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A Potent Seryl tRNA Synthetase Inhibitor SB-217452 Isolated from a *Streptomyces* species

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A potent inhibitor of seryl tRNA synthetase, designated SB-217452 has been isolated from *Streptomyces* sp. ATCC 700974. The fermentation, isolation, structure elucidation and some properties are described. SB-217452 showed inhibitory activity against both *Staphylococcus aureus* and rat seryl tRNA synthetases, with similar IC_{50} values of approximately 8 nM. The inhibitor is the serine linked nucleoside moiety of the antibiotic albomycin δ_2. In contrast to albomycin δ_2, SB-217452 showed only very weak antibacterial activity against a limited range of microorganisms. The compound has not been previously reported as a naturally occurring metabolite. In addition to SB-217452, albomycin δ_2 Fe^{3+} complex and the novel Al^{3+} complex were isolated from the fermentation. These complexes had no seryl tRNA synthetase inhibitory activity.

Aminoacyl-tRNA synthetases catalyze one of the most critical steps in protein biosynthesis. They attach one of twenty amino acids to one of about sixty tRNAs. Their specificities with regard to amino acids and tRNAs are responsible for the accurate primary structures of proteins. The enzymes are essential for cell viability, and are potential targets for antibacterial agents.

A program of screening for inhibitors of bacterial tRNA synthetases identified an inhibitor of seryl tRNA synthetase (SRS) produced by a Streptomycete culture ATCC 700974.

Here we report the production, isolation, identification, structure elucidation and biological properties of the novel metabolite, which proved to be related to albomycin δ_2. A novel metal complex of albomycin δ_2 was also isolated.

Materials and Methods

Assay Procedures for the Detection of SB-217452

The isolation of SB-217452 was monitored at all stages using the seryl tRNA synthetase inhibition assay.

The aminoacylation assay measures the enzyme mediated incorporation of radiolabelled serine into seryl tRNA. The *Staphyloccocus aureus* SRS enzyme used in the assay was from a crude enzyme preparation. The procedures used for both preparation of the crude bacterial enzyme, the rat enzyme and the aminoacylation assay are based on previously described methods.[1~4]

The enzyme and 20 μl of the test sample were pre-incubated for 5 minutes at 37°C. A pre-mixed solution (100 μl) of tRNA, radiolabelled amino acid and co-factors was added. This reaction mix comprised the following constituents: L[U-^{14}C] serine 3.125 μM [Amersham, specific activity 5.99 GBq/mmol]; bulk tRNA from *E. coli* strain MRE 600 0.5 mg; ATP 0.38 mg; $Mg(OAc)_2 \cdot 4H_2O$ 17.5 mM; dithiothreitol 3.5 mM; KCl 17.5 mM and Trizma [Sigma] pre-set buffer (pH 8.5) 50 mM.

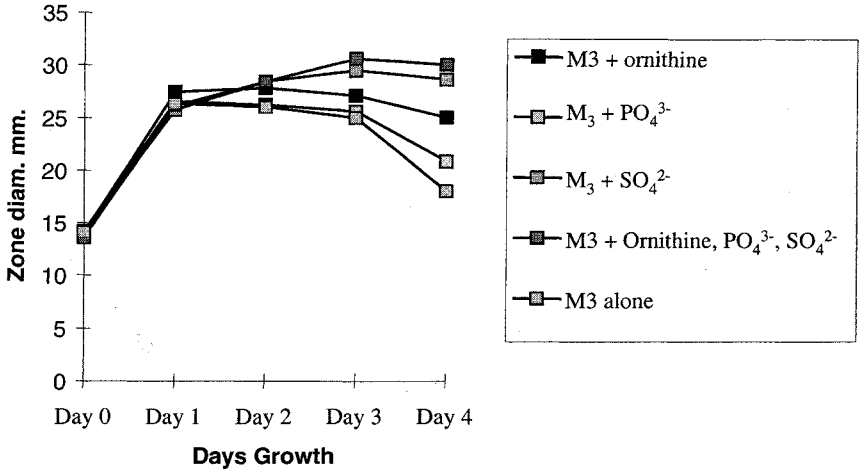
HPLC analysis was also performed, using a Waters Spherisorb ODS II 10 μm column (4.6×250 mm), mobile phase deionised water, flow rate 2 ml/minute, and monitoring UV absorbance at 225 nm. SB-217452 had a retention time of 5.0 minutes.

