containing 0.5 mM β-mercaptoethanol) and on QAE-Sephadex A-50 (25 mM TES buffer, pH 7.5, containing 1 mM β-mercaptoethanol).

The purified enzyme resembled the β-lactamase described by FARRAR and O'DELL[7], also isolated from a strain of *Chromobacterium violaceum*. Both enzymes exhibited cephalosporinase substrate profiles and were inhibited somewhat by *p*-chloromercuribenzoate (PCMB); incubation of the enzyme with PCMB for 10 minutes resulted in 20% inhibition, incubation for 60 minutes gave 60% inhibition. A pI greater than 9.5 was observed using isoelectric focusing (LKB Multiphor, PAG plates pH 3.5~9.5).

Fermentation

Seed culture was prepared by transferring a loopful of surface growth from an agar slant of *Chromobacterium violaceum* SC 11,378 into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: Oatmeal, 2.0% and tomato paste, 2.0% in tap water. The pH was adjusted to 7.0 (NaOH) before sterilization. The flasks were incubated at 25°C on a rotary shaker (300 rpm; 5 cm stroke) for approximately 24 hours. A 1% (v/v) transfer of this culture growth was used to prepare a second stage seed culture in 4-liter Erlenmeyer flasks containing 1.5 liters of the medium described above. The flasks were incubated for approximately 24 hours under the same conditions described above. A 1% (v/v) transfer of the second stage seed culture was used to inoculate a 380-liter stainless steel fermentation tank

containing 250 liters of the following production medium: yeast extract, 0.25%; glucose, 3.0%; NZ amine-A, 1.0% (pH 6.5) in tap water. The fermentation was continued for approximately 18~24 hours at 25°C with an agitation rate of 155 rpm and an air flow of 285 liters/minute. Antibiotic production was determined by a paper disc agar diffusion assay using *Bacillus licheniformis* SC 9262 as the test organism.

Biological Properties

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of SQ 26,180 against a wide range of bacteria was determined by a two-fold agar dilution method and the results are shown in Table 2. The compound exhibited weak antibacterial activity, showing the greatest effect against Gram-negative organisms, especially *Pseudomonas*.

Table 2. Antimicrobial activity of SQ 26,180.

Test organism	MIC[1] (μg/ml)
Staphylococcus aureus SC 1276	50
Staphylococcus aureus SC 2399	50
Staphylococcus aureus SC 2400	100
Staphylococcus aureus SC 10165	100
Streptococcus faecalis SC 9011	>100
Streptococcus agalactiae SC 9287	12.5
Micrococcus luteus SC 2495	25
Escherichia coli SC 8294	>100
Escherichia coli SC 10857	>100
Escherichia coli SC 10896	25
Escherichia coli SC 10909	50
Klebsiella aerogenes SC 10440	>100
Klebsiella pneumoniae SC 9527	>100
Proteus mirabilis SC 3855	>100
Proteus rettgeri SC 8479	>100
Proteus vulgaris SC 9416	>100
Salmonella typhosa SC 1195	>100
Shigella sonnei SC 8449	>100
Enterobacter cloacae SC 8236	>100
Enterobacter aerogenes SC 10078	>100
Citrobacter freundii SC 9518	100
Serratia marcescens SC 9783	25
Pseudomonas aeruginosa SC 9545	3.1
Pseudomonas aeruginosa SC 8329	50
Acinetobacter calcoaceticus SC 8333	25

[1] Minimum inhibitory concentrations were determined by a two-fold agar dilution method on DST agar (Oxoid). Final inoculum level was 10^4 colony-forming units.

Stability to β-Lactamases

All β-lactamase stability studies of SQ 26,180 were performed at 25°C in 0.1 M phosphate buffer, pH 7.0, as previously described[2]. As can be seen in Table 3, SQ 26,180 was quite stable in the presence of β-lactamases. The compound showed no significant affinity for the *Staphylococcus aureus*, K1 or TEM-2 β-lactamases, enzymes which exhibit primarily penicillinase activity. The β-lactamase from *Chromobacterium violaceum* also did not appear to bind SQ 26,180. However, binding to the P-99 β-lactamase by SQ 26,180 was not progressive and could be

Table 3. Susceptibility to and inhibition of β-lactamases by SQ 26,180[1].

