SQ 28,332, A NEW MONOBACTAM PRODUCED BY A *FLEXIBACTER* SP.

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL PROPERTIES

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A new monobactam SQ 28,332 has been isolated from fermentations of a *Flexibacter* sp. and the structure **3** was deduced from its spectroscopic properties. SQ 28,332 exhibits weak antibacterial activity.

The monobactams are a new class of bacterially produced antibiotics that were recently discovered independently by workers at Takeda Chemical Industries[1,2] and at the Squibb Institute for Medical Research.[3~5] This class consists of monocyclic β-lactam antibiotics that are *N*-acyl derivatives of (*S*)-3-aminomonobactamic acid (**1**) or (*R*)-3-amino-3-methoxymonobactamic acid (**2**). Both methoxylated and nonmethoxylated compounds are naturally produced.[6~8] We wish to report the isolation and characterization of a new monobactam, SQ 28,332 (**3**), produced by *Flexibacter* sp. SC 12,681. A brief description of the producing strain and its fermentation conditions is given. The biological properties of SQ 28,332 are also presented.

Taxonomy

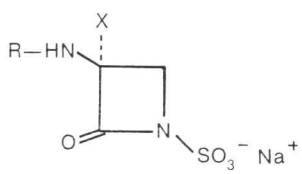
SQ 28,332 is produced by *Flexibacter* sp. SC 12,681. Although *Flexibacter* sp. were isolated occasionally on our various bacterial isolation media and in large numbers on specialized media (P.M.Y.A. II and skim milk - acetate agar) developed for the isolation of gliding bacteria,[9] this particular monobactam-producing strain was isolated on only one occasion from a sample of decaying fresh water swamp grass from the Great Swamp, New Jersey. The sample was stored at 4°C for approximately four weeks before being plated onto GFY agar consisting of (g/liter): glucose 5.0, fructose 5.0, yeast extract 5.0, $CaCO_3$ 3.0, vitamin B_{12} 0.002 and agar 17.5. *Flexibacter* sp. SC 12,681 has been deposited with the American Type Culture Collection (ATCC) under the accession number of ATCC 35208.

Morphology

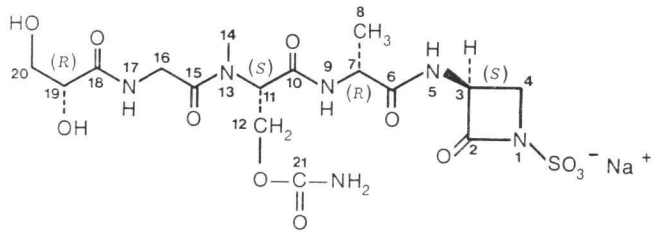
Vegetative cells of *Flexibacter* sp. SC 12,681 are Gram-negative long slender rods whose ends are blunt to rounded. There is no evidence of fruiting bodies. Motility is by gliding. The cells contain an intra-cellular yellowish-orange pigment soluble in hexane and ethanol that is presumed to be a carotenoid based on a positive test with $SbCl_2$. The hexane fraction exhibits a characteristic absorbance peak at 450 nm with a shoulder at 429 and 470 nm.

SC 12,681 is oxidative, catalase positive and H_2S positive by the lead acetate paper strip test with cysteine as the substrate. It is chitinase and cellulase negative; and exhibits no growth on marine agar.

Fig. 1. Structure of monobactams and SQ 28,332.



1 R=X=H
2 R=H, X=OCH_3



3 SQ 28,332

The mol percent G+C of the DNA is 40~42% as determined by the thermal denaturation method in 0.5×standard saline citrate (SSC=0.15 M sodium chloride - 0.15 M sodium citrate). Following the classification of LEWIN,[10] this organism is a Flexibacter.

Fermentation

Fermentation was initiated by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks, each containing 100 ml of sterilized yeast extract (0.5%) in distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm; 5-cm stroke) for approximately 24 hours. A 1.0% (v/v) transfer of this culture growth was used to inoculate a 75-liter Fermatron fermentor (New Brunswick Scientific, Edison, NJ) containing 50 liters of the same yeast extract medium described above. The fermentation was continued for approximately 24 hours at 25°C at an agitation rate of 200 rpm, and an air flow of 50 liters/minute. Antibiotic production and isolation were monitored by a paper disc agar diffusion assay using *Bacillus licheniformis* (SC 9262) as the test organism.

