# TWO NEW MONOBACTAM ANTIBIOTICS PRODUCED BY A *FLEXIBACTER* SP.

## I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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(Received for publication June 27, 1983)

Two new β-lactam antibiotics, namely SQ 28,502 and SQ 28,503, have been isolated from fermentations of a *Flexibacter* sp. They are demethoxy monobactams with oligopeptide side chains and have molecular weights of 1,462 and 1,446, respectively. These β-lactams show a high degree of stability to a variety of β-lactamases and act as potent irreversible inactivators of P99 β-lactamase from *Enterobacter cloacae*. They exhibit weak antibacterial activity.

Several β-lactam antibiotics produced by bacteria have recently been reported.[1] These compounds include the monobactams,[2,3] a carbapenem[4] and a cephalosporin.[5] During our continuing screening program developed to detect β-lactam antibiotics from bacteria, we have isolated two demethoxy monobactams from strains of *Flexibacter* sp.

These new monobactams, namely SQ 28,502 and SQ 28,503, both of which contain oligopeptide side chains, are described herein. This paper gives a brief description of the producing strain and the fermentation conditions leading to the isolation and characterization of these compounds. The biological properties of these antibiotics are also presented.

### Taxonomy

The monobactam-producing strains of Flexibacter were isolated infrequently from garden soil, leaf litter and water samples when plated onto standard isolation media used for the isolation of a wide variety of bacteria. The producing strains were isolated most frequently from water samples when plated onto colloidal chitin agar,[6,7] modified by eliminating $MnCl_2$ and adding yeast extract, 1.0 g/liter. Several members of producing, as well as non-producing, strains of Flexibacter were readily isolated from a wide variety of soil and water samples when plated onto either PMA II[8] or skim milk - acetate agar.[8] All producing cultures were isolated from fresh samples collected locally in New Jersey. *Flexibacter* sp. SC 11479, producing SQ 28,502 and SQ 28,503, has been deposited with the American Type Culture Collection (ATCC) under the accession number of ATCC 35103.

### Morphology

*Flexibacter* sp. SC 11479 is a Gram-negative bacillus. It grows as long slender rods and exhibits a gliding motility. Sheaths are absent, as are any signs of fruiting bodies. *Flexibacter* sp. SC 11479 metabolizes carbohydrates oxidatively, utilizing as a sole carbon source the following compounds: glucose, arabinose and galactose. Glycerol, xylose, fructose and lactose are not utilized. The organism is non-cellulolytic. The following test responses were positive: hydrolysis of aesculin and casein, production of $H_2S$ (lead acetate method) and catalase. Negative tests were obtained for urease. The

organism produces a carotenoid pigment which exhibits characteristic chromophoric peaks at 410, 428 and 450 nm in hexane. The molar percentage of guanine and cytosine from the DNA of this organism is 42.

Following the classification of LEWIN,[9] this organism is characterized as a Flexibacter. Assignment of species designation was not made as too few standard strains were available.

## Fermentation

The fermentation was initiated by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks, each containing 100 ml of the following sterilized medium: oatmeal 2.0% and tomato paste 2.0% in distilled water. The pH was adjusted to 7 and the flasks were incubated at 20°C on a rotary shaker (300 rpm; 5-cm stroke) for 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, New Jersey) containing 50 liters of the same oatmeal - tomato paste medium described above. The fermentation was continued for 22 ~ 24 hours at 20°C at an agitation rate of 200 rpm and an air flow of 50 liters/minute. Antibiotic production was determined by a paper disc diffusion assay using *Bacillus licheniformis* (SC 9262) as the test organism.

## Isolation

The isolation scheme is outlined in Fig. 1. The two antibiotics were separated by a combination of ion-exchange and reverse phase chromatography. Final separation of the two components was achieved by chromatography on MCI gel CHP20P using a gradient of $CH_3OH$ - $CH_3CN$ in pH 2.3 phosphate buffer. Phosphate salts were removed by desalting on CHP20P and the antibiotics were eluted with $CH_3CN$ - $H_2O$ - AcOH (50: 50: 1). Components SQ 28,502 ($Na^+$ salt) and SQ 28,503 ($Na^+$ salt) were obtained as white amorphous powders upon freeze drying.