Assay Procedure for the Albomycin δ_2 Fe^{3+} and Al^{3+} Complexes

The isolation of the two complexes was monitored at all stages using an antibacterial assay.

All samples were assayed for antibiotic activity using the

Graph 1. *E. coli* ESS antibacterial activity in fermentations of ATCC 700974 in supplemented media.



conventional agar hole-in-plate method. 400 ml of DST agar [Difco] were inoculated with 2 ml of overnight broth culture of *E. coli* (ESS). Test samples were added to wells cut in the agar and the plate incubated overnight at 37°C.

HPLC analysis was also performed on the albomycin δ_2 Fe^{3+} and Al^{3+} complexes using a Waters Spherisorb ODS II 10 μm C18 column (4.6×250 mm), mobile phase 12.5% methanol in water, flow rate 2 ml/minute, and monitoring UV absorbance at 280 nm. The albomycin δ_2 Fe^{3+} complex had a retention time of 10.2 minutes and the Al^{3+} complex had a retention time of 17.8 minutes.

Fermentation Conditions for SB-217452 Production

Culture ATCC 700974, is an unidentified grey-sporing streptomycete.

Medium M3 was used for both seed and production fermentations. M3 contained tomato puree 20 g, Collofilm dextrin 20 g, dried bakers yeast 10 g, $CoCl_2 \cdot 6H_2O$ 0.005 g in 1 liter of tap water. pH was adjusted to 7.3 prior to sterilization. In addition antifoam L81, 0.1 ml/liter was added to the 20 liter fermentations.

1 ml of vegetative stock of the culture was used to inoculate a 100 ml primary seed flask. Following incubation at 28°C, 240 rpm for 48 hours, secondary seed flasks containing 300 ml M3 medium in 2 liter flasks, were inoculated using a 5% inoculum and fermented under the same conditions as the primary stage. At 48 hours, 2× 300 ml aliquots were pooled and used to inoculate 5×20 liter fermenters containing 15 liters of M3 medium.

The fermenters were harvested at 68 hours. The five fermenters were pooled and centrifuged to yield 75 liters of culture supernatant.

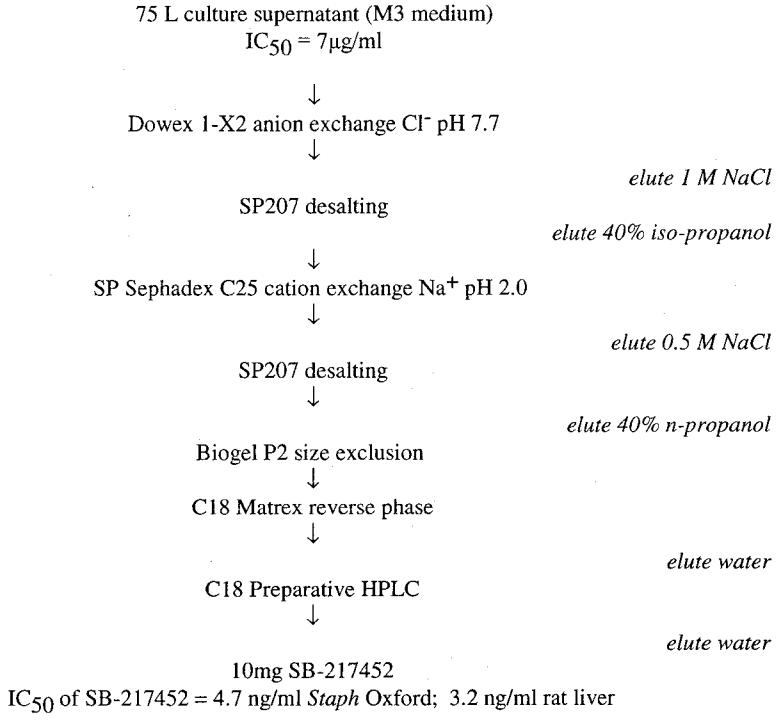
Fermentation Conditions for Albomycin δ_2 Fe^{3+} and Al^{3+} Complexes

