SQ 27,860, A SIMPLE CARBAPENEM PRODUCED BY SPECIES OF *SERRATIA* AND *ERWINIA*

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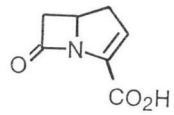
In our continued search for the production of β-lactam-containing molecules from bacteria, we report the isolation and structure determination of a simple carbapenem SQ 27,860, produced by species of *Serratia* and *Erwinia*. The antibiotic is highly unstable and isolation was achieved through the *p*-nitrobenzyl ester.

Over the past few years we have screened large numbers of bacteria from a wide variety of habitats and ecosystems, searching for β-lactam-producing organisms. In 1981 we reported the isolation of a series of monocyclic β-lactams and β-lactones from bacteria[1,2]. We now report on the first bacterially produced bicyclic β-lactam, SQ 27,860, a simple carbapenem having structure **1** (Fig. 1), which shows activity *in vitro versus* a broad spectrum of bacterial species.

Fig. 1. Structure of SQ 27,860.



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This paper describes the production, isolation and structure determination of SQ 27,860.

Strain Description

The capacity for bacteria to synthesize SQ 27,860 appears to be rather limited in nature. Two genera of producing organisms (*Serratia* sp. and *Erwinia* sp.) have been identified and the sources of these organisms are listed in Table 1.

Table 1. Collection sites of *Serratia* sp. and *Erwinia* sp. producing SQ 27,860.

	Sample	Collection site
Serratia sp.	*Salicornia alterniflora* roots and stems	Cheesequake Salt Marsh, New Jersey
	Channel water	Cheesequake Salt Marsh, New Jersey
	Phalophycea sp.	Salt Marsh, Germany
	Unidentified plant along river bank	Oceangate, New Jersey
Erwinia sp.	Rotting weeds	West Windsor, New Jersey
	Soil from market place	Morocco, North Africa
	Dried soil and leaves of eucalyptus tree	Morocco, North Africa
	Floating grass and debris in river	Oceangate, New Jersey
	Wood shavings	Seaside Park, New Jersey
	Algal mat in stream	Allaire State Park, New Jersey
	Forest leaf litter	Allaire State Park, New Jersey
	Leaves and stem of unidentified brier plant	Thompson Park, New Jersey

1. *Serratia* Species

The organism is a Gram-negative rod, motile by means of peritrichous flagella. On BENNETT's agar[3], colonies initially appear pasty white, developing a central pink pigment after about 48 hours. Pigment production was optimal between 23~28°C. On glycerol-peptone agar colonies were deeply pigmented from cherry red to reddish purple.

Characterization of the Pigment: The pigment was extracted from cells of *Serratia* sp. SC 11,482 grown on glycerol - peptone agar by the method of WILLIAMS, *et al.*[4]. The crude pigment extract, along with that obtained from a known strain of *Serratia marcescens*, was chromatographed on a silica gel thin-layer plate (Eastman Chromogram). The developing solvent was toluene - ethyl acetate (1: 1). The pigments of both SC 11,482 and *S. marcescens* resolved into two components: the main one appearing as a pinkish red spot with Rf 0.75; the minor component was pale lavender and remained at the origin. The main pigment fraction from the crude extract was eluted from a silica gel column with the above solvent, concentrated *in vacuo* and dissolved in 2 ml of ethanol acidified with 1 N HCl (10: 1 v/v). The main pigments of SC 11,482 and *S. marcescens* isolated in this way, both exhibited a sharp spectral peak at 535 nm, which is characteristic of prodigiosin.

Biochemical Characteristics of SC 11,482: SC 11,482 was positive for the following biochemical characters: ornithine decarboxylase; acetoin production; lipolysis on Tween 80; SIMMON's citrate; aesculin hydrolysis; acid from mannitol, sorbitol, xylose, arabinose, melibiose and sucrose; growth on Difco marine agar and nutrient agar containing 4% NaCl. It was negative for the following properties: cytochrome oxidase; DNase; lysine decarboxylase; arginine decarboxylase; methyl red; growth on KCN; production of H_2S on triple sugar iron agar and cysteine; gluconate; chitinase; acid from inositol, adonitol, arabinose and α-methyl glucoside.

