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SB-219383, a Novel Tyrosyl tRNA Synthetase Inhibitor from a *Micromonospora* sp.

I. Fermentation, Isolation and Properties

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A novel, potent and selective inhibitor of bacterial tyrosyl tRNA synthetase, designated SB-219383 has been isolated from *Micromonospora* sp. NCIMB 40684. The fermentation, isolation and some properties are described, whilst the structure determination is described in the succeeding paper[1]. SB-219383 showed competitive, inhibitory activity against a *Staphylococcus* tyrosyl tRNA synthetase, with an IC_{50} of <1 nM, and exhibited weak *in vitro* activity against some *Streptococcus* sp.

Aminoacyl tRNA synthetases are the key components of protein biosynthesis. They are responsible for maintaining the fidelity of transfer of genetic information from DNA into protein, playing a crucial role in this process by charging their cognate tRNA(s) with the correct amino acid[2]. The enzymes are essential for cell viability, and these enzymes are potential targets[3] for antibacterial agents, as demonstrated by the isoleucyl tRNA synthetase inhibitor, pseudomonic acid A, a naturally occurring antibiotic produced by *Pseudomonas fluorescens* NCIMB 10586[4].

During a programme of screening soil microorganisms for inhibitors of tyrosyl tRNA synthetase (YRS), a novel compound designated SB-219383 was detected. SB-219383 is a potent competitive inhibitor of bacterial tyrosyl tRNA synthetase, but exhibits only weak antibacterial activity.

This paper describes the fermentation conditions for the production of SB-219383 from a new strain of the genus *Micromonospora*, NCIMB 40684, together with the isolation, purification and biological properties.

Taxonomy of Producing Organism

Strain NCIMB 40684 was isolated from soil collected in close proximity to a White Ant nest in Longhope, South Africa. The culture was grown and morphologically examined after 7~14 days on ISP2 (yeast malt extract agar) and ISP3 (oatmeal agar) following the method of SHIRLING and GOTTLIEB[5]. The isolate NCIMB 40684 had orange substrate mycelium that darkens to brown/black with sporulation and eventually becomes mucoid in appearance. The spores were studied using a light microscope ×400 magnification and were arranged singly on short sporangiophores. Sporulation was observed from submerged culture samples. No pigment was produced on any agar. The cell wall was analysed for diaminopimelic acid content[6,7]. None was detected. A combination of physiological parameters were also examined using API strips 50CH and 20E. The results are shown in Table 1. Carbon utilisation studies were carried out in minimal medium as described by STEVENSON[8]. With the accumulated data NCIMB 40684 was classified as a *Micromonospora* sp. The Deutsche Sammlung von Mikroorganismen und Zellkulturen confirmed this identification.

Table 1. Morphological and physiological data for NCIMB 40684.

DAP type	none detected
Spores	single
Spore mass colour	brown
Diffusable pigment	-
Melanoid formation	-
Hydrolysis of starch	+
Liquification of gelatine	+
Utilisation of urea	-
Production of:	
nitrate reductase	-
H2S	-
Carbon utilisation:	
Adonitol	+
L-arabinose	-
Cellobiose	+
Fructose	+
D-Galactose	+
D-Glucose	+
Glycerol	-
Inositol	-
Inulin	-
Lactose	-
Maltose	+
Mannitol	-
D-Mannose	-
Melibiose	+
D-Raffinose	-
Rhamnose	-
Salicin	-
Trehalose	-
Xylitol	+
D-Xylose	-

Materials and Methods

Preparation of the tRNA Synthetases

Enzymes used for this work were crude tyrosyl tRNA synthetase (YRS) from *Staphylococcus aureus* Oxford for monitoring fermentation and extraction, and purified recombinant tyrosyl tRNA synthatase from *S. aureus* WCUH 29, overexpressed in *E. coli* for kinetic studies. Crude YRS isolated from rat liver was also prepared. The procedures used for preparation of the tRNA synthetases were essentially those from previously described methods[9~11].

