NOVEL POTENTIATORS OF β-LACTAM ANTIBIOTICS

ISOLATION OF SQ28,504 AND SQ28,546 FROM *CHROMOBACTERIUM VIOLACEUM*

RAYMOND COOPER†, J. SCOTT WELLS and RICHARD B. SYKES

The Squibb Institute for Medical Research
P.O. Box 4000, Princeton, New Jersey 08540, USA

(Received for publication December 11, 1984)

Two novel compounds, SQ28,504 and SQ28,546 are produced by *Chromobacterium violaceum*. These compounds enhance the antibacterial activity of β-lactam antibiotics against Gram-negative organisms. Both SQ28,504 and SQ28,546 induce morphological changes in the presence of β-lactam antibiotics. Only SQ28,546 has weak antimicrobial activity against several Gram-negative organisms.

The monobactam-producing strain[1] of *Chromobacterium violaceum* (ATCC 31532), produces two novel bacterial metabolites, SQ28,504 and SQ28,546. Both molecules have been identified as glycopeptides with molecular weights of 963 and 1,179 respectively. These compounds act synergistically with β-lactam antibiotics against Gram-negative organisms inducing unusual morphological changes. The morphological changes observed are similar to those reported for bulgecin[2], a glycopeptide (MW 591), that is co-produced with the monobactam sulfazecin.

Fermentation

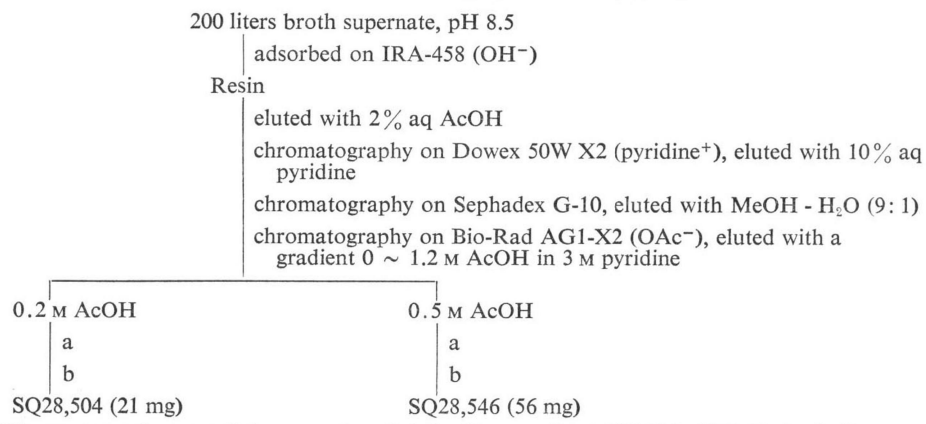
The microorganism used for the production of SQ28,504 and SQ28,546 was a strain of *C. violaceum* (ATCC 31532). The fermentation was initiated by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks, each containing 100 ml of the following sterilized medium: oatmeal 2% and tomato paste 2% in distilled water. The pH was adjusted to 7 and the flasks were incubated at 25°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 Fematron fermentor (New Brunswick Scientific, Edison, New Jersey) containing 50 liters of the following sterilized media: glucose 2.5 g, yeast extract 2.0 g, NZ Amine A 4.0 g per liter. The fermentation was continued for 24 hours at 25°C at an agitation rate of 200 rpm and an air flow of 50 liters/minute. At harvest the whole broth was centrifuged and the broth supernate was adjusted to pH 8.5 with NaOH and left standing at room temperature for 1 hour. Under these conditions the monobactam SQ28,180 that was present in the broth supernate was destroyed.

Isolation

SQ28,504 and SQ28,546 were recovered from the treated filtrate by adsorbing on Amberlite IRA-458 prepared in the OH^- form. Further purification steps leading to the isolation of SQ28,504 and SQ28,546 are presented in Scheme 1. The two components were separated on Bio-Rad AG1-X2 (OAc^-) resin using an acetate gradient in aqueous pyridine. Further purification of each component proceeded according to the steps shown in Scheme 1 to yield SQ28,504 and SQ28,546 as white amorphous solids

† Present address: Department of Antibiotics, Schering Corporation, 60 Orange Street, Bloomfield, NJ 07003, USA.