The production of prodigiosin in Gram-negative bacteria is limited presently to the genera *Alteromonas, Beneckia, Serratia* and *Vibrio*. SC 11,482 can be differentiated from *Alteromonas rubra* and *Beneckia gazogenes* in that the latter two are obligately halophilic polar flagellates. It differs from *Vibrio psychroerythrus* in that the latter is an obligately halophilic psychrophile (no growth above 20°C). SC 11,482 is mesophilic growing optimally between 25~30°C and while halotolerant (up to 4% NaCl) does not require salt for growth. The two properties are also shared by members of the genus *Serratia*. SC 11,482 is considered to belong in this genus.

Table 2. Biochemical characteristics of *Serratia* sp. SC 11,482 and comparison strains.

	S. marcescens		*S. marinorubra* ATCC 27,614	*S. plymuthica* ATCC 183
	SC 11,482	SC 12,360		
DNase	−	+	+	+
Gelatinase	−	+	+	+
Chitinase	−	+	−	+
Lysine decarboxylase	−	+	+	−
Ornithine decarboxylase	+	+	−	−
H_2S	−	+	−	+
Gluconate	−	+	+	+
Acid from adonitol	−	−	+	−
arabinose	+	−	+	−
melibiose	+	−	+	+

Within the genus *Serratia* three pigmented species are recognized: *S. marcescens*, *S. marinorubra* (synonym: *rubidaea*) and *S. plymuthica*[5]. Table 2 summarizes the key characters differentiating SC 11,482 from these species.

On the basis of the above, SC 11,482 is considered to be a species of *Serratia*. While the characters presented serve to differentiate it from the other pigmented species of the genus, it is premature to designate this strain as a new species in the absence of DNA homology studies with the other species.

Table 3. Biochemical properties of SQ 27,860-producing strains of *Erwinia*.

Character	SC 12,637	SC 12,638
Cytochrome oxidase	−	−
Pectinolytic*	+++	−
Gelatinase	+	−
Gluconate	−	+
DNase	−	−
Chitinase	NT	−
Pigment	−	Yellow
Aesculin hydrolysis	+	−

* Determined on a mixed salts - yeast extract pectin medium.

2. *Erwinia* Species

SQ 27,860 production has been detected in two species of *Erwinia*. Both organisms are fermentative Gram-negative peritrichous flagellated rods. On the basis of the biochemical properties listed in Table 3, SC 12,637 has been assigned to the *Erwinia carotovora* group and SC 12,638 to the *Erwinia herbicola* group.

Fermentation

Initial characterization studies were performed on SQ 27,860 produced by *Serratia* sp. SC 11,482. Seed culture was prepared by transferring a loopful of surface growth from an agar slant into 500 ml Erlenmeyer flasks containing 100 ml of Antibiotic Assay Broth (AAB) (Baltimore Biological Laboratory, Cockeysville, Maryland). The flasks were incubated at 25°C on a rotary shaker (300 rpm; 5.1 cm stroke) for approximately 24 hours. A 1% (v/v) transfer of this culture growth was used to inoculate 50 liters of AAB medium in a FM-75 Fermatron fermentor (New Brunswick Scientific, New Brunswick, NJ). The fermentation was continued for 18~22 hours at 25°C at an agitation rate of 200 rpm and an air flow of 51 liters/minute. Antibiotic production was determined by a paper-disc agar-diffusion assay using *Bacillus licheniformis* SC 9262 as the test organism.

Isolation and Structure Determination

SQ 27,860 is very unstable which made the isolation somewhat challenging. The antibiotic is most stable in the pH range of 7 to 9 but only survives at a detectable level for several days at 5°C. Freezing or concentration of the broth filtrate destroyed the activity. Paper electrophoresis at pH 7 in 0.05 M sodium phosphate buffer gave a mobility of 0.95 relative to *p*-nitrobenzenesulfonate (1.00) and vitamin B_{12} (0.0), showing that the antibiotic is an acidic substance. SQ 27,860 can be extracted into butanol at pH 3 but not at pH 5, indicating that the antibiotic is a weak acid. However, the stability at pH 3 is so poor that extraction was not useful to us for concentration.

SQ 27,860 can be adsorbed from the broth supernate onto charcoal and can be stored in this form at −90°C for extended periods without substantial loss of activity. Elution from the charcoal with acetone - water (7: 3) gave a concentrate that could be further purified by ion-exchange chromatography on Dowex 1-X8 resin. Other purification methods tried were beset by stability problems and did not give useful results. Therefore esterification of the acid was pursued in order to get a more tractable derivative for further purification and structural studies.