## Characterization and Spectroscopic Properties

Components SQ 28,502 and SQ 28,503 have Rf values of 0.65 and 0.55, respectively, on OPTI-UP $C_{12}$ (Fluka) reverse phase chromatography plates when developed with $CH_3CN$ - $CH_3OH$ - 1% AcOH (5: 1: 14). Positive reactions to RYDON, FOLIN, ninhydrin and phenanthrenequinone reagents were observed. Acid hydrolysates (6 N HCl, 17 hours, 107°C, $N_2$) of both SQ 28,502 and SQ 28,503 were analyzed by Stein-Moore analysis and GC-MS using the corresponding *N*-trifluoroacetyl *n*-butyl ester derivatives. The following amino acids were observed in both compounds: glycine, serine, isoleucine, methionine, arginine, glutamic acid, 2,3-diaminopropionic acid and an unidentified amino acid containing a *p*-hydroxy aromatic nucleus. Sulfate ion was detected in the acid hydrolysate using ion chromatography.

The electrophoretic mobilities for SQ 28,502 and SQ 28,503 are shown in Table 1. The compounds do not possess the typical strongly acidic character (electrophoretic mobility at low pH) normally associated with the monobactams isolated to date.[10,13] This is explained by the presence of arginine in the oligopeptide side chain; the positively-charged guanido function acts as a counter ion to the $-SO_3^-$ group.

The mass spectra of both antibiotics were obtained by the fast atom bombardment (FAB) technique. For SQ 28,502, peaks at *m/z* 1,463 $(M+H)^+$ and *m/z* 1,485 $(M+Na)^+$ were observed corresponding to a molecular weight of 1,462. For SQ 28,503 peaks at *m/z* 1,447 $(M+H)^+$ and *m/z* 1,469 $(M+Na)^+$ were observed indicating a molecular weight of 1,446.

Table 1. Electrophoretic mobilities of SQ 28,502 and SQ 28,503.

| Electrolyte | pH | Mobility* | |
|---|---|---|---|
| | | SQ 28,502 | SQ 28,503 |
| $HCO_2H$ - $CH_3COOH$ - $H_2O$ (1:3:36) | 2.0 | −0.30 | −0.20 |
| 0.05 M Sodium dihydrogen phosphate | 4.5 | −0.15 | −0.10 |
| Sodium 0.05 M phosphate | 7.0 | 0.00 | 0.00 |
| Sodium 0.05 M carbonate - bicarbonate | 9.2 | +0.15 | +0.15 |

* On Whatman Grade 2 paper; 12 V/cm, 1 hour; mobilities relative to vitamin $B_{12}$ (0.00) and *p*-nitrobenzenesulfonate (1.00).

The IR spectra (KBr) of both SQ 28,502 and SQ 28,503 have an absorption band at 1760 $cm^{-1}$ indicative of a $\beta$-lactam carbonyl. Peaks at 3400 (NH/OH) and 1660 (amide) $cm^{-1}$ are present. Evident, though poorly resolved, are peaks in the 1300~1200 $cm^{-1}$ region and at 1045 $cm^{-1}$, indicating the presence of an $-SO_3^-$ group. Further characterization of the SO stretching vibration region that allowed identification of the 2-oxoazetidine-1-sulfonate moiety of the monobactams was accomplished in aqueous solutions; these results are presented in the following communication.[11)]

Fig. 1. Isolation of SQ 28,502 and SQ 28,503.