Scheme 1. Isolation of SQ28,504 and SQ28,546.



a. Chromatography on cellulose powder, eluted with a gradient CH_3CN - H_2O (9: 1 ~ 1: 1).
b. Chromatography on Bio-Rad 50WX2 (pyridine⁺), eluted with a gradient of 0 ~ 0.5 M pyridine in 1 M HCOOH.

upon lyophilization.

Characterization and Spectroscopic Properties

SQ28,504 and SQ28,546 are stable in aqueous solution between pH 2 and pH 9 at room temperature. They are water soluble, amphoteric compounds. Electrophoresis data, presented in Table 1, show that SQ28,504 and SQ28,546 are cationic at pH 2 and pH 4.5 and anionic at pH 7 and above, with isoelectric points near pH 4.5. The two components are resolved on several TLC systems (Table 2). The two compounds give positive reactions with Rydon, ninhydrin and fluoram reagents. The UV and IR spectra of SQ28,504 and SQ28,546 are virtually identical. The UV spectra showed no characteristic absorptions at wavelengths greater than 210 nm. The IR spectra, taken in KBr, exhibited bands at 3400 broad (NH/OH), 1660 broad and 1550 broad (CONH), 1250, 1050 and 650 (S=O) cm^{-1}. The IR spectrum of SQ28,504 is shown in Fig. 1.

The molecular weight of SQ28,504 was determined by fast atom bombardment (FAB) mass spectrometry[3]. In the positive ion mode, the protonated molecular ion M+H was at *m/z* 964. In the negative ion mode the ion $(M-H)^-$ was at *m/z* 962, implying a molecular weight of 963 for SQ28,504.

Table 1. Electrophoresis of SQ28,504 and SQ28,546.

Buffer (pH)	Mobility[a]	
	SQ28,504	SQ28,546
HCO_2H - HOAC - H_2O (1: 3: 36) (2.1)	−0.25	−0.25
0.05 M Sodium dihydrogen phosphate (4.5)	−0.03	−0.03
0.05 M Sodium phosphate (7.0)	+0.30	+0.30
0.05 M Sodium carbonate (9.2)	+0.60	+0.80

[a] On Whatman No. 1 paper, 12 V/cm, 1 hour; mobilities relative to vitamin B_{12} (0.00) and *p*-nitrobenzenesulfonate anion (1.00).

Table 2. TLC properties of SQ28,504 and SQ28,546.

Phase (solvent)	Rf	
	SQ28,504	SQ28,546
Silica gel Merck (1-PrOH - conc NH_4OH, 7: 3)	0.3	0.2
Reverse phase Opti C_{12}* (0.01 M TBA in H_2O, pH 7)	0.6	0.5
Whatman 3 mm paper (MeOH - 2-PrOH - H_2O, 4: 3: 3)	0.7	0.6

* Fluka, N.Y.

Fig. 1. The IR spectrum of SQ28,504 (KBr).

