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SB-203207 and SB-203208, Two Novel Isoleucyl tRNA Synthetase Inhibitors from a *Streptomyces* sp.

I. Fermentation, Isolation and Properties

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Two novel inhibitors of isoleucyl tRNA synthetase designated SB-203207 and SB-203208 have been detected in the culture of a new *Streptomyces* species. The fermentation, isolation and some properties of the inhibitors are described.

The transformation of the information encoded in genetic material into a functional protein structure is one of the most important of all processes in living organisms. The aminoacyl tRNA synthetases play a crucial role in this process by charging their cognate tRNA(s) with the correct amino acid.[1] These enzymes are potential targets[2] for antibacterial agents as demonstrated by the isoleucyl tRNA synthetase inhibitor, pseudomonic acid A (mupirocin), a naturally occurring antibiotic produced by *Pseudomonas fluorescens* NCIB 10586.[3]

During a programme of screening soil micro-organisms for inhibitors of isoleucyl tRNA synthetase (IRS), two novel compounds designated SB-203207 and SB-203208 were detected. The compounds were found to be structurally related to altemicidin, an acaricidal and antitumour compound.[4,5] SB-203207 and SB-203208 are potent competitive inhibitors of bacterial and mammalian isoleucyl tRNA synthetase but exhibit only weak antibacterial activity.

Evidence in this paper indicates that SB-203208 is a natural product but that SB-203207 may be a degradation product of SB-203208.

This paper describes the fermentation conditions for the production of SB-203208 from a new strain of the genus *Streptomyces*, NCIMB 40513. Methods for extraction and purification, and for the conversion of SB-203208 to SB-203207 *via* controlled hydrolysis are described.

Despite attempts to improve the titre of SB-203208, yields remained unchanged at <1 mg/liter. The possibility that the production of SB-203208 was limited due to inhibition of isoleucyl tRNA synthetase within cells of the producing organism was investigated, and is described in this paper.

Some structural studies and physico-chemical and biological properties of SB-203207 and SB-203208 are also reported.

Materials and Methods

Preparation of the tRNA Synthetases

The procedures used for preparation of the tRNA synthetases were adapted from previously described methods for the preparation of bacterial tRNA synthetases.[6,7]

For the preparation of *Staphylococcus aureus* tRNA synthetases, *Staphylococcus aureus* Oxford was grown in a 20 liter fermenter containing 15 liters 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 IRS), was diluted 1 in 20 with 50 mM Tris buffer pH 7.7 immediately prior to use in the assay (see below).

Preparations of tRNA synthetases from *Pseudomonas fluorescens* NCIB 10586, *Candida albicans* 3153A and *Streptomyces* sp. NCIMB 40513 and homogenised rat liver were processed essentially as described for the preparation of the *Staphylococcus aureus* Oxford tRNA synthetases.

Assay Procedures and Detection Methods

Isoleucyl tRNA synthetase activity was assayed by measuring the enzyme-mediated incorporation of radiolabelled amino acid into tRNA (the aminoacylation method). This measures amino acid-tRNA as trichloroacetic acid-precipitable radioactivity made from labelled amino acid in the presence of tRNA and ATP. The procedure used was adapted from a previously described method.[8]

tRNA was supplied by Boehringer-Mannheim, D-68298 Mannheim, Germany. The radiolabelled amino acid was from Amersham Life Science, Little Chalfont, Buckinghamshire, UK. All other reagents were supplied by Sigma Chemical Company Ltd., Poole, Dorset, UK. Precipitates were harvested using a Skatron Cell Harvester [Camo Ltd., Studlands Park Ave., Newmarket, Suffolk, UK] onto glass fibre filters [printed filtermat B, double thickness, Pharmacia Wallac, Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks, UK]

20 μl of test sample was added to 50 μl of crude enzyme prepared and diluted as described above. The enzyme and test sample were pre-incubated for 5 minutes at 37°C. Pre-mixed reaction mix (100 μl) was added. This reaction mix comprised the following constituents: L[U-^{14}C] isoleucine 0.25 μCi, 315 mCi/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 125 mM and Trizma pre-set buffer (pH 8.5) 50 mM.

After addition of the reaction mixture, the assay samples were incubated at 37°C for 20 minutes and the reaction quenched with 5% trichloroacetic acid (2 ml per reaction) and cooled on ice for 30 minutes. The precipitates were collected onto glass fibre filters which were washed successively with 5% trichloroacetic acid (6 ml) and ethanol (6 ml). The filters were dried at 70°C for 1 hour and the levels of radioactivity measured by scintillation spectrometry.

The isoleucyl tRNA synthetase inhibition assay was used at all stages of the work described in this paper.