Conditions suitable for phase-transfer esterification of prostaglandins[6] failed even though the antibiotic does undergo ion-pair extraction to a moderate extent. Esterification was achieved by absorption of the antibiotic on Bio-Rad AG MP-1 resin and treatment of the dry loaded resin with *p*-nitrobenzyl bromide[7]. Acetonitrile was a suitable solvent for this reaction in small scale runs but gave erratic results on a larger scale, perhaps due to residual water on the resin. Dimethylformamide was found to be superior and gave consistently good results with a small sacrifice in convenience from its lower volatility. Benzyl and *o*-nitrobenzyl esters were also prepared by this technique but were not fully characterized. The isolation of SQ 27,860 as the crystalline *p*-nitrobenzyl (PNB) ester **2** is outlined in Fig. 2.

Fig. 2. Isolation of SQ 27,860 as the *p*-nitrobenzyl ester.

SC 11,482 broth supernate (pH 7.2)
↓ Stir with Amberlite XAD-2 and filter.
Filtrate
↓ Stir with charcoal and filter.
Loaded charcoal
↓ Stir with acetone - water (7:3) at pH 7.7 and remove acetone from eluate.
Aqueous concentrate
↓ Chromatograph on Dowex 1-X8, Cl^- form, eluting with aqueous NaCl.
Active effluent
↓ Desalt with charcoal as above and sorb antibiotic on Bio-Rad AG MP-1 resin. Wash resin with acetonitrile and dry *in vacuo*.
Loaded resin
↓ Treat with *p*-nitrobenzyl bromide in DMF. Chromatograph resulting ester on silicic acid and recrystallize from ether - acetone.
SQ 27,860 - *p*-nitrobenzyl ester (**2**).

Before isolation of the crystalline PNB ester was achieved, a structure for SQ 27,860 was derived by degradation of the crude noncrystalline ester that was prepared as shown in Fig. 2 with the omission of the ion-exchange chromatography step. The PNB ester has a peak at 1783 cm^{-1} ($CDCl_3$) in the IR spectrum indicating a β-lactam structure in accord with expectations from the microbiological characterization. Since the PNB ester is also rather unstable, especially to acid, the ester was subjected to base-catalyzed methanolysis in the expectation of opening the β-lactam ring to give a methyl PNB diester that would be more stable.

Treatment with methanol containing triethylamine followed by purification of the methanolysis product by chromatography gave a colorless oil, **3**, for which a ^1H NMR spectrum in CD_3CN was obtained (Table 4). However, upon attempting to obtain a spectrum in CD_3OD, further methanolysis took place, namely a facile ester interchange that released *p*-nitrobenzyl alcohol and gave the mixed CH_3, CD_3 diester

Table 4. ^1H NMR data for SQ 27,860-derived compounds.

Position	Chemical shift (ppm downfield from Me_4Si)		
	2 in $CDCl_3$	**3** in CD_3CN	**4** in CD_3OD
3	6.59 (t, 2.7 Hz, 1H)	2.8 (m, 2H)	2.9 (m, 2H)
4	2.89 (m, 2H)	1.4~2.4 (m, 2H)	1.7 (m, 1H) 2.3 (m, 1H)
5	4.32 (m, 1H)	4.29 (m, 1H)	4.54 (m, 1H)
6	3.01 (dd, J=5.4, 16.6 Hz, 1H) 3.53 (dd, J=3.1, 16.6 Hz, 1H)	2.59 (dd, J=7.5, 15.8 Hz, 1H) 2.64 (dd, J=7, 15.8 Hz, 1H)	2.62 (dd, J=7.7, 16.0 Hz, 1H) 2.22 (dd, J=6.2, 16.0 Hz, 1H)
8	5.23 (d, J=13.7 Hz, 1H) 5.50 (d, J=13.7 Hz, 1H)	5.37 (s, 2H)	
9	7.61 (d, J=8.8 Hz, 2H)	7.63 (d, J=9 Hz, 2H)	
10	8.23 (d, J=8.8 Hz, 2H)	8.23 (d, J=9 Hz, 2H)	
11		3.65 (s, 3H)	3.69 (s, 3H)

Fig. 3. Methanolysis of the *p*-nitrobenzyl ester of SQ 27,860.