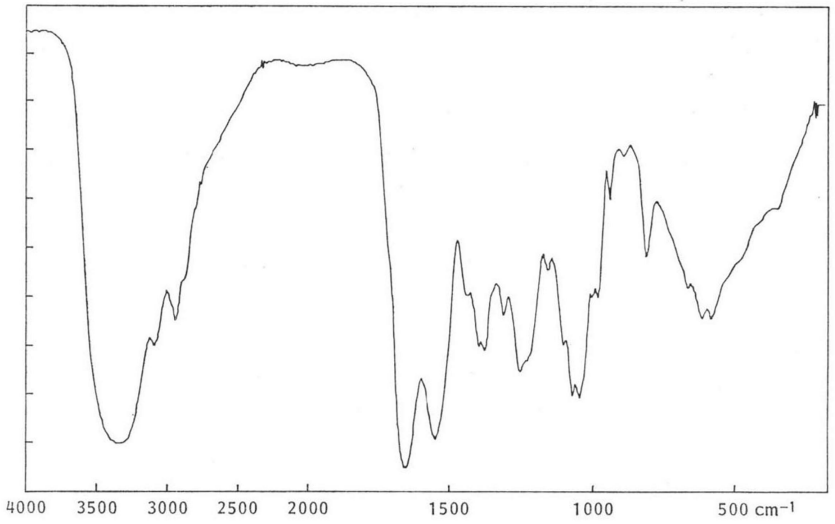
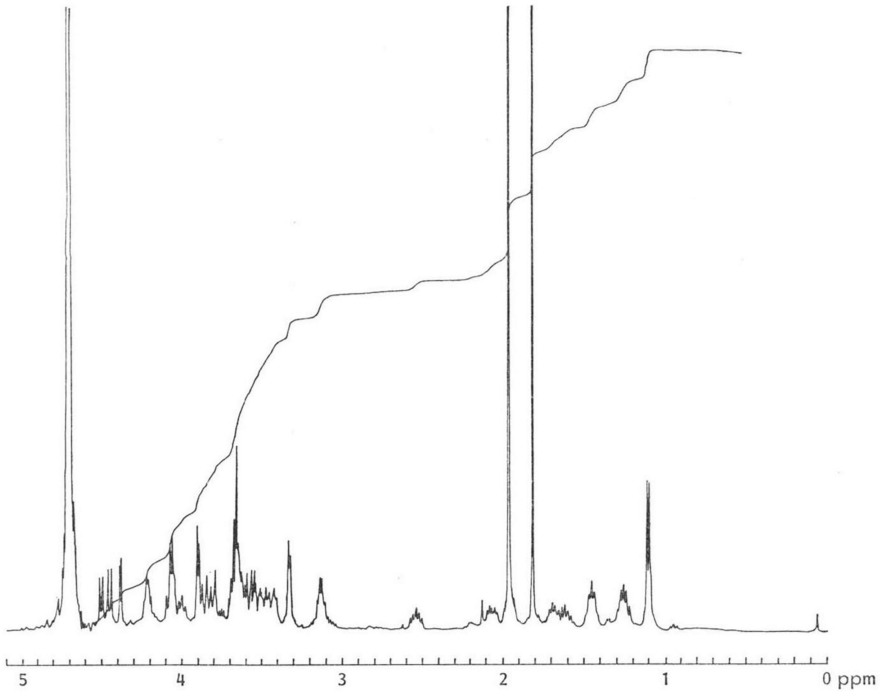


Fig. 2. 400 MHz ^{1}H NMR spectrum of SQ28,504 in D_2O.



Analysis of the acid hydrolysate of SQ28,504 (6 N HCl, 110°C, 17 hours) indicated the presence of threonine, serine and lysine in a 1: 1: 1 ratio. The configurations of threonine and serine as the 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[4] derivatives were determined by HPLC using peak enhancement with authentic samples. Both amino acids were shown to have the L-form. The GITC diastereoisomers from DL-lysine were not resolved under these conditions. Analysis of the acid

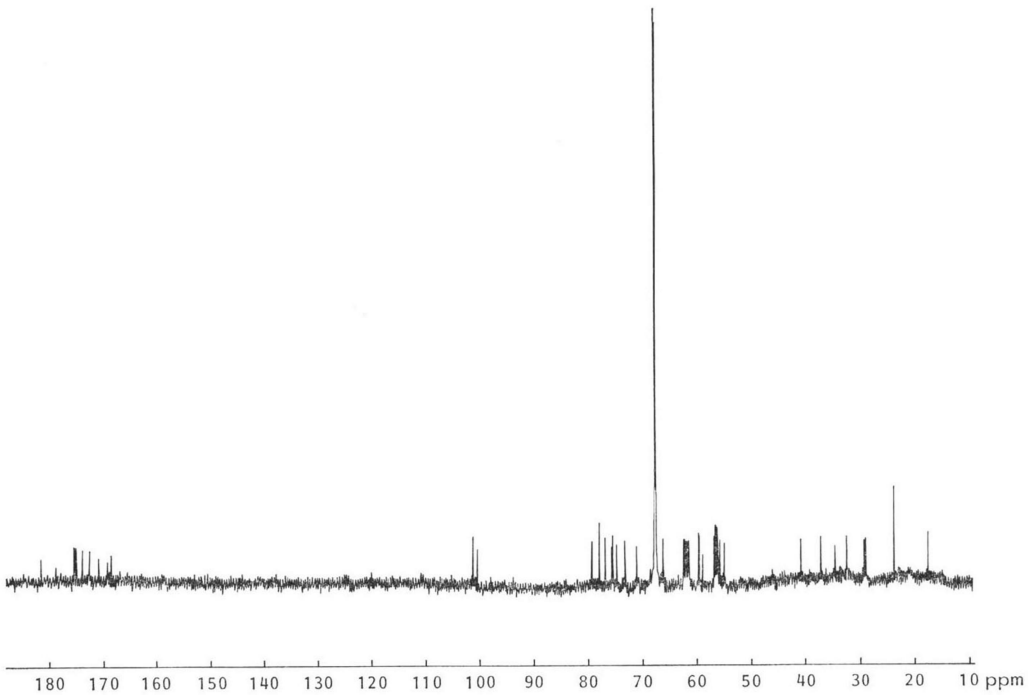
hydrolysate of SQ28,504 (2 N HCl, aqueous MeOH, 5 hours) indicated the presence of a sugar identified as glucosamine. Sulfate ion (precipitated as the Ba salt) was also present in the hydrolysate.