For IC_{50} assays, test compounds were dissolved in methanol, and subsequently serially diluted with water to the required concentrations. IC_{50} values were determined by computer-generated fitting of measured inhibition data to curves of:

$$\%I = \frac{(I_{max} - I_{min})}{(1 + ([I]/IC_{50})^s)}$$

where s=slope at mid-point, [I]=inhibitor concentration

HPLC analysis of SB-203207 and SB-203208 was carried out using a Waters Nova-pak C18, 60A, 4 μm, 3.9×150 mm column. The solvent system was solvent A=0.05 M NH_4OAc, solvent B=MeOH, with a gradient of 0% to 100% B in 20 minutes. Detection was at 299 nm. Flow rate was 1 ml/minute. Under these conditions SB-203207 had a retention time of 11.6 minutes, and SB-203208, 18.8 minutes

During hydrolysis of SB-203208 to SB-203207, levels of SB-203207 and β-methylphenylalanine were monitored using 50 mM NaH_2PO_4 pH 5.0 containing 4% CH_3CN as mobile phase. SB-203208 was monitored using a mobile phase of 50 mM NaH_2PO_4 pH 5.0 containing 16% CH_3CN.

Basic Hydrolysis of SB-203208 to SB-203207

SB-203208 was hydrolysed to SB-203207 and β-methylphenylalanine by incubation of a 5 mg/ml solution of SB-203208 adjusted to pH 10 with ammonium hydroxide at 37°C for 24 hours. Analytical HPLC (see below) indicated that the conversion was substantially complete at this stage.

The resulting solution was purified on a Diaion HP20 column eluted with 50% aqueous propan-2-ol. The percolate, water-wash and eluate were collected as three bulks and were freeze dried. The products were analysed by reversed phase C18 HPLC. The HPLC analysis indicated that the percolate contained pure SB-203207, the water wash contained SB-203207 contaminated with β-methylphenylalanine and the eluate contained a mixture of SB-203207, β-methylphenyl alanine and SB-203208 in the ratio 3 : 3 : 1.

Taxonomy of Producing Organism

Strain NCIMB 40513 was isolated from an agricultural

soil sample. It was grown and morphologically examined after 7~14 days on ISP2 (yeast extract-malt extract agar), ISP3 (oatmeal agar), ISP6 (peptone yeast extract iron agar) and ISP7 (tyrosine agar) following the method of SHIRLING and GOTTLIEB.[9] The isolate NCIMB 40513 had white aerial and substrate mycelium with grey spores. The spores were studied using a light microscope ×400 magnification and were arranged in spirals containing 10 or more spores. No pigment was produced on any of the agars including no melanoid pigment on ISP6 and ISP7. The cell wall was analysed for diaminopimelic acid content and type[10,11], which was shown to be L-DAP. A combination of physiological parameters was also examined using API strips 50CH and 20E. The results are shown in Table 1. Carbon utilisation studies were carried out using a minimal medium as described by STEVENSON.[11] With the accumulated data NCIMB 40513 was classified as a *Streptomyces* sp.

Table 1. Morphological and physiological data for NCIMB 40513.

DAP type	L-DAP
Spore chain	*Spirales*
Spore mass colour	Grey
Diffusible pigments	-
Melanoid formation	-
Growth at 45°C	-
Hydrolysis of starch	+
Liquefacation of gelatine	-
Utilisation of urea	+
Production of:	
nitrate reductase	-
catalase	+
cytochrome oxidase	-
H_2S	-
acetoin	+
Carbon utilisation:	
Adonitol	+
L-Arabinose	-
Cellobiose	+
D-Fructose	+
D Galactose	+
D-Glucose	+
Glycerol	+
Inositol	+
Inulin	+
Lactose	+
Maltose	+
Mannitol	+
D-Mannose	+
Melibiose	+
D-Raffinose	+
Rhamnose	+
Salicin	-
Trehalose	+
Xylitol	+
D-Xylose	+

Fermentation Conditions for *Streptomyces* sp. NCIMB 40513

0.5% of frozen vegetative stock was used to inoculate 100 ml of G1F medium in 500 ml flasks. Medium G1F consisted of bacteriological peptone (Oxoid) 7 g, Collofilm dextrin 30 g and black treacle 30 g in 1 liter of deionised water. The medium was adjusted to pH 6.9 with sodium hydroxide prior to sterilisation. The flasks were incubated at 28°C for 72 hours on a gyratory shaker at 240 rpm.