For the preparation of bacterial YRS, *Staphylococcus aureus* Oxford was grown in a 20 litre fermenter containing 15 litres of Nutrient Broth inoculated with 1% overnight broth culture. Cells were harvested during exponential growth at 2.5 hours by centrifugation at 5,000 *g* for 1 hour at 4°C. The cells were resuspended in 80 ml of 10 mM Tris-HCl (pH 7.8) and re-centrifuged at 10,000 *g* for 20 minutes at 4°C. The cells were taken up in 15 ml of 10 mM Tris-HCl (pH 7.8) and sonicated for 5×30 seconds with 30 seconds cooling between bursts. The cell paste was centrifuged at 15,000 *g* for 20 minutes at 4°C. DNase (final concentration 3 μg/ml) was added to the supernatant and the solution incubated for 20 minutes at room temperature. The mixture was then ultracentrifuged at 100,000 *g* for 3 hours at 4°C. The resulting S_{100} enzyme preparation was dialysed overnight against 10 mM Tris-HCl buffer (pH 7.8) and recentrifuged at 35,000 *g* for 15 minutes at 4°C. Glycerol (33% v/v) was added to the preparation, which was subsequently stored at −20°C. The enzyme preparation (an unfractionated mixture of tRNA synthetases including YRS), was diluted 1 in 20 with deionised water immediately prior to use in the assay (see below).

Recombinant *S. aureus* YRS from *E. coli* BL21(DE3) carrying the pDB575 : pBROC610 plasmid was supplied from a 150 litre fermentation. The recombinant *S. aureus* YRS was purified to near homogeneity (~98% as judged by SDS-PAGE) using a three stage chromatographic process. The cell lysate was loaded onto Q-Sepharose and eluted using a 0~650 mM NaCl gradient over 15 bed volumes. The pooled fractions from the Q-Sepharose were made up to 2 M $(NH_4)_2SO_4$ [by the addition of 4 M $(NH_4)_2SO_4$] and the precipitate was removed by centrifugation. The supernatant was loaded on to a Phenyl Sepharose High Performance column; this was eluted using a gradient of 2 M~1 M $(NH_4)_2SO_4$ over 5 bed volumes, followed by 1 M~0 M $(NH_4)_2SO_4$ over 15 bed volumes. The pooled fractions from the Phenyl Sepharose High Performance were buffer exchanged using Sepharose G25M, and then loaded onto a Source 15Q column. The column was eluted using a NaCl gradient, 0~500 mM, over 60 bed volumes. The fractions containing the highly purified YRS were concentrated by ultrafiltration and stored in 50% glycerol at 2.5 mg/ml.

Tyrosyl tRNA Synthetase Inhibition Assays

YRS activity was measured by aminoacylation or PPi/ATP exchange activity using modifications to previously described methods[11].

Aminoacylation assays were performed at 37°C in a

mixture containing (final concentrations) 100 mM Tris/Cl pH 7.9, 50 mM KCl, 16 mM $MgCl_2$, 5 mM ATP, 3 mM DTT, 4 mg/ml *E. coli* MRE 600 tRNA (Boehringer Mannheim) and 10 μM L-tyrosine (0.3 μM L-[$^{3,5\text{-}3}$H] tyrosine [Amersham, Specific activity: 1.78 TBq/mmol], 10 μM carrier) and 0.2 nM YRS. Reactions were initiated with enzyme and terminated using 7% trichloroacetic acid as previously described[12]. The rate of reaction in all experiments was linear with respect to protein and time with less than 50% total tRNA acylation.

Steady-state pyrophosphate/ATP exchange in the presence of supersaturating substrates[11] was monitored at 37°C in (final concentrations) 2 mM [^{32}P]PPi (specific activity = $\sim 5\times10^4$ cpm nmole^{-1}), 2 mM L-tyrosine, 5 mM ATP, 100 mM Tris/Cl pH 7.9, 50 mM KCl, 12 mM $MgCl_2$ and 3 mM DTT. All reactants (except the tracer amounts of [^{32}P]PPi) were pre-incubated for 2 hours at 37°C prior to the initiation of isotopic exchange by addition of isotopically labelled PPi. Reactions were then quenched after 5 minutes using 0.4 M Pyrophosphate in 7% perchloric acid and harvested as previously described[11]. All least squares data fitting was performed using Grafit[13].