Broth supernate, adjust to pH 3

1. Adsorb on Dowex 50WX2 ($H^+$), elute with aq. 2 M NaCl - *iso*-PrOH - AcOH (700: 300: 1).
2. EtOAc extraction, aq. layer retained.
3. Chromatography on CHP20P (75~150 $\mu$) at pH 3 eluting with $H_2O$ - $CH_3CN$ gradient.
4. Chromatography on SP-Sephadex C-25 ($Na^+$) at pH 3, eluting with a 0~0.5 M NaCl gradient.
5. Chromatography on CHP20P (75~150 $\mu$) at pH 3, eluting with a 0~30% $CH_3OH$ - $CH_3CN$ (1:2) gradient.
6. Chromatography on Sephadex G-25 eluting with 1% aq. AcOH.
7. Chromatography on CHP20P (37~75 $\mu$), eluting with a 0 to 20% $CH_3OH$ - $CH_3CN$ (4:1) gradient in sodium 0.05 M phosphate, pH 2.3.

Desalt → SQ 28,502 | Desalt → SQ 28,503

The location of the $-SO_3^-$ group in both SQ 28,502 and SQ 28,503 was elucidated by acid hydrolysis in 2 N HCl, converting the monobactam to the ring opened secondary sulfamic acid. Treatment of this acid hydrolysate with $BaCl_2$/2 N HCl and $NaNO_2$[12)] caused precipitation of $BaSO_4$. In the absence of prior acid hydrolysis of the demethoxy monobactam, addition of $BaCl_2/NaNO_2$ did not give $BaSO_4$ precipitation. Further details of this reaction are provided in the accompanying communication.[11)]

The $^1H$ NMR (400 MHz) spectrum of SQ 28,502 is presented in Fig. 2. Due to the complexity of the signals in the region of $\delta$ 3.0 to $\delta$ 5.0, the chemical shifts of the demethoxy monobactam were not immediately apparent. Extensive $^1H$ NMR decoupling studies gave the following information: the H-3 proton of the azetidinone resonates at $\delta$ 4.80 (in Fig. 2, this proton is hidden under the $H_2O$ peak) and is coupled to the two H-4 protons. These protons have chemical shifts of $\delta$ 3.55 (dd, $J$=3.0 and 5.8 Hz) and of $\delta$ 3.80 (t, $J$=5.8 Hz). These values are in agreement with previously reported data for a demethoxy monobactam.[13)] The $^{13}C$ NMR spectrum (Fig. 3) is typical of a peptide molecule. Seventeen carbonyl resonances are observed. The amide C=O signal at 167 ppm is assigned to the $\beta$-lactam C=O, in close agreement with the assignment for the $\beta$-lactam C=O (166.5 ppm) for the demethoxy monobactam SQ 26,770.[13)]

## Biological Properties

Both SQ 28,502 and SQ 28,503 exhibit weak antimicrobial activity. The minimal inhibitory concentration values (MIC's) are presented in Table 2.

Fig. 2. $^{1}H$ NMR (400 MHz, ($D_2O/CD_3COOD$) spectrum of SQ 28,502.