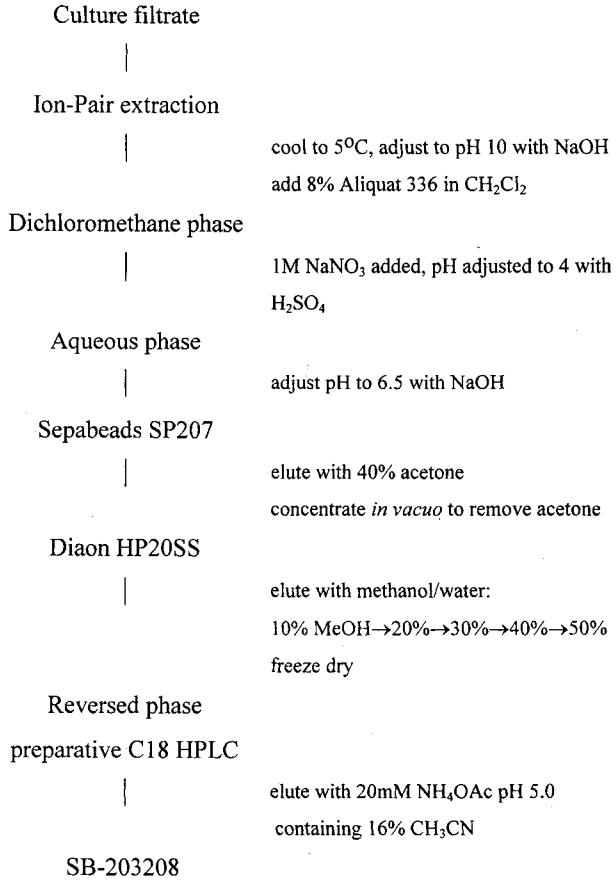
This primary seed was inoculated at 5% into secondary seed flasks which also contained G1F medium and which were prepared and incubated under the same conditions as the primary seeds.

The secondary seed was inoculated at 5% into 2 liter flasks containing 400 ml of G1F medium and incubated under the same conditions for 48 hours. The final stage fermentation was carried out in a 300 liter fermenter using G1F medium prepared with tap water and to which 0.5 ml PPG2000 antifoam had been added. The vessel was sterilised at 121°C for 60 minutes with the agitator running at 100 rpm after the medium had been adjusted to pH 6.9 with sodium hydroxide. The fermenter was inoculated with 5% of the tertiary seed and run under the following conditions which remained constant throughout the 67 hours incubation time: agitator speed 100 rpm, 28°C and 150 liter/minutes airflow with an overpressure of 0.2 bar.

Purification of SB-203208

The following media were used in the purification procedure for SB-203208 (Fig. 1): Aliquat 336 ion-pairing reagent from General Mills, Kankakee, Illinois, USA; Diaion HP20SS and Sepabeads SP207, non-ionic styrene divinyl benzene adsorption resin from Mitsubishi Chemical Industries Ltd, Japan. Preparative HPLC used a Microsorb C18 5 μm column (21.4×250 mm) from Ranin Instruments USA.

Fig. 1. The isolation of SB-203208 from *Streptomyces* sp. NCIMB 40513.



Isolation of SB-203208 from *Streptomyces* sp. NCIMB 40513

The isolation procedure for SB-203208 is shown in Fig. 1.

Results and Discussion

The isolation of SB-203208 was problematic due to the low fermentation titres, typically 800 μg/liter. In addition, initial isolation studies suggested that the active metabolite was only partially bound to a number of adsorption resins and performance was variable with ensuing poor recoveries. The activity initially bound to Diaion HP20 adsorption resin, but on subsequent purification with the same resin, some of the activity was not retained. This was later found to be due to the conversion of the fermentation metabolite to a second compound, SB-203207. The isolation and identification of β-methylphenylalanine within the partially purified matrix lead to the identification of SB-203207 as an analogue of SB-203208 with loss of a β-methylphenylalanyl moiety.

Investigation of the conditions under which SB-203208 converted to SB-203207 revealed slow, partial conversion at room temperature under basic conditions. Heating to 37°C and adjustment of the pH to 10 led to complete and irreversible conversion within twenty-four hours. Conversely SB-203208 was stable and unchanged at low pH and low temperatures. Since SB-203208 is considerably less polar than SB-203207, and thus easier to isolate, efficient purification procedures involved the use of low temperatures and maintaining the pH below 7 wherever possible. During the initial stage of ion-pair extraction (carried out at pH 10), the degradation of SB-203208 was prevented by using low temperatures and rapid re-adjustment of the organic phase. Following the isolation of SB-203208, subsequent hydrolysis to SB-203207 was readily achieved using the method described in Materials and Methods.

Table 2. Inhibition of isoleucyl tRNA synthetase (IRS) from various sources by SB-203207 and mupirocin.