Isolation

SQ 28,332 was isolated from fermentation broths as outlined in Fig. 2. The antibiotic was extracted into dichloromethane containing a tetraalkylammonium salt, cetyldimethylbenzylammonium chloride being effective for this purpose. Back extraction of SQ 28,332 into water was accomplished by transforming the ion-pair into the sodium salt with sodium thiocyanate. The resulting aqueous extract was desalted on Darco granular charcoal. Further purification was accomplished by a combination of adsorption, reverse-phase and ion-exchange chromatography to give SQ 28,332.

Fig. 2. Isolation of SQ 28,332.

Broth supernate

1. Ion-pair extraction into dichloromethane 0.05 M in cetyldimethylbenzylammonium chloride.
2. Back extraction into water 1 M in NaSCN.
3. Desalting on Darco charcoal, eluting with pyridine - H_2O (1: 1).
4. Chromatography on cellulose powder, eluting with CH_3CN - H_2O gradient (4: 1~1: 1).
5. Chromatography on MCI gel CHP20P, eluting with a H_2O~MeOH gradient.
6. Chromatography on BioRad AG1-X2 (OAc^-) eluting with a 0.2~2.0 M pyridine - AcOH gradient.
7. Chromatography on cellulose powder eluting with CH_3CN - H_2O gradient (9: 1~1: 1).

SQ 28,332

Physicochemical Properties of SQ 28,332

SQ 28,332 is a water soluble, strongly acidic antibiotic having a mobility of 0.42 at pH 2.3 and pH 7.0 on paper electrophoresis relative to vitamin B_{12} (0.00) and *p*-nitrobenzenesulfonate anion (1.00). SQ 28,332 has an Rf of 0.30 on silica gel, EtOAc - BuOH - AcOH - H_2O (1: 1: 1: 1). Color reactions are as follows: positive in RYDON-SMITH and sodium periodate - silver nitrate tests and negative in the ninhydrin test. SQ 28,332 has an optical rotation of $[\alpha]_D^{25}$ −9.5° (*c* 0.21, H_2O).

The molecular weight and empirical formula were determined by fast atom bombardment (FAB)[11] mass spectrometry. Both positive and negative ion spectra led to the conclusion that the molecular weight, as the sodium salt, was 548. The exact mass observed for $C_{16}H_{26}N_6O_{12}SNa$ (M+H) was 549.127 (theory 549.122). The UV spectrum of SQ 28,332 showed only end absorption and the IR spectrum in KBr exhibited bands at 1760 (β-lactam carbonyl), 1715 (carbamate), 1650 (secondary amide) and 1250 and 1030 cm^{-1} (sulfamic acid).

Hydrolysis of SQ 28,332 in 6 N HCl at 110°C for 15 hours gave a mixture that was separated by ion-exchange chromatography into D-glyceric acid, D-alanine, glycine, *N*-methyl-L-serine[12] and L-2,3-diaminopropionic acid. The configurations of alanine and diaminopropionic acid were determined as the *N*-pentafluoropropionyl isopropyl esters,[13] by gas chromatography using a chiral column.*[14] By peak enhancement with authentic samples, *N*-pentafluoropropionylalanine isopropyl ester and *N*-pentafluoropropionyldiaminopropionic acid isopropyl ester from the hydrolysis were shown to be D and L, respectively. The configuration of glyceric acid was determined by comparison of its optical rotation with authentic D-glyceric acid. The configuration of *N*-methylserine, as the 2,3,4,5-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate[15] (GITC) derivative, was determined by HPLC using a Waters C_{18}-μBondapak column in a Waters Z-module. By peak enhancement with authentic *N*-methyl-L-serine[12] GITC derivative, the configuration of *N*-methylserine was shown to be L. This assignment was also confirmed by comparison of its optical rotation with that of authentic *N*-methyl-L-serine.

The ^1H NMR data for SQ 28,332 (structure **3**) is given in Table 1. The β-lactam protons at C-3

Table 1. ^1H NMR data for SQ 28,332 in D_2O and DMSO-d_6 and Ac-SQ 28,332 in D_2O.