HPLC Analysis

HPLC analysis of SB-219383 was carried out using a Waters Spherisorb ODS II 10 μm C18 column (4.6× 250 mm), monitoring UV absorbance at 225 nm and eluting at 2 ml/minute with distilled water at pH 2.0 (adjusted with HCl). Under these conditions SB-219383 has a retention time of 3.9 minutes.

Fermentation Conditions

Micromonospora sp. NCIMB 40684 was maintained as frozen vegetative stock. 1ml of frozen vegetative stock was used to inoculate M3 seed medium (100 ml) contained in a single 500 ml Erlenmeyer flask. M3 contained tomato puree 20 g, Collofilm dextrin 20 g, bakers yeast 10 g, $CoCl_2\cdot6H_2O$ 0.005 g in 1 litre of deionised water adjusted to pH 7.3 with sodium hydroxide prior to sterilisation.

The flask was incubated on a gyratory shaker at 240 rpm and 28°C for 48 hours. For the secondary seed, 100 ml aliquots of M3 medium in 500 ml Erlenmeyer flasks were each inoculated with 4% of the primary seed and incubated for a further 48 hours at 240 rpm and 28°C.

For the tertiary seed 2-litre shake flasks containing 300 ml of M3 were inoculated with 4% of the secondary seed and incubated at 240 rpm and 28°C for 48 hours.

For the quaternary seed stage 100 litres of M3 medium (made up with tap water)+0.01% SAG471 antifoam were sterilised at 121°C for 30 minutes in a Braun 150-litre fermenter. After cooling the vessel was then inoculated with 4.2 litres of tertiary seed and then incubated for 49 hours at 28°C and maintained at 0.5 bar overpressure with an agitator speed of 260 rpm (2.15 m/s tip speed) and an air flow rate of 50 litres/minute.

For the final stage fermentation 3000 litres of M3 medium (again made up in tap water+0.01% SAG471 antifoam) were sterilised in a Bioengineering 4500-litre fermenter for 45 minutes at 121°C. After cooling to 28°C the fermenter was inoculated with the contents of the quarternary seed fermenter and incubated at 28°C and maintained at 0.4 bar overpressure with an agitator speed of 82 rpm (2.23 m/s tip speed) and an air flow rate of 1500 litres/minute. The airflow rate was decreased to 1000 litres/minute after 30 hours incubation to prevent excess foaming and then increased back to 1500 litres/minute after a further 9 hours. Broth (1500 litres) was harvested from the fermenter after 67 hours incubation, the titre was 0.3 μg/ml. The remaining 1500 litres were incubated as above, with an air flow rate of 2400 litres/minute and were harvested after a further 26 hours incubation. Titre at harvest was 0.63 μg/ml. Subsequent fermentation development will be the subject of a future paper.