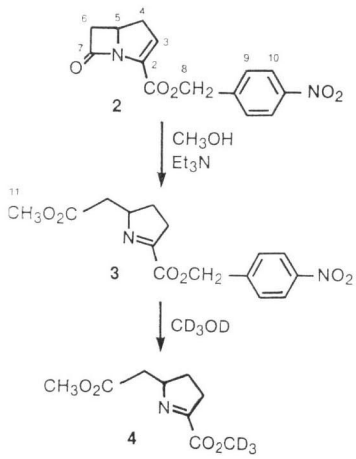

Table 5. Antibacterial activity*1 of SQ 27,860.

Test organism	Inhibition zone (mm)	
	SQ 27,860*2	Ampicillin*3
Staphylococcus aureus SC 1276 Pen^S	10.6	39.5
Staphylococcus aureus SC 2400 Pen^R	10.3	16.5
Escherichia coli SC 10,439 TEM^-	13.3	23.0
Escherichia coli SC 10,404 TEM^+	≤6.0	≤6.0
Enterobacter cloacae SC 10,441 $P99^-$	10.5	20.0
Enterobacter cloacae SC 10,435 $P99^+$	11.0	≤6.0

*1 Determined on DST agar (Oxoid Laboratories, Basingstoke, England).
*2 60 μl of column eluate (see experimental section) on 6 mm discs.
*3 10 μg ampicillin per disc.

4. This ester was purified by chromatography, giving a colorless oil that had an M+1 peak in the CI mass spectrum (H_2O>1 torr) at *m/z* 203. Upon heating a sample of **4** in methanol, an additional peak at *m/z* 200, corresponding to the undeuterated dimethyl ester, was observed as expected. A reasonable structure for **4** was easily postulated from the ^1H NMR spectrum (Table 4). The structures of the primary methanolysis product **3** and the PNB ester **2** of SQ 27,860 follow as shown in Fig. 3 and establish **1** as the structure of SQ 27,860.

Biological Properties

SQ 27,860 showed a broad spectrum of activity *in vitro* against Gram-positive and Gram-negative bacteria (Table 5). It was equally active against penicillin-sensitive and penicillin-resistant staphylococci. Among the Gram-negative organisms, SQ 27,860 was equally active against β-lactamase P99-producing *Enterobacter cloacae* and the lactamase-negative strain ($P99^-$). However, SQ 27,860 was inactive against the β-lactamase TEM-producing *E. coli* strain at the concentration tested.

Discussion

Discovery of the cephamycins in 1970[8,9] was the first reported isolation of new naturally occurring β-lactam antibiotics since the discovery of cephalosporin C in 1955[10]. The cephamycins were soon to be followed by other naturally occurring β-lactams, including the nocardicins[11], olivanic acids[12], clavulanic acid[13], thienamycin[14] and a host of other carbapenems[15~18]. In contrast to the penicillins and cephalosporins these molecules are produced exclusively by members of the actinomycetes.

In 1981, the first reports of bacterially-produced β-lactams were published[1,19]. These molecules are characterized by having the 2-oxoazetidine-1-sulfonic acid moiety. Isolation from nature of 7-oxo-1-azabicylo[3·2·0]hept-2-ene-2-carboxylic acid (1-carba-2-penem-3-carboxylic acid, **1**) is the first indication that bacteria are capable of producing bicyclic β-lactam antibiotics. This antibiotic has been prepared synthetically[20,21] but has not previously been reported as a naturally-occurring substance. Full spectroscopic characterization of **1** has not been reported because of the poor stability, but characterization of the synthetic racemic PNB ester has been reported[22]. The preparation of the *d* and *l* enantiomers of **2** has also been reported[23] but with no indication of optical purity or melting point. The crystalline

PNB ester of SQ 27,860, obtained as shown in Fig. 2, has spectroscopic properties consistent with the data in Reference 22. Synthetic **2** has been used as an intermediate in the preparation of olivanic acid analogs that lack substituents at C-6[24].

A novel source of β-lactam antibiotics is of enormous interest to the pharmaceutical industry. That bacteria produce both monocyclic and bicyclic β-lactam antibiotics begs one to ask the question, what else do they produce? We hope to answer this question in due course.

Experimental

NMR spectra were recorded on a Varian Associates XL-100-15 spectrometer; chemical shifts (δ) are given in ppm downfield from internal Me_4Si. Mass spectra were determined with an Extranuclear Laboratories modified Simulscan instrument. IR and UV spectra and optical rotations were determined on Perkin-Elmer Model 621, Model 202 and Model 141 instruments, respectively. Melting points were measured in soft glass capillaries with a Thomas-Hoover apparatus and are uncorrected. Thin-layer chromatography was done on Merck silica gel 60 F-254 plates. Acidic impurities were removed from $CDCl_3$ with basic alumina, and dimethylformamide was dried over Type 3A Molecular Sieves.