Position number	D_2O		Position number	DMSO-d_6[d]
	SQ 28,332 δ (J Hz)[a]	Ac-SQ 28,332 δ (J Hz)[a]		SQ 28,332 δ (J Hz)[e]
3	4.80 (2.9, 6.0)	4.81 (3.3, 6.1)	5 (NH)	8.55 (7.6)[f]
4α	3.82 (5.8, 5.8)	3.84 (5.8, 5.8)		8.58 (7.6)
4β	3.60 (3.3, 5.8)	3.62 (3.0, 5.8)	9 (NH)	8.09 (8.2)[f]
7	4.25 (7.3, 7.3, 7.3)	4.2~4.55 (m)[c]		8.78 (8.2)
8	1.28 (7.3)	1.30 (7.3)	17 (NH)	7.75 (4.8, 4.8)
11	5.02 (4.8, 7.9)	5.01 (5.2, 7.9)		7.81 (4.8, 4.8)
12	4.34 (8.2, 11.9); 4.38 (5.1, 11.9)	4.2~4.55 (m)[c]	19 (OH)	5.75 (5.4) 5.80 (5.4)
14	3.00 (s)[b]	3.02 (s)[b]	20 (OH)	4.61 (6.1, 6.1)
16	4.08 (17.0); 4.18 (17.0)	4.15 (s)		4.61 (6.1, 6.1)
19	4.22 (m)	5.34 (3.7, 3.9)	21 (NH_2)	6.45~6.75 (br. s)
20	3.71 (4.8, 11.9); 3.74 (3.6, 11.9)	4.2~4.55 (m)[c]		

[a] ppm downfield from TMS using HDO (4.73 ppm) as internal standard.
[b] Minor peaks at δ 2.78 and 2.79 (C-14H) are attributed to the rotational isomer for SQ 28,332 and Ac-SQ 28,332, respectively.
[c] Decoupling experiments indicated that the C-19 proton (δ 5.34) was coupled to the C-20 protons (δ 4.32).
[d] Chemical shifts of exchangeable protons are given; the rotational isomers existed in a 1 : 1 ratio in DMSO-d_6.
[e] ppm downfield from TMS using DMSO (2.49 ppm) as internal standard.
[f] Assignments may be interchanged.

* Column: Altech Associates 25 m glass WCOT column with RSL-007 stationary phase.

(δ 4.80, dd, 2.9, 6.0 Hz) and C-4 (α, δ 3.82, tr, 5.8 Hz; β, δ 3.60, dd, 3.3, 5.8 Hz) have chemical shifts and coupling constants that are in accord with those reported for the corresponding positions in other monocyclic β-lactams.[7,18] Decoupling experiments indicated that the alanyl methyl protons (δ 1.28) were coupled to the alanyl methine proton (δ 4.25), the C-11 proton (δ 5.02) was coupled to the C-12 protons (δ 4.34 and 4.38) and the C-19 proton (δ 4.22) was coupled to the C-20 protons (δ 3.71 and 3.74).

Acetylation of SQ 28,332 (pyridine - acetic anhydride, room temperature, 2 hours) gave a diacetyl derivative. The ^{1}H NMR of diacetyl-SQ 28,332 indicated that the C-20 protons had shifted from δ 3.71 and 3.74 to 4.32 and the C-19 proton had shifted from δ 4.22 to 5.34, which are in accord with the chemical shift differences observed in primary and secondary alcohols on acylation.[17]

The ^{1}H NMR spectrum of SQ 28,332 in DMSO-d_6 (20°C) was complex due to the existence of rotational isomers (*ca.* 1: 1 ratio). The chemical shifts of the exchangeable protons are listed in Table 1. The C-11 proton (δ 4.62 and 5.12) and C-14 protons (δ 2.72 and 2.92) showed the greatest difference in the chemical shift for the two rotational isomers as expected for a mixture of rotamers at the tertiary amide. A temperature-dependent NMR experiment indicated that the rotational isomers persist on the NMR time scale at 60°C, above which the antibiotic is unstable.

The ^{13}C NMR data is presented in Table 2. The multiplicities of the carbon were determined using the INEPT[19] technique. The minor peaks observed for glycyl, *N*-methylserinyl and alanyl carbons are attributed to the rotational isomer. Corresponding minor peaks for glyceryl and β-lactam carbons were not observed implying greater distance from the tertiary amide linkage and consistent with the sequence of amino acids shown (Fig. 1) for SQ 28,332. This contiguity is further substantiated by a difference NOE experiment. Irradiation of the C-14 protons (δ 3.00) showed a strong NOE for the C-16 protons (δ 4.08 and 4.18), the C-11 proton (δ 5.02) and the C-12 protons (δ 4.34 and 4.38).