Once the SRS inhibitor was determined to be related to albomycin δ_2, we were alerted to the fact that the other metabolites produced by ATCC 700974 could also be albomycins. The fermentation and isolation of albomycin δ_2 has been studied extensively.[5] Production of albomycin δ_2 is dependent on the ornithine, phosphate and iron concentration in the medium; thus M3 was supplemented with ornithine, phosphate, and ferrous sulfate. The antibacterial fermentation profiles are shown in Graph 1. The single addition of phosphate, ornithine or ferrous sulfate increased the production of *E. coli* ESS antibacterial activity. The addition of all three supplements together was the most beneficial. After 3 days incubation, antibacterial activity was 4-fold greater than that produced by M3. The stability of this production was sustained such that after 4 days incubation the differential between M3+supplements and M3 alone was greater than 16-fold.

The supplemented M3 medium used for production of albomycin δ_2 Fe^{3+} and albomycin δ_2 Al^{3+} complexes, contained in addition to the ingredients described for SB-217452 production above: ornithine 15 g, Na_2HPO_4 (anhydrous) 10.7 g and $FeSO_4 \cdot 7H_2O$ 0.28 g per liter of tap water. pH was adjusted to 7.3 prior to sterilization.

50 flasks (500 ml Erlenmeyer) containing 100 ml each of supplemented M3 medium were inoculated (3%) from

Fig. 1. Isolation procedure for SB-217452.



day 3 seed flasks (240 rpm, 28°C) grown in 100 ml M3. After 3 days at 28°C and 240 rpm (final pH 6.2) the 50 flasks were bulked and harvested by centrifugation to yield 4.5 liters of supernatant.

Extraction of SB-217452

The SRS inhibitor SB-217452, is a polar, amphoteric compound. The compound was isolated from the culture supernatant using the procedure shown in Fig. 1. Evaluation of the efficiency of the extraction process was carried out by measuring the SRS inhibitory activity of the product from each chromatographic step. The overall purification achieved was 1,500-fold compared to the culture supernatant.

Isolation of Albomycin δ_2 Fe^{3+} and Al^{3+} Complexes

Supernatant from the culture grown in supplemented M3 medium, was loaded onto a Diaion HP 20 adsorption column and eluted with 20% *iso*-propanol. Fractions with *E. coli* ESS antibacterial activity were further purified on a SP Sephadex C25 cation exchange column (Na^+ form), loaded at pH 2 and eluted with 0.2 M NaCl at neutral pH. Active fractions were bulked and adjusted to pH 7 prior to loading onto a Biogel P2 size exclusion column, eluted with water. The metabolites were purified further on a C18 Matrex reversed phase column eluted with 15% MeOH in water, followed by preparative C18 reversed phase HPLC. The two metabolites were collected separately and both subjected to further C18 HPLC, eluting with 10% MeOH in water. 3.2 mg of metabolite A (albomycin δ_2 Fe^{3+} complex), and 3.9 mg of metabolite B (Al^{3+} complex), were obtained.