Isolation of 7-Oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic Acid as the *p*-Nitrobenzyl Ester (2)

The isolation of SQ 27,860 was monitored by antibacterial assay with *E. coli* SC 12,155. Broth supernate from a 50-liter fermentation was stirred with 3 liters of Amberlite XAD-2 resin at 5°C for 1 hour. The mixture was filtered and the filtrate was stirred with 1.2 kg (dry weight) of Fisher cocoanut charcoal (50~200 mesh) at 5°C for 1.5 hours. The charcoal was separated, washed with cold water, and then stirred with 8 liters of acetone - water (7: 3) at room temperature for 1 hour, maintaining the pH at 7.7 by the addition of 6 N NaOH. The mixture was filtered and the filtrate concentrated *in vacuo* to 1.5 liters at 20°C. The resulting concentrate was chromatographed at 5°C on a 2.5×41 cm column of Dowex 1-X8 resin (Cl^- form, 200~400 mesh), eluting at 10 ml/minute with 50 ml of water followed by a linear gradient prepared from 3.7 liters of water and 3.6 liters of 1 M NaCl, and collecting 20-ml fractions. Active fractions (78~98) were combined and shaken with 21 g of Fisher cocoanut charcoal at 5°C for 1.5 hours, maintaining the pH at 7 with 1 N HCl. The charcoal was separated and stirred at 0°C with 200 ml of acetone - water (7: 3) for 1 hour (pH 7.50). The mixture was filtered and the filtrate concentrated *in vacuo* at 20°C to 76 ml. This solution was applied at 5°C and 2 ml/minute to a 1.1×21 cm column of Bio-Rad AG MP-1 resin (Cl^- form, 100~200 mesh). The resin was washed with 50 ml of cold water and 100 ml of acetonitrile and was then dried *in vacuo* (20°C) giving 5.36 g of loaded resin. SQ 27,860 can be stored in this form at −90°C. Samples for biological studies were eluted with 0.5 M NaCl from resin prepared as above but with the omission of the ion-exchange chromatography.

The loaded resin (5.36 g) was mixed with a solution of 5.4 g of *p*-nitrobenzyl bromide in 10 ml of dry dimethylformamide. After 2 hours at 20°C, the slurry was mixed with xylenes (mixture of isomers), filtered, and the filtrate was taken to dryness *in vacuo* (20°C), removing dimethylformamide azeotropically with xylenes. The residue, in 50 ml of toluene, was chromatographed on a 2.5×7 cm column of Mallinckrodt silicic acid (100 mesh), eluting at 5°C with 50 ml toluene followed by 300 ml of toluene - ether (14: 1) and collecting 20-ml fractions. Fractions 13~15 contained **2** (TLC, ether, Rf 0.23) and were combined and concentrated *in vacuo*, giving 13.4 mg of crystalline residue. Three recrystallizations from acetone - ether gave 5.7 mg of *p*-nitrobenzyl 7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylate (**2**) as a pale-yellow solid: m.p. 119~121°C; UV_{max} (CH_3CN) 270 nm (ε 13,000); $[\alpha]_D^{22}$ +104° (*c* 0.3, toluene); IR ($CDCl_3$) 1783, 1729, 1609, 1525, 1349, 1319, 1276, 1257, 1209, 1161, 1104 and 1011 cm^{-1}.

Anal. Calcd. for $C_{14}H_{12}N_2O_5$: C 58.34, H 4.20, N 9.72.
Found: C 58.48, H 4.40, N 9.50.

Methanolysis of 2

Crude **2** from a 50-liter fermentation, produced essentially as described above but omitting the ion-exchange chromatography step, was dissolved in 100 ml of methanol and 10 ml of triethylamine and kept at 20°C for 1 hour. The solution was concentrated *in vacuo* and the methanolysis product (TLC, ether, Rf 0.28) was purified by chromatography on Whatman LP-1 silica gel eluting with ether to give 4.5 mg

of **3** as a colorless oil (^{1}H NMR, Table 4).

A solution of **3** in CD_3OD was kept at 35°C for 1.5 hours. The product (TLC, ether, Rf 0.20) was purified by chromatography on silica gel eluting with ether to give 2.5 mg of **4** as a colorless oil: IR ($CHCl_3$) 1733 and 1630 cm^{-1} (^{1}H NMR, Table 4).

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