The ^{1}H and ^{13}C NMR spectral data for SQ28,504 indicate a glycopeptide structure. The ^{1}H NMR spectrum of SQ28,504 is shown in Fig. 2. Assignments were made with the aid of decoupling experiments. The two anomeric protons resonate at δ 4.50 (d, J=7.9 Hz) and δ 4.45 (d, J=8.6 Hz) respectively. A doublet, centered at δ 1.10 (3H, d, J=6.1 Hz) that is coupled to the proton at δ 4.20 (1H, multiplet), is assigned to the threonine methyl. A single peak at δ 1.97 (6H, s) is assigned to two equivalent *N*-acetyl methyl groups. In the ^{13}C NMR spectrum of SQ28,504 the signals at 101.38 and 100.67 ppm are assigned to the two anomeric carbon atoms. The signal at 23.43 ppm is assigned to the two *N*-acetyl methyl carbons.

FAB-mass spectral data support the presence of an SO_3 group and *N*-acetylglucosamine in the molecule of SQ28,504. Prominent fragment ions observed in the positive ion mode are as follows: m/z (M+H) 964, 884 and 681 amu (atomic mass unit). A mass analyzed ion kinetic energy (MIKE) spectrum[5] of the peak at m/z 964 resulted in the fragment ion m/z 884. This loss of 80 mass units is due to the cleavage of SO_3 from the molecule. These observations were used to characterize the monobactams, that are β-lactams possessing an SO_3 group[6]. The ion m/z 884 further fragments by loss of *N*-acetylglucosamine, resulting in the fragment ion m/z 681.

The molecular weight of SQ28,546 is 1,179, determined from FAB-mass spectral data. A protonated molecular ion M+H m/z 1,180 was observed. The addition of one more serine and a glutamic acid residue accounts for the difference in molecular weight between SQ28,546 (MW 1,179) and SQ28,504 (MW 963). Amino acid analysis of the acid hydrolysate of SQ28,546 indicated the presence of threonine, serine, lysine and glutamic acid in a ratio of 1: 2: 1: 1. Sulfate ion was also present in the hydroly-

Fig. 3. 100 MHz ^{13}C NMR spectrum of SQ28,546 in D_2O.



sate. Elemental sulfur analysis obtained on SQ28,546 was in agreement with the presence of one sulfur atom in the molecule. From elemental analysis and ^{13}C NMR data for SQ28,546 a molecular formula of $C_{43}H_{73}N_9O_{27}S$ is suggested.

The fragmentation of SQ28,546 in the FAB-mass spectrum is similar to that of SQ28,504. The loss of 80 mass units ($M+H-SO_3$) was observed for SQ28,546, resulting in the fragment ion m/z 1,100. This ion underwent further cleavage due to the loss of *N*-acetylglucosamine, resulting in the fragment ion m/z 897 amu.

The ^{13}C NMR spectrum of SQ28,546 is shown in Fig. 3. The spectrum is similar to SQ28,504 with the addition of carbon signals of an additional serine and a glutamate residue. In this ^{13}C NMR spectrum the signals at 101.25 and 100.33 ppm are assigned to the anomeric carbon atoms, indicating the presence of two sugars in the molecule.

Biological Properties

Both SQ28,504 and SQ28,546 potentiate the activity of β-lactam antibiotics against Gram-negative bacteria. Disks containing the test compounds (30 μg) were placed adjacent to each other on the surface of nutrient agar seeded with a test organism. Augmentation of the zones of inhibition of the β-lactams in the presence of the test compound was observed, such enhancement being evidence for potentiation of the antibiotic activity. This potentiating activity inhibiting the Gram-negative bacteria when combined with β-lactam antibiotics is presented in Table 3. Potentiating activity was observed with cell-wall active components *e.g.* vancomycin, but not for protein synthesis inhibitors *e.g.* gentamicin and chloramphenicol. No combination was active against *Staphylococcus aureus* or *Streptococcus faecalis*.