Methods for Structure Elucidation

FAB-MS and HRFAB-MS were carried out on VGZAB IF and JEOL SX-102 spectrometers. Mass spectral deuteration experiments were run on a Finnigan MAT TSQ 70 spectrometer. Optical rotation was determined with an Optical Activity Ltd. polaar 3000. UV spectra were taken with a Beckman DU68 UV-visible spectrophotometer. NMR spectra were recorded on Bruker AM400 and AVANCE400 spectrometers. For dimethylsulfoxide solutions, NMR chemical shifts were referenced to an internal standard of tetramethylsilane at 0.00 ppm. For deuterium oxide solutions, proton chemical shifts were referenced to the residual HOD signal at 4.80 ppm whilst carbon-13 chemical shifts were referenced to an external standard of dioxan at 67.4 ppm.

Method for the Amino Acid Analysis

Acid hydrolysis of SB-217452 (20 μg dried into a 6× 50 mm borosilicate hydrolysis tube) was carried out *via* a vapour phase method, using constant boiling HCl/0.1% phenol, (250 μl) at 110°C, under N_2 for 24 hours. The resulting hydrolysate was evaporated to dryness.

N-isobutyryl-L-cysteine-*o*-phthalaldehyde (IBLC-OPA) chiral derivatisation was carried out as follows. Dried hydrolysate or amino acid reference standard was dissolved in borate buffer (100 μl of 0.15 M sodium borate, adjusted to pH 10.4 with 1 M NaOH) and vortex mixed. To a 5 μl aliquot of this solution was added 85 μl borate buffer, 5 μl OPA, (1 mg/ml in borate buffer) and 5 μl IBLC, (4 mg/ml in borate buffer). The solution was vortex mixed and the reaction allowed to proceed for 5 minutes at room temperature.

HPLC for amino acid analysis was carried out using a Waters Alliance 2690 chromatograph and a Waters NovaPak reverse phase column (ODS, 60 Å, 4 μM, 3.9× 150 mm, flow rate 1.0 ml/minute). An autosampler was used to inject 5 μl aliquots of solution. Sodium acetate (0.023 M), pH 6.0/methanol/acetonitrile was utilised for gradient elution of amino acid derivatives. Detection was carried out using a fluorescence detector, Ex. 230 nm, Em. 445 nm.

Results

Structure Elucidation of SB-217452

The molecular formula was established as $C_{19}H_{24}O_9N_6S_1$ by high-resolution fast atom bombardment mass (HRFAB-MS) and ^{13}C NMR data. Mass spectrometric deuteration experiments had indicated the presence of 10 exchangeable protons. The structure of SB-217452 shown in Table 1 was deduced from COSY-45, HMQC and HMBC NMR experiments run in D_2O and DMSO-d_6 (Table 1 and 2).

Physical and spectroscopic data obtained were very similar to those reported for the serine linked nucleoside moeity of albomycin δ_2[6] (Table 3).

The compound was subjected to acid hydrolysis followed by chiral derivatisation and chromatography with fluorescence detection, which confirmed the L-serine configuration. The retention times of reference AA-IBLC derivatives were as follows (minute): L-Ser-IBLC, 13.38 minutes; D-Ser-IBLC, 14.71 minutes. A peak with retention time, 13.26 minutes was observed on analysis of SB-217452 hydrolysate, corresponding to L-Ser. Spiking of SB-217452 hydrolysate with L-Ser resulted in only one peak whilst spiking with D-Ser resulted in two peaks.

The relative stereochemistry at the four chiral centres around the five membered ring has been shown to be identical to that of the literature compound. This follows from the nOe and rOe data for the compound in aqueous and dimethylsulphoxide-d_6 solution. A definitive nOe (and rOe) observed between H1′ and H3′ confirmed their syn-1,3 relationship and hence the relative stereochemistry at these two centres. Evidence for the relative stereochemistry at C4′ follows from the weaker rOe's observed between H1′ and H4′ as well as between H5′ and H6. The relative stereochemistry at C2′ therefore follows from the weak rOe observed between H2′ and H5′.