The final proof of the sequence of side chain residues was determined by FAB mass spectrometry. Under a variety of conditions SQ 28,332 did not give sequence ion fragmentation that often results from the cleavage of peptide bonds.[19] The major fragments observed were the ones resulting from the loss of SO_3,[20] NH_3, NH_2CO_2H, CH_2O *etc.* However, partial hydrolysis of SQ 28,332 (Dowex 50

Table 2. ^{13}C NMR data for SQ 28,332 in D_2O.

Carbon	Chemical shifts (δ)[a]	Carbon	Chemical shifts (δ)[a]
2	166.4	12	64.3[b]
3	56.1[d]	14	33.7[b]
4	48.8	15	170.2[b]
6	172.0[c]	16	42.2[b]
7	50.6[b]	18	175.3[c]
8	17.5	19	73.3
10	175.2[c]	20	62.3
11	58.8[b,d]	21	159.0

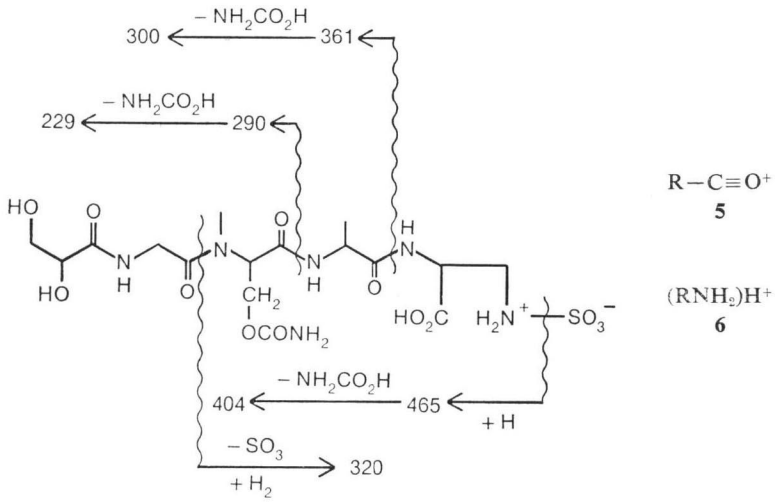
[a] ppm downfield from TMS, using dioxane (67.6 ppm) as internal standard.
[b] Minor peaks at δ 31.3 (C-14), 41.8 (C-16), 50.7 (C-7), 59.4 (C-11), 62.1 (C-12) and 169.1 (C-15) are attributed to the existence of a rotational isomer.
[c,d] Assignments may be interchanged.

Table 3. Antibacterial activity (MIC) of SQ 28,332.

Organism	SC No.	SQ 28,332 (μg/ml)
Staphyloccus aureus	1276	12.5
S. aureus	2400	25.0
Streptococcus agalactiae	9287	6.3
Escherichia coli	8294	>50
E. coli	10857	50
Klebsiella aerogenes	10440	>50
Proteus mirabilis	3855	>50
P. vulgaris	9416	>50
Enterobacter cloacae	8236	>50
Serratia marcescens	9783	>50
Pseudomonas aeruginosa	9545	>50
P. aeruginosa	8329	>50

Agar dilution: 10^4 cfu.

Fig. 3. Mass spectrometric fragmentation of compound **4**.



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W-X2, H^+) resulted in a β-lactam hydrolyzed compound **4**, that gave a small peak at *m/z* 545 (M+H) and a large peak at 465 (M+H−SO_3) in the positive-ion FAB mass spectrum. A mass-analyzed ion kinetic energy (MIKE) spectrum[21] of the fragment peak *m/z* 465 resulted in two kinds of sequence ions, acylium ions **5** and the protonated amine **6** (Fig. 3). The sequence ions of type **5** occur at *m/z* 290 (229) and 361 (300). These establish the sequence X-alanyldiaminopropionic acid (Fig. 3). A sequence ion of type **6** occurs at *m/z* 320 establishing the sequence Y-*N*-methylserinylalanyldiaminopropionic acid.

Biological Properties

SQ 28,332 exhibits weak antibacterial activity (Table 3). Hydrolysis studies of SQ 28,332 using TEM-2, K1 and P99 β-lactamase were performed spectrophotometrically at 267 nm as described previously.[22] SQ 28,332 was slightly hydrolyzed by K1 β-lactamase and not hydrolyzed appreciably by P99 or TEM-2 β-lactamases. SQ 28,332 is a non-progressive weak inhibitor of P99 cephalosporinase with an I_{50} value of 190 μg/ml. No inhibition of TEM-2 and K1 broad spectrum β-lactamase was observed at 100 μg/ml (in all studies 1.0 mM cephaloridine was used as the substrate).

