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SB-253514 and Analogues; Novel Inhibitors of Lipoprotein-Associated Phospholipase A_2 Produced by *Pseudomonas fluorescens* DSM 11579

I. Fermentation of Producing Strain, Isolation and Biological Activity

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Two new classes of inhibitors of $LpPLA_2$ have been identified in fermentations of *Pseudomonas fluorescens*. The two structurally isomeric series differ in the geometry of closure of the bicyclic carbamate and comprise a range of compounds varying only in length of their lipophilic sidechain. The most abundant species were extracted from the cells and purified by silica and C18 chromatography. Members of the more stable class were shown to be potent and selective competitive inhibitors of $LpPLA_2$.

Lipoprotein-associated phospholipase A_2 ($LpPLA_2$) is responsible for the conversion of phosphatidylcholine to lysophosphatidylcholine and oxidised free fatty acids during the conversion of low density lipoprotein to its oxidised form[1]. Both products are potent chemoattractants for circulating monocytes.[2] Lysophosphatidylcholine results in macrophage proliferation[3] and the endolithial dysfunction[4,5] observed in patients with atherosclerosis. Inhibition of $LpPLA_2$ would be expected to stop plaque build-up and provide an attractive strategy in the treatment of atherosclerosis and related disorders.

Screening chemical libraries against recombinant $LpPLA_2$ resulted in β-lactam hits. Elaboration of these structures resulted in the synthesis of the first β-lactam inhibitors of this enzyme.[6] These compounds only possessed relatively modest levels of activity and so in addition to further screening of chemical libraries, a high throughput screen of our microbial broth extract library was performed. This screen resulted in the detection of potent activity in extracts of *Pseudomonas fluorescens* DSM 11579.

In this article we describe the fermentation of the producing strain and the isolation and biological properties of the most abundant actives isolated from cultures of DSM 11579. The structural elucidation[7] and biotransformation[8] of these compounds is described in the accompanying articles.

Materials and Methods

Producing Strain

The bacterium DSM 11579 was isolated from a soil sample from Texas (USA). It was taxonomically classified as *Pseudomonas fluorescens* by the Deutsche Sammlong von Mikroorganism und Zellkulturen GmbH (DSMZ).

Materials

1-Decanoyl-2-(*p*-nitrophenylglutaryl)phosphatidylcholine (DNPG) was synthesised as published.[9]

Assay for $LpPLA_2$ Inhibition

$LpPLA_2$ was assayed in 96 well microplates using a Molecular Devices Tmax plate reader. Plates of increasing sample concentration were made up in buffer (50 mM Hepes, 150 mM NaCl, pH 7.4). Enzyme (100 pM) then substrate (20 μM DNPG) was added and the reaction monitored for 20 minutes at 405 nm and 37°C. Reactions were additionally carried out with 10 minutes pre-incubation of inhibitor with enzyme prior to substrate addition.

Determination of Compounds by HPLC and TLC

HPLC analysis was performed on a 4.6×150 mm Waters Spherisorb C8 column. The column was eluted with 50% aqueous MeCN at 1.5 ml per minute. The eluent was monitored at 254 and 205 nm, and by positive ion APCI-MS. For analysis of crude fermentation broth, samples (3 ml) were prepared by vortexing with MeOH (3 ml) and loading onto a pre-equilibrated C8 SPE cartridge containing 100 mg of sorbent. The cartridge was washed with 50% MeOH (3 ml) then the analytes eluted with 100% MeOH (3 ml). TLC analysis was carried out on silica plates (Kieselgel $60F_{254}$) eluted with CH_2Cl_2 - MeOH. 13 : 1 (v/v). Plates were visualised by UV (254 nm) and staining with 10% ammonium molybdate in 2 M H_2SO_4 followed by heat. The active components ran closely together at Rf 0.22~0.25.