Extraction and Isolation

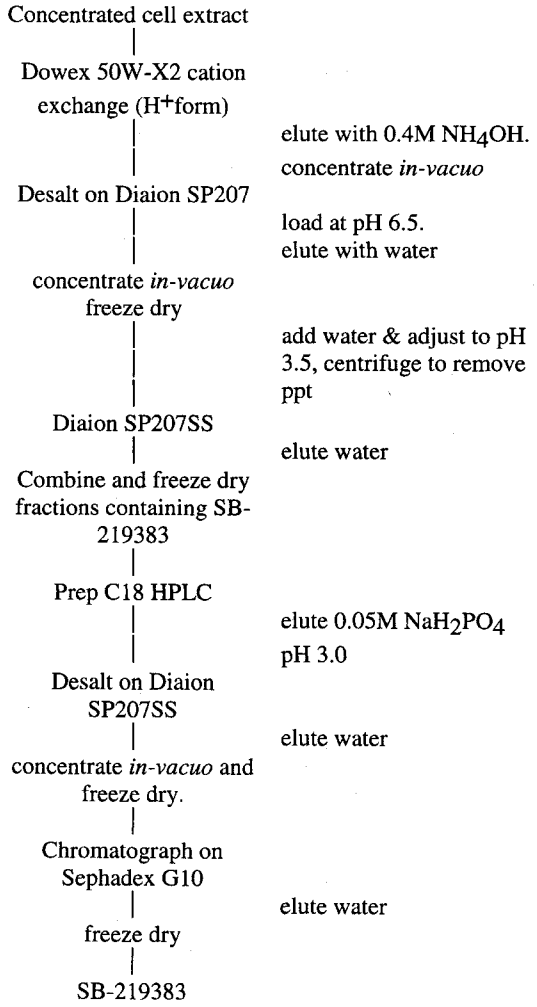
SB-219383 is intracellular, and may be recovered from the cells by heat treatment (70°C, 15 minutes) or methanol extraction (add methanol to 60% v/v), followed by centrifugation to remove the cell debris.

The cell extract was concentrated using a wiped film evaporator and filtered through Dicalite 478, before ultrafiltration on an Alpha Laval UFB-2 unit, (PM 500, Romicon, Rohm Hass). Further steps in the isolation procedure for SB-219383 are shown in Fig. 1.

The following chromatography media were used. Dowex 50W-X2 cation exchange resin supplied by Dow Chemical Company, Diaion SP207 and SPS07ss polymeric adsorbents, supplied by Mitsubishi Chemical Industries, and Sephadex G10 supplied by Pharmacia.

Preparative HPLC was carried out using a Microsorb C18 5 μ column (2.1×25 cm) from Rainin Instruments, operating at 30°C, eluting with 0.05 M NaH_2PO_4 pH 3.0 at a flow rate of 20 ml/minute and monitoring at 275 nm.

Fig. 1. The isolation of SB-219383 from *Micromonospora* sp. NCIMB 40684.



Results and Discussion

Properties of SB-219383

The physio-chemical properties of SB-219383 are shown in Table 2. Further detailed physio-chemical properties are described in part II of this paper[1].

Tyrosyl tRNA Synthetase Inhibition

SB-219383 was found to be a highly potent time-dependent competitive inhibitor of *S. aureus* YRS (Fig. 2A; closed symbols). Pre-incubation of SB-219383 with YRS for 10 minutes prior to measurement of the tRNA aminoacylation reaction in the presence of a tyrosine concentration equivalent to $\approx K_m^{Tyr}$, yielded an IC_{50} of 0.6± 0.4 nM (mean±SEM, n=3) for inhibition by SB-219383. This value was close to the enzyme concentration required in aminoacylation assays (0.2 nM YRS) and therefore may not have reflected true equilibrium constant for inhibitor binding. To address this, we examined inhibition of the PPi/ATP exchange YRS partial reaction measured at steady-state (2 hours incubation) in the presence of supersaturating substrate concentrations ([Tyr]=2 mM $\approx 100.K_m^{Tyr}$). We have previously demonstrated that this is a useful technique for determining the true inhibition constants of other highly potent tRNA synthetase inhibitors[11,12]. Primary data from a typical experiment of this type is shown in Fig. 2A (open symbols). A series of similar experiments yielded an IC_{50} for inhibition of PPi/ATP exchange under these conditions of 53.2±1.9 nM (mean±SEM, n=3). This was consistent with the value obtained for inhibition of aminoacylation by SB-219383 (since, to a first approximation for a competitive inhibitor, $IC_{50}=K_i(1+[S]/K_m)$) and confirms that the overall K_i for binding of SB-219383 to YRS is around 0.5 nM. SB-219383 selectively inhibits the bacterial YRS. The IC_{50} value using a preparation of the rat liver enzyme was 22 μM.