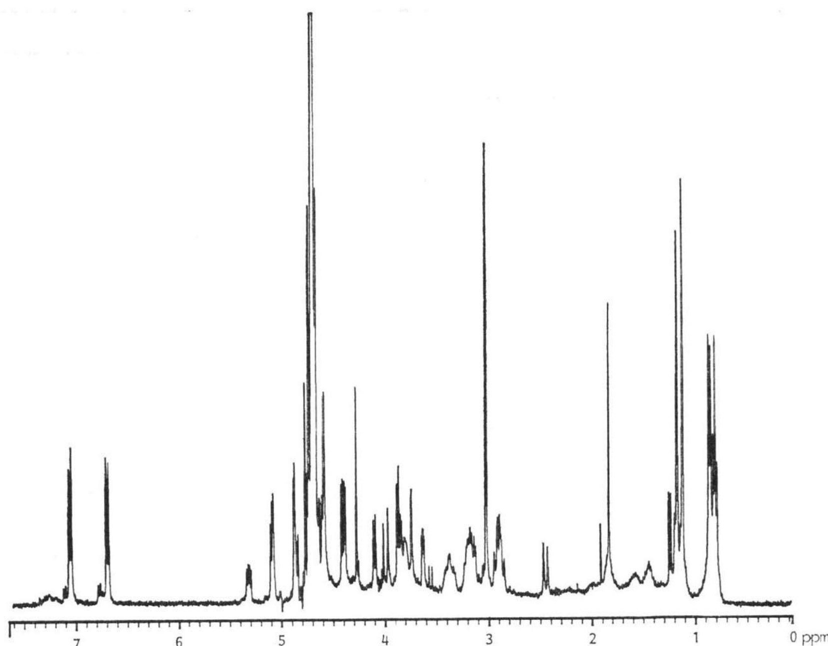
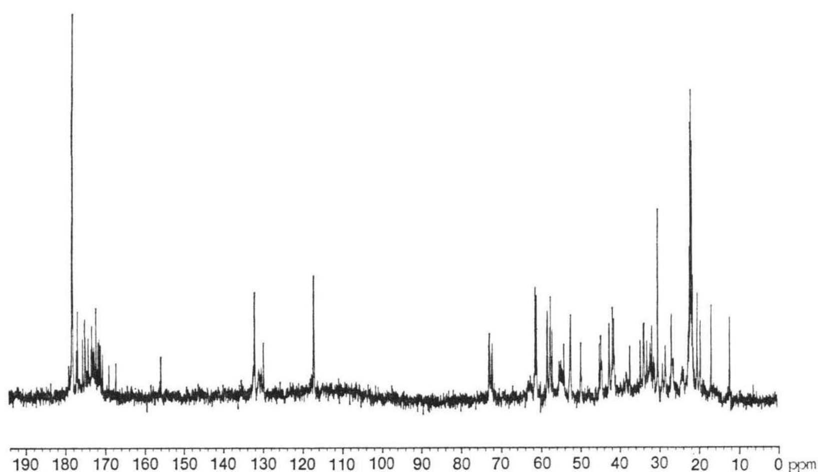



Fig. 3. $^{13}C$ NMR spectrum ($D_2O/CD_3COOD$) of SQ 28,502.




Hydrolysis studies of SQ 28,502 and SQ 28,503 using TEM-2, K1 and P99 $\beta$-lactamases were performed spectrophotometrically at 267 nm as described previously.[14] Relative rates of hydrolysis were $<0.02\%$ of those observed using cephaloridine as a reference substrate. It should be noted that under these same conditions another new naturally-occurring demethoxy monobactam containing a peptide side chain (SQ 28,332, MW 548, P. SINGH, unpublished results) was hydrolyzed slightly. However, the demethoxy monobactam SQ 26,396 bearing a simple *N*-acetyl side chain in the 3-position was readily hydrolyzed by TEM-2 and K1 $\beta$-lactamases and slowly hydrolyzed by P99 $\beta$-lactamase.[8] These results indicate that the oligopeptide side chain of SQ 28,502 and SQ 28,503 clearly contributed to conferring stability to $\beta$-lactamases.

Table 2. Antimicrobial activity of SQ 28,502 and SQ 28,503.

| Test organism | SC# | MIC* (μg/ml) | |
|---|---|---|---|
| | | SQ 28,502 | SQ 28,503 |
| *Staphylococcus aureus* 209P | 1276 | >100 | 50 |
| *Streptococcus agalactiae* | 9287 | 25 | 6.3 |
| *Micrococcus luteus* | 2495 | 25 | 12.5 |
| *Escherichia coli* | 8294 | 100 | 25 |
| *E. coli* | 10857 | 25 | 6.3 |
| *Proteus mirabilis* | 3855 | >100 | >100 |
| *Salmonella typhosa* | 1195 | 50 | 12.5 |
| *Serratia marcescens* | 9783 | >100 | >100 |
| *Pseudomonas aeruginosa* | 9545 | >100 | 100 |

* Minimum inhibitory concentrations were determined by a two-fold dilution method on DST agar (Oxoid). Final inoculum level was $10^4$ cfu.

Table 3. Inhibition of β-lactamases by SQ 28,502 and SQ 28,503.