Inoculum Preparation

The contents of a cryovial of *Pseudomonas fluorescens* strain DSM 11579 was grown on Oxoid Blood Agar Base in Petri dishes at 30°C. After one day, a loopful of well grown culture was used to inoculate 30 ml of Oxoid Tryptone Soya Broth in a 250 ml plain shake flask. The flasks were incubated at 28°C, 250 rpm (5 cm throw) for 24 hours then the culture was mixed 1 : 1 with cryopreservative (20% glycerol, 10% lactose) and stored in cryovials at −80°C.

Small Scale Fermentation

250 ml shake flasks containing 30 ml of YM medium (Bacto Peptone 0.5%, Difco Yeast Extract 0.3%, malt extract 0.3%, glucose 1%) were inoculated (4~5%) with cell suspension prepared as above, then incubated at 28°C, 250 rpm (5 cm throw) for 7 days.

Large Scale Fermentation

A 500 ml shake flask containing 100 ml L-broth seed medium (Difco Yeast Extract 0.5%, Difco Tryptone 1%, NaCl 1%, pH 7.4) was inoculated with 0.5 ml from a cryovial and incubated at 28°C, 240 rpm (5 cm throw) for 24 hours. This culture was used to inoculate (4~5% v/v) five, 2 liters plain shake flasks containing 400 ml L-broth seed medium which were incubated for 24 hours at 28°C, 240 rpm (5 cm throw).

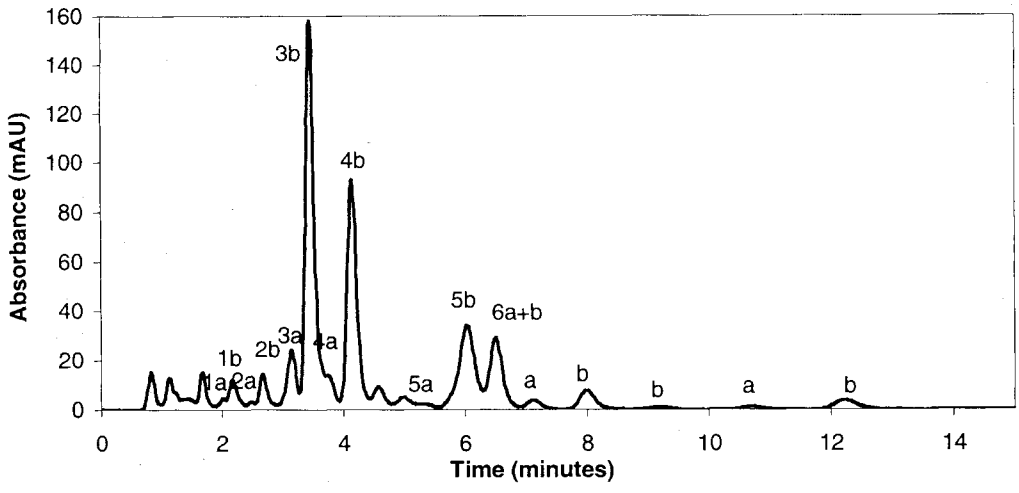
40 liters of F12 production medium (glucose 20%, Arkasoy 1%, spray dried corn steep liquor 0.125%, $(NH_4)_2SO_4$ 0.9%, $CaCO_3$ 2%, $MgSO_4 \cdot 7H_2O$ 0.05%, $NaHPO_4$ 0.325%, pH 7.0) together with 0.1% v/v SAG471 antifoam was sterilized in a 75 liters stainless steel fermenter at 121°C for 45 minutes using *in situ* steam sterilisation. After cooling to 26°C, the fermenter was inoculated with 2 liters of a 24 hours seed culture. Fermenters were incubated at 26°C, 0.5 bar overpressure with an airflow of 0.75 vessel volumes per minute and a rotor speed of 400 rpm. After 78 hours the culture was adjusted to pH 4.0~4.5 with *ortho*-phosphoric acid (9 M), heated to 70°C for 1 hour then cooled to 8°C with gentle agitation.