As albomycin δ_2 complexes were isolated from the same culture fermentation, and the optical rotation observed for SB-217452 is close to the optical rotation reported in the literature, we propose that the stereochemistry of the two remaining chiral centers C5′ and C6′ in SB-217452 is as shown in Table 3.

Antibacterial Activity of SB-217452

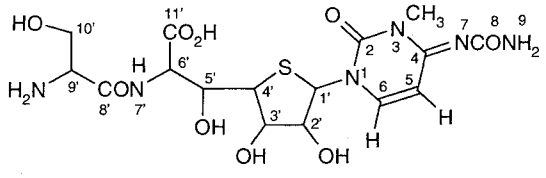
Using a microtitre broth dilution method, the antibacterial activity of SB-217452 was determined against the following organisms: *Escherichia coli* ESS, *Klebsiella pneumoniae* E70, *Pseudomonas aeruginosa* K799 61, *Moraxella catarrhalis* 1502, *Staphylococcus aureus* Oxford, and *Streptococcus pyogenes* CN 10. Although SB-217452 is a very potent inhibitor of *Staphylococcus aureus* Oxford seryl tRNA synthetase, whole cell antibacterial activity against this organism was very poor (MIC= 256 μg/ml). Of the other organisms tested, activity was detected against the *E. coli*, *M. catarrhalis* and *S. pyogenes* (MIC=256 μg/ml, 8 μg/ml and 16 μg/ml respectively). The poor whole cell antibacterial activity might reflect poor penetration of SB-217452 through the cell wall without the benefit of a siderophore moiety.

Structure Elucidation of Albomycin δ_2 Fe^{3+} and Al^{3+} Complexes

As SB-217452 is related to albomycin δ_2, the possibility that the other metabolites were albomycins in the free or complexed form guided the structure elucidation work. The spectroscopic data were compared with published data on albomycins.[7]

Mass experiments on metabolite A using the electrospray technique (+ve and −ve modes) gave strong molecular ions corresponding to a MW of 1045 suggesting the presence of the ferric complex of albomycin δ_2. Addition of oxalic acid in the sample induces decomplexation and

Table 1. NMR data on SB-217452 (400 MHz, D_2O).



Position	δH (m, J)	COSY-45	δC (HMQC)	DEPT	HMBC (1)
2	-	-	155.31	C	-
3 N-CH_3	3.40 (s)	-	32.74	CH_3	155.31, 158.31
4	-	-	158.31	C	-
5	6.25 (d, 8.3)	8.25	99.97	CH	140.95, 158.31
6	8.25 (d, 8.3)	6.25	140.95	CH	66.23, 99.97, 155.31, 158.31
8	-	-	170.37	C	-
1'	6.00 (d, 5.8)	4.58	66.23	CH	82.24, 140.95, 155.31
2'	4.58 (dd, 5.8, 7.4)	6.00, 4.45	82.24	CH	-
3'	4.45 (dd, 6.5, 7.4)	4.58, 3.80	77.80	CH	-
4'	3.80 (dd, 3.8, 6.5)	4.50, 4.45	53.23	CH	77.80
5'	4.50 (dd, 6.7, 3.8)	4.55, 3.80	71.87	CH	-
6'	4.55 (d, 6.7)	4.50	60.29	CH	-
8'	-	-	178.51	C	-
9'	3.90 (m)	-	58.30	CH	-
10'	3.90 (m)	-	65.08	CH_2	58.30
11'	-	-	174 (2)	C	-

(1) Carbon atoms detected following irradiation of protons from column δ_H

(2) Weak signal

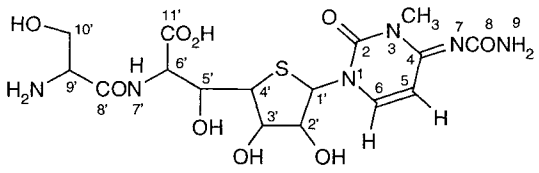
provides strong molecular ions corresponding to a MW of 992 agreeing with the presence of albomycin δ_2 in its free form.