SB-219383 is a time-dependent reversible inhibitor (data not shown). To analyse the mechanism of initial binding, three-dimensional competition experiments were performed in which the effect of different concentrations of SB-219383 upon L-tyrosine activation of tRNA aminoacylation were studied (*e.g.* Fig. 2B). The data obtained were best fitted to a competitive model of inhibition, yielding the following parameters for initial binding to YRS; K_i=271±39 nM, K_m^{Tyr}=17.6+2.9 μM. SB-219383 is therefore a tyrosine competitive inhibitor, with an initial K_i in the hundreds nM range, but where the initial E.I complex undergoes an isomerisation resulting in an overall K_i of <1 nM.

Antibacterial Properties

Using a microtitre broth dilution method, the antibacterial activity of SB-219383 was determined against the following range of organisms: *Escherichia coli*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes*. Although SB-219383 is a very potent inhibitor of tyrosyl tRNA synthetase, prepared from *Staphylococcus aureus*, no whole cell antibacterial activity (MIC >64 μg/ml) against this organism was detected. Of the organisms tested, activity was only detected against some of the *Streptococcus* strains (MIC=32 μg/ml). The absence of whole cell antibacterial activity against the other organisms

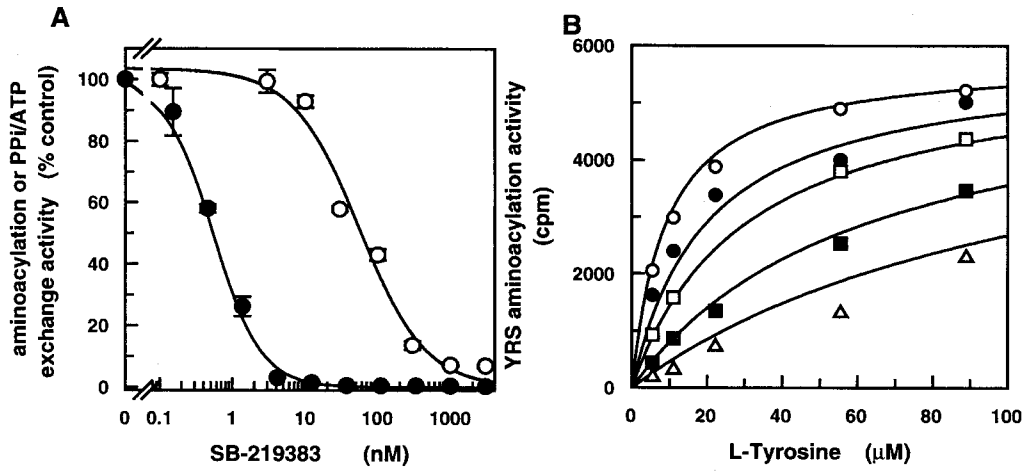
Table 2. Physico-chemical properties of SB-219383.

Appearance	colourless powder
UV λ max in H_2O nm	225, 275
Molecular formula	$C_{17}H_{23}N_3O_9$
Fab-MS positive ion	414 $(M+H)^+$
Molecular weight measured	413.1435
calculated	413.1434
IR (KBr) cm^{-1}	2950-2850, 1673, 1617, 1517, 1385, 1249, 1154, 1078, 1000
$[\alpha]_D$ [a]	$+25.3^o$
HPLC [b] (Rt minutes)	3.9

[a] $[\alpha]_D$ at 22.5 °C was determined on the ethyl ester, prepared from an ethanolic solution of SB-219383 mixed with 4 M HCL and left for 1 hr at room temperature.

[b] HPLC: column, Spherisorb ODSII C18, 10um, 4.6 x 250 mm. Solvent A = Distilled Water at pH 2.0 (adjusted with HCL). Detection wavelength 225nm Flow rate 2ml / min.

Fig. 2. Inhibition of YRS aminoacylation and super-saturating PPi/ATP exchange activity by SB-219383 and competitive mechanism of inhibition.