Isolation of SB-253514 (**3b**) and Analogues

40 liters of fermentation broth was passed through a disk stack centrifuge and the resulting cell slurry diluted with MeOH (70% final concentration) and stirred for 1 hour at 8°C. The extract was passed through a tubular bowl centrifuge to yield a clear amber solution. 15 liters of this solution was evaporated to dryness to yield a sticky gum (100 g). The gum was taken up in MeOH (400 ml) and added to 2.4 liters of EtOAc with stirring. The resulting white precipitate was removed by filtration and the supernatant concentrated *in vacuo* to yield a brown oil (23 g).

This oil was purified by chromatography on a 6×30 cm silica Flash-75™ cartridge (Biotage UK, Ltd.), eluting with DCM - MeOH 13 : 1 (v/v) at 110 ml/minute. Fractions (250 ml) containing the active components (as judged by TLC) were combined and concentrated to yield a 4.6 g fraction containing 1.5 g of **3b**. This fraction was further purified on a 4×15 cm C18 Biotage Flash-40™ column, eluting with a 50~90% MeCN gradient (15 ml/minute). Fractions (20 ml) containing the partially separated active components were collected and analysed by HPLC. The fractions rich in **3b** were further purified by C18 HPLC (44.6×250 mm) eluting with 65% MeCN for 17 minutes then 80% MeCN (30 ml/minute) to yield 980 mg of **3b** (29 minutes retention time). The other active components were similarly purified

Fig. 1. HPLC analysis of cell extracts.



from the other C18 Biotage fractions.

Results

Discovery and Fermentation Development

A high throughput screening of more than 35,000 microbial extracts resulted in the identification of $LpPLA_2$ inhibitors in extracts of *Pseudomonas fluorescens* strain DSM 11579. HPLC analysis of these extracts indicated that a number of related active species were present at low levels and accordingly, a fermentation development program was undertaken to increase the productivity of the organism. Initial shake flask experiments examined six different final stage incubation factors in eight media. This work identified several nutritional and environmental combinations giving significantly increased titers over the control. Optimization of carbon and nitrogen source levels identified a medium capable of giving a 20-fold improvement in detected inhibition activity in shake flasks and was subsequently scaled up to 40 liters fermentations.

Production of SB-253514 and Related Species

LCMS analysis (Fig. 1) of shake flask fermentations indicated that the actives fell into two classes, differentiated by chromophore and MS fragmentation characteristics. Within each class the various species differed in mass by 28 or 26 amu, suggesting that they incorporated varying lengths of fatty-acids (Table 1). The most abundant class, series 'b', exhibited a UV chromophore with maxima at 210 and 254 nm. Eleven members of this class were identified and six (**1**~**6b**) were produced with sufficient titer to target for isolation. The minor class, series 'a', comprised an apparently structurally isomeric series which exhibited UV maxima at 200 and 280 nm and a dominating M-44 fragmentation in the APCI mass spectrum. Mass ions corresponding to the parent molecular weight were observed by the milder ESI technique. Six species were identified with these characteristics but only two (**3a**, **4a**) were present in significant amounts. Subsequent fermentation development work, identified a medium which biased production almost entirely to the 'a' series (data not published here).

Further shake flask experiments indicated that the titer of the major inhibitor, SB-253514 (**3b**), increased up to day 4 then slowly decreased. Throughout production approximately 85% of the titer of **3b** was associated with the cells and insolubles whilst only 15% was soluble in the supernatant. Accordingly an isolation process was developed based on centrifugal recovery and extraction of the small but high yielding cell/solid fraction. Stability trials indicated that the actives were relatively stable to heat and low pH, but were unstable under alkaline conditions, series 'a' particularly so.

A 40 liter fermentation of strain DSM 11579 was carried out in a 75 liter vessel to provide material for isolation of the major actives. After a 20 hour lag phase the titer of **3b** steadily increased and was accompanied by a drop in pH (Fig. 2). Packed cell volume remained essentially constant

Table 1. Identified metabolites.