Mass data on metabolite B after acidification indicates a MW of 992 suggesting once more the presence of albomycin δ_2. Prior to acidification, the molecular weight was 1016. The difference in MW before and after acidification is 24 mass units. An aluminum (mass 27) complex of albomycin δ_2 is proposed for B. Other siderophores such as ferrichrome which complex Fe^{3+} ions, are also known to complex Al^{3+} and Ga^{3+} ions.[8]

The metabolites were analysed by X-ray energy spectroscopy, which confirmed the presence of iron and aluminum respectively in the samples.

The proposed structures for metabolites A and B are the ferric and aluminum complexes of albomycin δ_2 respectively. The latter compound is novel.

Table 2. NMR data on SB-217452 (400 MHz, DMSO-d_6).



Position	δH (m,J)	COSY-45	δC (HMQC)	DEPT	HMBC (1)
2	-	-	152.5	C	-
3 N-Me	3.25 (s)	-	30.9	CH_3	152.5, 155.0
4	-	-	155.0	C	-
5	6.22 (d, 8.3)	8.30	97.9	CH	139.0, 155.0
6	8.30 (d, 8.3)	6.22	139.0	CH	97.9, 152.5, 155.0
8	-	-	165.3	C	-
9	6.67 (br)	-	-	-	-
1'	5.75 (d, 5.0)	4.23	6.51	CH	82.0, 139.0, 152.5
2'	4.23 (dd, 6.2, 5.4)	5.75, 3.96	82.0	CH	-
3'	3.96 (dd, 6.2, 6.2)	4.23, 3.50	77.0	CH	65.1, 71.7, 82.0
4'	3.50 m	3.96, 4.12	55.1	CH	-
5'	4.12 (dd, 4.8, 7.0)	3.88, 3.50	71.7	CH	-
6'	3.88 (dd, 7.0, 7.0)	8.17, 4.12	57.3	CH	71.7, 175.9
7'	8.17 (d, 7.0)	3.88	-	-	175.9, 173.8, 55.1
8'	-	-	175.9	C	-
9'	3.26 (2)	-	58.6	CH	175.9
10'	3.51 (2)	-	65.7	CH_2	-
	3.48 (2)	-			175.9
11'	-	-	173.8	C	-

(1) Carbon atoms detected following irradiation of proton from column δ_H

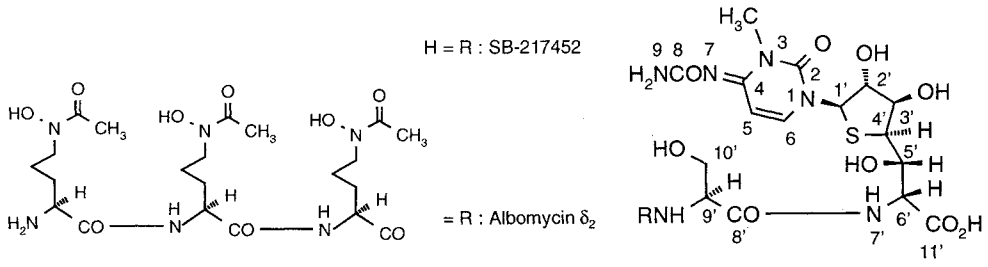
(2) Seryl fragment hidden under HDO

Discussion

SB-217452 is a novel seryl tRNA synthetase inhibitor related to albomycin δ_2. Structure elucidation indicated that the inhibitor was identical to the serine linked nucleoside moiety of albomycin δ_2. This compound has previously been described[6] as an enzymatic cleavage product of albomycin δ_2 but has not been reported as a naturally occurring metabolite.

The similarity in structure and spatial configuration between SB-217452 and seryl adenylate (a seryl tRNA synthetase inhibitor) could be an explanation for the SRS

Table 3. Physical and spectroscopic data of SB-217452.