Experimental

NMR spectra were determined on Varian Associates model XL-100-15 and Joel Ltd. GX400 spectrometers. Infrared spectra were recorded on a Perkin-Elmer model 621 spectrometer. Rotations were measured on a Perkin-Elmer model 141 polarimeter. Mass spectra were determined on VG Analytical Ltd. model ZAB 1F spectrometer.

Isolation of SQ 28,332

At harvest, the cells from a 50-liter tank fermentation of *Flexibacter* sp. SC 12,681 were separated by centrifugation. The supernate (pH 7.5) was extracted with 0.05 M cetyldimethylbenzylammonium chloride in CH_2Cl_2 (17 liters). The layers were separated and the CH_2Cl_2 layer was extracted with 1 M aqueous NaSCN (6 liters). The aqueous NaSCN layer was loaded onto a 630-ml column of Darco granular charcoal. The column was washed with 5 liters of water and the activity was eluted with pyridine - H_2O (1: 1, 5 liters) to give 7.2 g of crude SQ 28,332. The crude SQ 28,332 was chromatographed on a 450-ml cellulose column (Whatman CC-31) eluting with CH_3CN - H_2O, (4: 1, 2 liters), to give 3 g of crude SQ 28,332. This was further purified by reverse-phase chromatography on a 400-ml column of

MCI gel CHP20P (75~100 μ, Mitsubishi Chemical Ind.) eluting with water (400 ml) followed by a linear gradient of H_2O~MeOH (2.4 liters) to give 1.14 g of a solid. This solid was chromatographed on a 150-ml column of BioRad AG1-X2, OAc^-, 200~400 mesh, eluting with a linear gradient of 0.2~2.0 M pyridine - AcOH (pH 5.1, 1.2 liters) to give 101.0 mg of crude SQ 28,332. This was dissolved in H_2O, adjusted to pH 5 with 1 N NaOH and chromatographed on a 60-ml column of cellulose (Whatman CC-31) eluting with a linear gradient of CH_3CN - H_2O, 9: 1~1: 1 (500 ml) to give 30.5 mg of SQ 28,332 (sodium salt, Fig. 1).

Hydrolysis of SQ 28,332

A solution of 16.7 mg of SQ 28,332 in 1 ml of 6 N HCl was heated at 100°C for 15 hours and then concentrated *in vacuo*. The residue was chromatographed on a 2.5×18 cm column of BioRad AG50W-X2 resin (200~400 mesh, pyridinium form) eluting with a linear gradient prepared from 5% AcOH and 2 M pyridinium acetate, giving glyceric acid, a mixture of alanine and glycine, *N*-methylserine and diaminopropionic acid. The glyceric acid was converted to its potassium salt by passing a solution of glyceric acid through a column of Dowex 50W-X2, K^+, 200~400 mesh. The optical rotation of glyceric acid potassium salt was, $[\alpha]_D^{25}$ +11.2° (*c* 0.62, H_2O). The optical rotation of *N*-methylserine was, $[\alpha]_D^{25}$ +12.0° (*c* 0.2, 6 N HCl). The rotations of authentic D-glyceric acid potassium salt and *N*-methyl-L-serine under these conditions were +14.0° and +12.5°, respectively. The NMR spectra of the hydrolysis products were identical to those of authentic material.

Acetylation of SQ 28,332

A solution of SQ 28,332 (2 mg) in pyridine (0.5 ml) and acetic anhydride (0.5 ml) was stirred at room temperature for 3 hours and then concentrated *in vacuo*. The crude material was purified on a MCI gel CHP20P (75~100 μ, 1.1×20 cm) column eluting with a linear gradient of H_2O~MeOH (200 ml) to give acetyl-SQ 28,332 (0.7 mg) in 30% yield: FAB-MS (positive-ion mode) 655 $(M+Na)^+$ and 633 $(M+H)^+$.

Partial Hydrolysis of SQ 28,332

A solution of SQ 28,332 (3 mg) in water (0.2 ml) was passed through a 1-ml column of Dowex 50W-X2, H^+, 200~400 mesh and the column was washed with 2.4 ml of water. The aqueous solution was concentrated to dryness *in vacuo* to give partially hydrolyzed product **4** in quantitative yield. The FAB-MS is described in the text.

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