In the presence of β-lactams, both SQ28,504 and SQ28,546 induce morphological changes. These

Table 3. Enhancement of antibacterial activity of β-lactam antibiotics in combination with SQ28,504 and SQ28,546 (30 μg/disc).

Compound	Concentration (μg/disc)	Interaction[a]			
		Escherichia coli SC 8294[b]		*Proteus rettgeri* SC 8479[b]	
		SQ28,504	SQ28,546	SQ28,504	SQ28,546
Aztreonam	30	S	S	S	S
Azlocillin	30	—	A	—	S
Ampicillin	10	S	S	S	S
Cephalothin	30	A	A	S	S
Cefamandole	30	S	A	S	S
Cefotaxime	30	S	A	S	S
Cefoxitin	30	S	A	S	S
Benzylpenicillin	60	S	A	S	S
Piperacillin	30	S	S	S	S
Mecillinam	10	A	A	S	S
Moxalactam	30	S	S	S	S
N-Formimidoylthienamycin	30	A	A	S	S
Fosfomycin	30	S	A	S	S
Diumycin	30	S	A	S	S
Vancomycin	30	—	A	—	A
Gentamicin	30	A	A	A	A
Chloramphenicol	30	A	A	A	A

[a] A=Indifference, S=enhancement of activity, — not tested,
[b] Culture collection of E.R. Squibb & Sons, Inc.

morphological effects were assessed from either agar scrapings or broth samples. These effects on *Escherichia coli* SC8294 appear to be similar to those described for bulgecin[2]. Bulge formation (central swelling) in the elongated cells was observed and culture growth was inhibited. This effect of central swelling was observed with SQ28,546 at concentrations of ≥ 6.3 μg/ml.

Table 4. Antibacterial activity of SQ28,546.

Organism	MIC (μg/ml)
Staphylococcus aureus SC 1276[a]	>100
Proteus mirabilis SC 3855	25
P. rettgeri SC 8479	12.5
P. vulgaris SC 9416	12.5
Salmonella typhosa SC 1195	25

[a] Culture collection of E.R. Squibb & Sons, Inc.

SQ28,546 has weak antimicrobial activity against several Gram-negative organisms. The results, obtained by conventional agar-dilution assays, are presented in Table 4.

Conclusion

Two novel sulfonated glycopeptide metabolites, SQ28,504 and SQ28,546 were isolated and characterized. These compounds induce unusual morphological changes in the presence of β-lactam antibiotics and enhance the antibacterial activity of β-lactams against Gram-negative bacteria.

Acknowledgments

The authors wish to thank Mr. P. A. PRINCIPE and Mr. R. HUGILL for fermentation broths, Dr. S. K. TANAKA for obtaining MIC values and for the potentiation studies, Dr. S. UNGER for obtaining mass spectral data, and Dr. M. PORUBCAN and Ms. A. KAHLE for NMR data. Helpful discussions with Dr. W. L. PARKER are gratefully acknowledged.

References

1) WELLS, J. S.; W. H. TREJO, P. A. PRINCIPE, K. BUSH, N. GEORGOPAPADAKOU, D. P. BONNER & R. B. SYKES: SQ 26,180, a novel monobactam. I. Taxonomy, fermentation and biological properties. J. Antibiotics 35: 184~188, 1982
2) IMADA, A.; K. KINTAKA, M. NAKAO & S. SHINAGAWA: Bulgecin, a bacterial metabolite which in concert with β-lactam antibiotics causes bulge formation. J. Antibiotics 35: 1400~1403, 1982
3) BARBER, M.; R. S. BORDOLI, R. D. SEDGWICK & A. N. TYLER: Fast atom bombardment (F.A.B.): A new source for mass spectrometry. J. Chem. Soc., Chem. Commun. 1981: 325~327, 1981
4) KINOSHITA, T.; Y. KASAHARA & N. NIMURA: Reversed phase high-performance liquid chromatographic resolution of non-esterified enantiomeric amino acids by derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate and 2,3,4-tri-*O*-acetyl-β-D-arabino pyranosyl isothiocyanate. J. Chromatogr. 210: 77~81, 1981
5) COOKS, R. G.; J. H. BEYNON, R. M. CAPRIOLIC & G. R. LESTER: Metastable Ions. Elesevier, New York, 1973
6) COOPER, R.: Chemical and spectroscopic characterization of monobactams. J. Antibiotics 36: 1258~1262, 1983