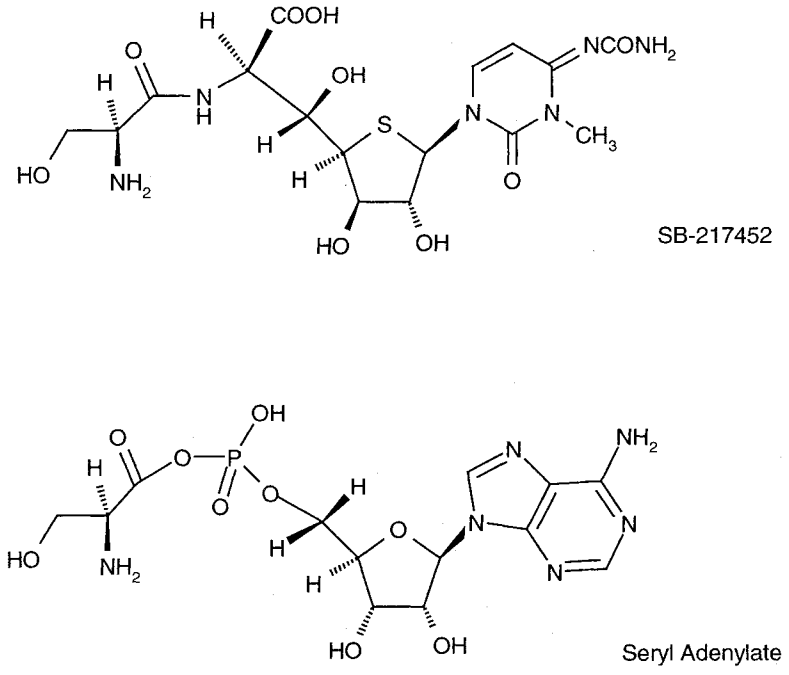
	Literature Data	Our Data
$[\alpha]_D$ (H_2O)	-15.56 (c = 1.025)	-14.88 (c= 0.216)
UV (1N HCl)	λ_{max} (ε) 213	215
	233	229
	306	306
FAB-MS (m/z)	477	477
^{1}H NMR (D_2O)	(250 MHz)	(400 MHz)
	δ_H (m, J)	δ_H (m, J)
3- NMe	3.35 (s)	3.40 (s)
5	6.20 (d, 8.3)	6.25 (d, 8.3)
6	8.26 (d, 8.3)	8.25 (d, 8.3)
1'	5.94 (d, 5.3)	6.00 (d, 5.8)
2')		4.58 (dd, 5.8, 7.4)
3')	4.34 - 4.54 (m)	4.45 (dd, 6.5, 7.4)
5')		4.50 (dd, 6.7, 3.8)
6')		4.55 (d, 6.7)
4'	3.78 (dd, 4.4, 6.0)	3.80 (dd, 3.8, 6.5)
9'	4.21 (dd, 5.6, 4.3)	3.90 (m)
10'	3.96 (dd, 12.2, 5.6)	3.90 (m)
	4.04 (dd, 12.2, 4.3)	

inhibitory activity of SB-217452 (Fig. 2).

Albomycin δ_2 has good Gram-positive and -negative antibacterial activity. The compound is actively taken up into bacterial cells *via* the transport system for the structurally similar iron complex, ferrichrome. Albomycin is cleaved and the antibacterial moiety is released into the cytoplasm, whereas the iron carrier moiety appears in the medium.

We propose, based on the evidence that SB-217452, the seryl linked nucleoside moiety of albomycin δ_2 is an inhibitor of seryl tRNA synthetase, that inhibition of seryl tRNA synthetase could contribute to the mode of action of the albomycin family of antibiotics, after siderophore mediated transport into the bacterial cells.

Fig. 2. Similarites in structure between SB-217452 and seryl adenylate.



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