Compound	Mass	λ_{max} (nm)	Ret^n Time (min)
1a	526.6	200, 280	2.0
1b (SB-291071)	"	210, 254	2.1
2a	552.6	200, 280	2.5
2b (SB-291072)	"	210, 254	2.7
3a (SB-315021)	554.7	200, 280	3.1
3b (SB-253514)	"	210, 254	3.4
4a	580.7	200, 280	3.8
4b (SB-253517)	"	210, 254	4.1
5a	582.7	200, 280	5.4
5b (SB-253518)	"	210, 254	6.0
6a	608.7	200, 280	7.2
6b (SB-291073)	608.7	210, 254	7.2

Fig. 2. A 40 liter fermentation profile.

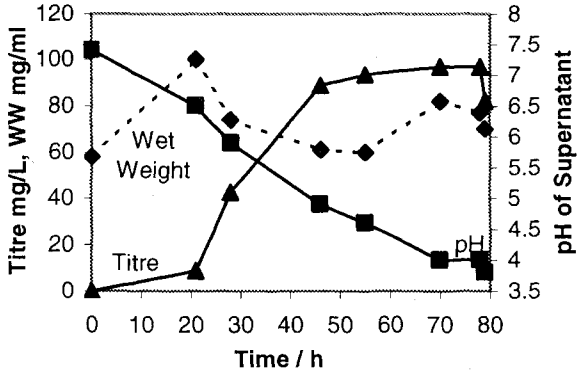
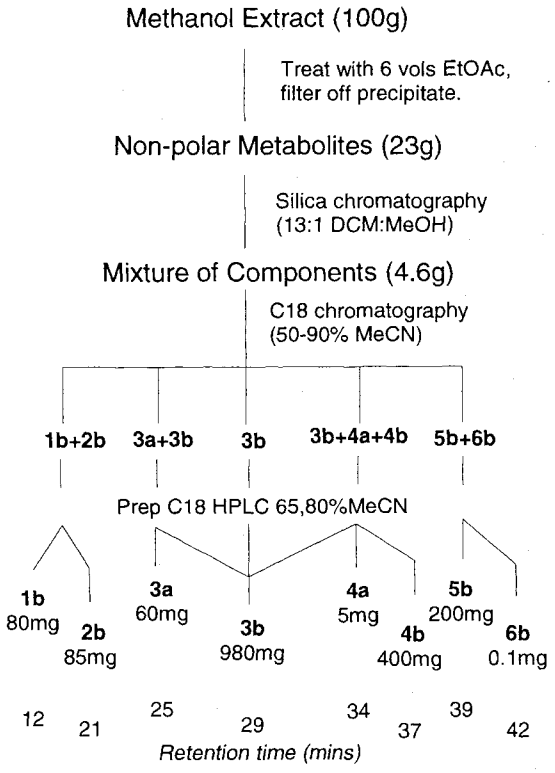


Fig. 3. Isolation of actives.



due to the highly particulate composition of the media and the dissolution of the $CaCO_3$ as pH dropped. Following a plateau in accretion of **3b** at 78 hours, the culture was pasteurised by heating to 75°C, then the broth was cooled and centrifuged to recover the cells and media solids. The resulting cell slurry was diluted with methanol to 70% v/v, stirred for 1 hour, then re-centrifuged to generate a clear amber coloured extract.

Isolation and Purification

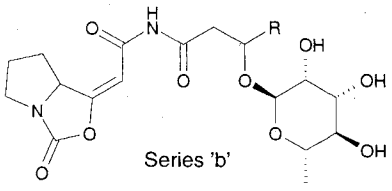
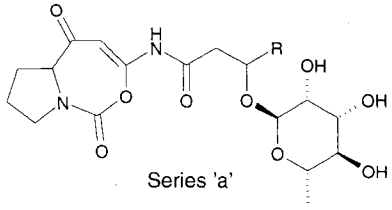
The major active components (**1**~**6b**, **3a**, **4a**) produced by fermentation of *Pseudomonas fluorescens* DSM 11579 were isolated from the methanolic cell extract by chromatography as shown (Fig. 3).