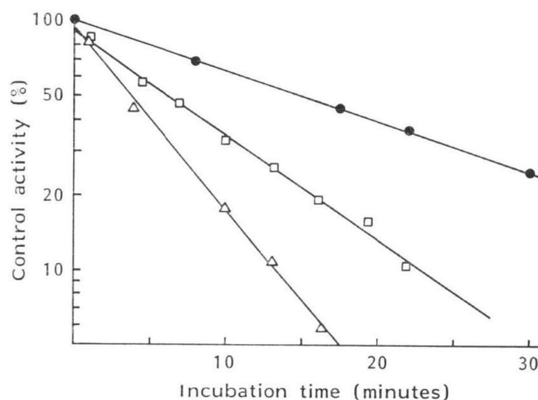
| Inhibitor | Preincubation time (minutes) | $I_{50}$ (μM) for β-lactamase type | | |
|---|---|---|---|---|
| | | TEM-2 | K1 | P99 |
| SQ 28,502 | 0 | >150 | >150 | 200 |
| | 5 | >150 | >150 | 0.07 |
| SQ 28,503 | 0 | >150 | >150 | 220 |
| | 5 | >150 | >150 | 0.7 |

$I_{50}$ values were determined in 0.1 M phosphate buffer, pH 7.0 at 25°C using 1.0 mM cephaloridine as substrate. β-Lactamases were purified as described previously.[14] Final enzyme concentrations were 26 nM for TEM-2, <30 nM for K1 and 5 nM for P99 β-lactamase. Assays not involving preincubation were initiated by addition of enzyme (1~5 μl) to substrate and inhibitor (or buffer). For other studies enzyme and inhibitor or buffer were preincubated 5.0 minutes in volumes of 22~102 μl (25°C) before 1.0 ml substrate was added.

Neither SQ 28,502 nor SQ 28,503 inhibited TEM-2 or Kl β-lactamase at levels of 0.15 mM. However, both compounds are potent inhibitors of P99 β-lactamase, as shown in Table 3. Inhibition of the P99 enzyme was time- and concentration-dependent for both SQ 28,502 and SQ 28,503, as shown in Fig. 4. It was observed that with inhibitor (SQ 28,502) to enzyme ratios as low as 2.5 total inhibition of P99 β-lactamase could be obtained. Second order rate constants for inactivation were $3.1 \times 10^4$ liter/mol/minute for SQ 28,502 and $3.2 \times 10^2$ liter/mol/minute for SQ 28,503. When samples of totally inhibited enzyme were dialyzed overnight, no significant recovery of enzyme activity was observed. When the dialyzed samples were incubated for 30 hours at 25°C, no recovery of enzymatic activity was evident.

Fig. 4. Inactivation of P99 β-lactamase by SQ 28,502.

Homogeneous P99 β-lactamase (1.2 μM) was incubated at 25°C with 3.2 μM (●), 8.5 μM (□) or 13.8 μM (△) SQ 28,502, in a volume of 100 μl. Samples were assayed periodically by diluting 5 μl of the incubation mixture into 1.0 ml cephaloridine (1.0 mM).



Similar behavior was observed for both SQ 28,502 and SQ 28,503. Therefore, these compounds may be considered as irreversible inactivators of P99 β-lactamase. Such characteristics were not observed in previous studies involving inhibition of P99 β-lactamase with third generation cephalosporins or monobactams.[14] It should be noted that compounds such as aztreonam or latamoxef, which are also potent inhibitors of P99 β-lactamase, are eventually hydrolyzed by the enzyme with half-times of less than 7 hours.[14]

In conclusion, two novel demethoxy monobactams, SQ 28,502 and SQ 28,503, have been isolated from a bacterial culture. These monobactams do not demonstrate significant affinity for the Kl and TEM-2 broad spectrum β-lactamases. They also exhibit poor antibacterial activity. However, they are potent inactivators of the P99 cephalosporinase.

Acknowledgments

The authors wish to thank R. Hugill for assistance with the fermentation, S. Fisher for assistance with the biological assays, Dr. S. K. Tanaka for obtaining MIC values, Dr. S. Unger for obtaining mass spectra and Dr. M. Porubcan and A. Kahle for $^1$H and $^{13}$C NMR data.

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