	IC_{50} nM	
IRS	SB-203207	Mupirocin
Staphylococcus aureus Oxford	1.7	1.5
Streptomyces sp. NCIMB 40513	401	>200,000
Pseudomonas fluorescens NCIB 10586	1.4	>200,000
Candida albicans 3153A	1.84	2,700
Rat liver	<2	~20,000

Table 3. Antibacterial activity of SB-203207 and SB-203208.

	MIC (ug/ml)	
Organism	SB-203207	SB-203208
Escherichia coli DC2	>32	>128
Proteus mirabilis C889	>32	>128
Haemophilus influenza Q1	>32	32
Moraxella catarrhalis 1502	8	4
Pseudomonas aeruginosa 10662	>32	>128
Staphylococcus aureus Oxford	>32	>128
Staphylococcus epidermidis 100724	>32	>128
Enterococcus faecalis I	>32	>128
Streptococcus pyogenes CN10	>32	32
Streptococcus pneumoniae 1761	Not tested	8

Tests were carried out by serial dilution in nutrient broth by microtitre. Inoculum was prepared by dilution of an overnight broth culture to give the equivalent of approx 10^6 cells/ml.

Biological Properties

Using the enzyme inhibition assay described in the Materials and Methods section, the IC_{50} values for SB-203207 and mupirocin against isoleucyl tRNA (IRS) from *Staphylococcus aureus* Oxford, *Pseudomonas fluorescens* NCIB 10586, *Candida albicans* 3153A, rat liver and *Streptomyces* sp. NCIMB 40513 were determined.

The inhibition data shown in Table 2 demonstrate that SB-203207 is a potent inhibitor of the IRS from all the tested sources. Interestingly, against IRS from its own producing organism, the IC_{50} of SB-203207 was two orders of magnitude higher than for the other enzymes tested. In contrast, mupirocin is a highly potent inhibitor of the staphylococcal IRS, whereas it is a poor inhibitor of the other enzymes tested, including its own producing organism, *P. fluorescens* NCIB 10586 (IC_{50}>200,000 nM compared with ~1.5 nM for mupirocin-sensitive staphylococcal IRS).

SB-203207 was used in inhibition studies in preference

Table 4. Physico-chemical properties of SB-203207 and SB-203208.

	SB-203207	SB-203208
Appearance	colourless powder	colourless powder
UV (H_2O) λ_{max} nm	299	297
Molecular formula	$C_{19}H_{31}N_5O_8S$	$C_{29}H_{42}N_6O_9S$
Fab-MS positive ion	490 $(M+H)^+$	651 $(M+H)^+$
Molecular weight measured	489.1911	650.2738
calculated	489.1893	650.2734
IR ν_{max} (KBr) cm^{-1}	3420, 3363, 3225, 2963, 2930, 2875, 1636, 1509, 1460, 1384, 1338, 1285, 1211, 1117, 1080, 852, 770, 605.	3420, 3385, 3225, 2967, 2935, 2875, 1744, 1637, 1526, 1453, 1396,1340, 1289, 1262, 1211, 1112, 1069, 850, 766, 704, 655.
HPLC[a] (Rt minutes)	11.6	18.8

[a] HPLC: column, Waters Nova-pak C18, 60A, 4um, 3.9 x 150 um.
Solvent A = 0.05M NH_4OAc, solvent B = MeOH. Gradient 100% A / 0%B to 0 %A 100%B in 20 min. Detection wavelength 299nm Flow rate 1ml / min.

to SB-203208 because of its stability. Inhibition data obtained with SB-203208 suggested that both compounds give very similar levels of inhibition.

The concentration at which SB-203207 could be predicted from its IC_{50} to inhibit its own cellular synthetase completely, was close to the measured titre limit of SB-203208 in *Streptomyces* sp. NCIMB 40513 fermentations. It was therefore possible that inhibition of IRS by SB-203208 was limiting protein synthesis in the organism and further production of SB-203208.

The antibacterial activity of SB-203207 and SB-203208 was determined against a range of organisms by the microtitre method shown in Table 3. The activity was weak and limited in spectrum. Although SB-203207 is a very potent inhibitor of isoleucyl tRNA synthetase prepared from *Staphylococcus aureus* Oxford, no whole cell activity against the organism was detected. This might reflect poor penetration of SB-203207 through the staphylococcal cell wall.

During our screening programme for inhibitors of isoleucyl tRNA synthetase, SB-203208 has been isolated and identified from a number of streptomycete cultures, often co-produced with the polyether antibiotic nigericin. Although a ubiquitous metabolite, the poor and limited spectrum of antibacterial activity of SB-203208 is the probable reason for its having been only recently discovered using a target based screen.

Physico-chemical Properties

Physico-chemical properties are summarised in Table 4. Further detailed physico-chemical properties are described in part II of this paper.[5]

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