Spectroscopic studies on the purified SB-253514 (**3b**) and SB-315021 (**3a**) indicated that series 'b' incorporated a novel 5:5 bicyclic carbamate moiety, whilst series 'a' incorporated a 7:5 carbamate moiety as found in cyclocarbamide a/b[10]. Structures are indicated in Table 2. Full details of the structural elucidation of these compounds is presented in the accompanying paper.[7]

Biological Properties

The isolated compounds were assayed against recombinant $LpPLA_2$. Measurements were made both with

Table 2. Inhibition of $LpPLA_2$ by isolated metabolites.

	minutes pre-incubation	IC_{50} R = C_9H_{19} 1a/b	IC_{50} R = $C_{11}H_{21}$ 2a/b	IC_{50} R = $C_{11}H_{23}$ 3a/b	IC_{50} R = $C_{13}H_{25}$ 4a/b	IC_{50} R = $C_{13}H_{27}$ 5a/b	IC_{50} R = $C_{15}H_{29}$ 6a/b
 Series 'b'	**SB-**	**291071**	**291072**	**253514**	**253517**	**253518**	**291073**
	0	880 nM	1180 nM	530 nM	280 nM	96 nM	260 nM
	10	30 nM	20 nM	51 nM	28 nM	6 nM	10 nM
 Series 'a'				**315021**			
	0			3000 nM			
	10			400 nM			

and without a 10 minutes pre-incubation as the inactivation appeared time dependent (Table 2). The 5 : 5 (series 'b') compounds were competitive *vs.* DNPG and IC_{50} varied with the length of the side chain. LCMS experiments indicated the formation of a 1:1 covalent complex between $LpPLA_2$ and **3b** and further kinetic analysis indicated very slow reactivation ($T_{1/2}$>24 hours). Due to the instability of the 7 : 5 nucleus only the most abundant member of series 'a' (**3a**) was tested and this gave poor inhibition, presumably due its instability under the assay conditions.

Potent $LpPLA_2$ inhibition was similarly demonstrated by **3b** using isolated rabbit and human plasma. Good inhibitory activity was observed when **3b** was dosed intravenously to Watanabe heritable hyperlipidaemic rabbit, however, when dosed orally, no inhibition was observed. In order to determine its selectivity **3b** was screened against porcine elastase, trypsin, chymotrypsin, thermolysin, fungal aspartic protease, bacterial type IX metalloprotease and the herpes protease enzymes (CMV, VZV, HSV-2). No inhibition of these enzymes was observed, however, moderate inhibition of PAF acetylhydrolase II did occur (IC_{50} 485 nm, 10 minute pre-incubation). When tested against a panel CYP450 isozymes, **3b** showed no inhibition.

Discussion

The metabolites produced by *Pseudomonas fluorescens* strain DSM 11579 are present as two series of structurally isomeric 'twins', sharing the same fatty-acid derived backbones but differing in the geometry of closure of the bicyclic carbamate. Under the fermentation conditions used for this work, the more stable 5 : 5 series was predominant. The structural similarities between the series suggests that the two classes share a series of common precursors, possibly an acyclic β-ketoimide. Of the species characterised so far, a range of fatty acids from 12 to 18 carbons in length are utilized but, a number of other more lipophilic species are present in these fermentations.

The cross screening results, and LCMS observation of a covalent adduct between $LpPLA_2$ and **3b** indicate that in addition to being potent inhibitors of $LpPLA_2$, these compounds are selective and irreversible acylators. Whilst **3b** was not orally active, enzymatic deglycosylation[8] of this compound resulted in an orally active metabolite.

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