β-Lactamase[2]	Compound	Relative V_{max}	I_{50} (mM)
Staphylococcus aureus	Benzylpenicillin	100	—
	SQ 26,180	<0.02	>2.0
TEM-2	Benzylpenicillin	100	—
	SQ 26,180	<0.01	>2.0
K-1	Benzylpenicillin	100	—
	SQ 26,180	<0.02	>2.0
P-99	Cephaloridine	100	—
	SQ 26,180	<0.05	0.22
Chromobacterium violaceum	Cephaloridine	100	—
	SQ 26,180	N.D.	>0.5

[1] Studies performed as previously described[2].

[2] β-Lactamases were prepared from *Staphylococcus aureus* SC 10165, *Escherichia coli* (TEM) SC 11101, *Klebsiella aerogenes* (K-1) SC 10436 and *Enterobacter cloacae* (P-99) SC 10,435.

Table 4. Binding of SQ 26,180 to pencillin-binding proteins of *Escherichia coli*.

Organism	Amount (μg/ml) to inhibit penicillin binding completely to PBPs						MIC (μg/ml)
	PBP1a	PBP1b	PBP2	PBP3	PBP4	PBP5/6	
Escherichia coli SC 8294	10	>100	>100	>100	10	10	>100

Table 5. Binding of SQ 26,180 to penicillin-binding proteins of *Staphylococcus aureus*.

Organism	Amount (μg/ml) to inhibit penicillin binding completely				MIC (μg/ml)
	PBP1	PBP2	PBP3	PBP4	
Staphylococcus aureus SC 2399	100	>100	>100	2.0	100

reversed after the enzyme-inhibitor complex was dialyzed. Therefore, SQ 26,180 appeared to act as a reversible competitive inhibitor (*Ki* of 80 μM).

Binding to Penicillin-Binding Proteins

SQ 26,180 was tested for its ability to bind essential penicillin binding proteins (PBPs) of *Escherichia coli* and *Staphylococcus aureus*. Solubilized (2% triton X-100) membranes of sonicated *E. coli* SC 8294 and *S. aureus* SC 2399 were incubated ($\sim$100 μg of protein) with the compound at 30°C for 10 minutes in a total volume of 50 μl. After the addition of 10 nmole of [^{14}C]benzylpenicillin (Amersham-Searle; specific activity 51 μCi/μmole), the incubation was continued for another 10 minutes. Protein was precipitated with four volumes of cold acetone and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by fluorography[11]. Protein-bound [^{14}C]penicillin was determined by visual examination of the X-ray film.

The results of the binding experiment are shown in Tables 4 and 5. As expected from the MIC data, binding of SQ 26,180 to the essential PBPs of *E. coli* and *S. aureus* was poor. However, good binding to PBPs 1a, 4 (=DD-carboxypeptidase 1B)[12] and 5/6 (=DD-carboxypeptidase 1A)[13] of *E. coli* and PBP4 (=DD-carboxypeptidase)[14] of *S. aureus* was observed.

Inhibition of DD-Carboxypeptidase

The ability of SQ 26,180 to inhibit DD-carboxypeptidase was tested using partially purified *Streptomyces* R61 enzyme[8]. The compound was preincubated together with the enzyme at 30°C for 0 and 30 minutes in a total volume of 20 μl. Two nanomoles of substrate, [^{14}C]diacetyl-L-Lys-D-Ala-D-Ala[9], were added, and the incubation was continued for 30 minutes. The reaction was stopped by the addition of 5 μl of 0.25 M HCl, and the hydrolysis product, [^{14}C]diacetyl-L-Lys-D-Ala, was separated from the substrate by high voltage paper electrophoresis at pH 3.5[10]. The percentage of hydrolysis (allowed not to exceed 15%) was determined from liquid scintillation counting of the two radioactive spots. SQ 26,180 appeared to have good inhibitory activity against *Streptomyces* R61 DD-carboxypeptidase since the amount of compound required to cause 50% inhibition of the enzyme (I_{50}) was 3 μM at no preincubation and 1.0 μM at 30 minutes of preincubation.

In addition to SQ 26,180 we have also isolated a related antibiotic SQ 26,445 having a dipeptide group at the 3-position[2]. SQ 26,445, produced by strains of *Gluconobacter* or *Acetobacter*, is relatively common in nature and is identical with sulfazecin isolated from a strain of *Pseudomonas acidophila*[1].

Agrobacterium radiobacter produces a mixture of monocyclic β-lactams and an account of these

strains will be forthcoming.

The monobactams, monocyclic β-lactam antibiotics produced by bacteria, have opened up a new era of β-lactam research. As with other naturally-occurring β-lactam molecules *e.g.* penicillins and cephalosporins, molecular modification leads to compounds with superior properties over the naturally-occurring molecules. One such compound, SQ 26,776 has been identified and is presently being tested in the clinic.

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