A: Dose response curves for inhibition of YRS aminoacylation activity measured at $[Tyr]=K_m^{Tyr}$ and after a 10 minute pre-incubation (●), or steady-state PPi/ATP exchange measured at $[Tyr]\approx 100.K_m^{Tyr}$ and after 2 hours pre-incubation (○). **B**; Competitive inhibition of initial YRS aminoacylation activity by SB-219383 with respect to tyrosine activation. L-Tyrosine was present at the concentration indicated and SB-219383 at 0 (○), 56 (●), 111 (□), 278 (■) or 556 (△) nM. Solid lines show a global fit of all the data to three-dimensional equation describing competitive inhibition and yielding the following parameters; $K_m^{Tyr}=17.6\pm2.9\,\mu M$, $K_i=271\pm39$ nM.

might reflect poor penetration of SB-219383 through the cell wall.

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References

1) HOUGE-FRYDRYCH, C.; S. READSHAW & D. BELL: SB-219383, a novel tyrosyl tRNA synthetase inhibitor from a *Micromonospora* sp. II. Structure determination. J. Antibiotics 53: 351~356, 2000
2) SCHIMMEL, P.: Aminoacyl tRNA synthetases: General scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. Ann. Rev. Biochem. 56: 125~158, 1987
3) SCHIMMEL, P.; J. S. TAO & J. HILL: Aminoacyl tRNA synthetases as targets for new anti-infectives [Review]. FASEB Journal 12 (15): 1599~1609, 1998 Dec.
4) FULLER, A. T.; G. MELLOWS, M. WOODFORD, G. T. BANKS, K. D. BARROW & E. B. CHAIN: Pseudomonic acid: an antibiotic produced by *Pseudomonas fluorescens*. Nature 234: 416~417, 1971
5) SHIRLING, E. & D. GOTTLIEB: Methods for characterisation of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
6) BERGEY's Manual Systematic Bacteriology. Volume 4. *Eds*., S. T. WILLIAMS *et al*., pp. 2344~2347 and 2469~2472, Williams and Wilkins, 1989
7) LECHEVALIER, M. & H. LECHEVALIER: The Chemotaxonomy of Actinomycetes. *In* DIETZ and THAYERS (Editors), Actinomycete Taxonomy, Special Publication 6, Society for Industrial Microbiology, Arlington, VA, pp. 227~291, 1980
8) STEVENSON, I.: Utilisation of aromatic hydrocarbons by *Arthrobacter* spp. Can. J. Microbiol. 13:205, 1967
9) HUGHES, J. & G. MELLOWS: Interactions of pseudomonic acid A with *Escherichia coli* B isoleucyl tRNA-synthetase. Biochem. J. 191: 209~219, 1980
10) HUGHES, J. & G. MELLOWS: Inhibition of Isoleucyl-transfer ribonucleic acid synthetase in *Escherichia coli* by pseudomonic acid. Biochem. J. 176: 305~318, 1978
11) POPE, A. J.; K. J. MOORE, M. MCVEY, L. MENSAH, N. BENSON, N. OSBOURNE, N. BROOM, M. J. B. BROWN & P. J. O'HANLON: Characterization of isoleucyl tRNA synthetase from *Staphylococcus auerus*. II. Mechanism of inhibition by reaction intermediate and pseudomonic acid analogues studied using transient and steady-state kinetics. Biol. Chem. 273(48): 31691~31701, 1998
12) POPE, A. J.; J. LAPOINTE, L. MENSAH, N. BENSON, M. J. B. BROWN & K. J. J. MOORE: Characterization of isoleucyl tRNA synthetase from *Staphylococcus aureus*. I. Kinetic mechanism of the substrate activation reaction studied by transient and steady-state techniques. Biol. Chem. 273 (48): 31680~31690, 1998
13) LEATHERBARROW, R. J.: *Gafit* Version 3.0, Erithacus Software Ltd., Staines, UK (1992)