VOL. 53 NO. 7, JULY 2000 THE JOURNAL OF ANTIBIOTICS pp. 733 — 735

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

III. Biotransformation Using Naringinase

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(Received for publication January 11, 2000)

Lipoprotein associated phospholipase A_2 ($LpPLA_2$) is responsible for the conversion of phosphatidylcholine to lysophosphatidylcholine and oxidised free fatty acids, both of which are potent chemoattractants for circulating monocytes.[1,2] Build-up of lysophosphatidylcholine results in macrophage proliferation[3] and endolithial dysfunction[4,5] and so inhibition of $LpPLA_2$ provides an attractive strategy in the treatment of atherosclerosis and related disorders.

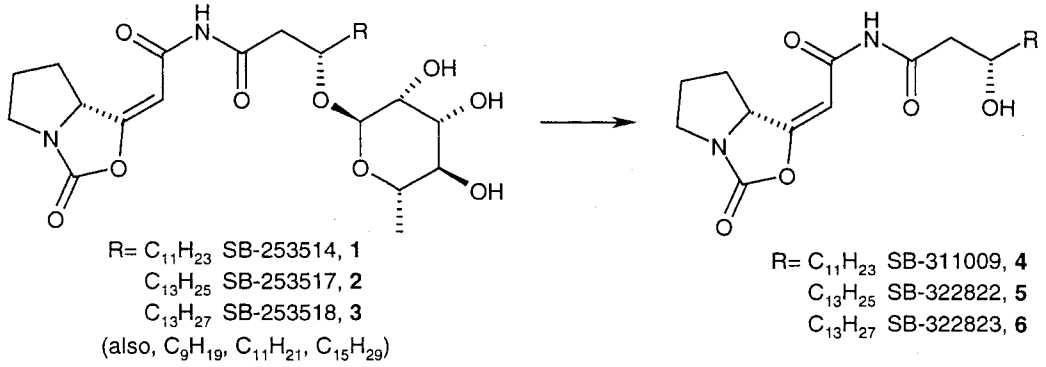
In the course of screening microbial extracts for inhibitors of lipoprotein associated phospholipase A_2 ($LpPLA_2$), two isomeric series of inhibitors were isolated from culture broths of *Pseudomonas fluorescens* DSM 11579.[6] The most active series of compounds are based on a 5:5 bicyclic carbamate function with a range of rhamnose substituted β-hydroxy fatty acid derived side chains (Fig. 1).

The most abundant inhibitor, SB-253514 (**1**) was shown to be a highly selective competitive inhibitor of $LpPLA_2$, and was active when administered iv to Watanabe heritable hyperlipidaemic (WHHL) rabbit. However, when dosed po this compound showed no inhibition. Additionally, spectroscopic studies[7] on **1** and related compounds had failed to conclusively indicate the stereochemistry of this compound as it had not been possible to obtain crystals for X-ray crystallography.

Deglycosylation of this compound† and two longer chain species, SB-253517 (**2**) and SB-253518 (**3**), was attempted in the hope that the products would be crystalline, and also demonstrate improved bioavailability.

Acid and base catalysed hydrolysis protocols for removal of the sugar were unsuccessful due to the sensitivity of the carbamate, and so enzymatic methods were sought. Six enzymes; naringinase (a crude preparation from *Penicillium decumbens* containing α-rhamnosidase activity), β-amylase, β-glucosidase, α-glucosidase, β-glucosidase, β-mannosidase were screened by incubation of **1** with the enzyme in buffer containing MeOH or DMSO as a co-solvent to partially solubilise the substrate.†† Only naringinase showed the desired activity, demonstrating 15% conversion to the deglycosylated compound, SB-311009 (**4**). Optimisation of the reaction parameters was carried out using a designed array experiment and resulted in the identification of a superior system using 1 mg/ml substrate

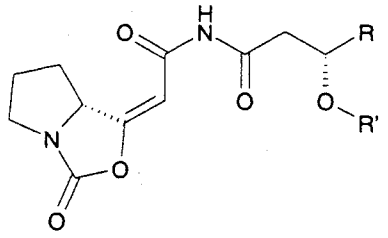
Fig. 1. Biotransformation of major actives from DSM 11579.



† Trace amounts of **4** were identified by LCMS in fermentations of strain DSM 11579 but attempts to increase the levels of these deglycosylated compounds by manipulating the culture conditions were unsuccessful.

†† 10% MeOH in buffer was found to be the optimal trade off between solubility and enzyme inactivation. The use of calcium alginate immobilised naringinase[8] in toluene and EtOAc was investigated as an alternative. However, whilst prolonged enzyme activity was seen, the rates of conversion were very low.

Table 1. Inhibition of $LpPLA_2$ (no pre-incubation).



IC_{50}	$R=C_{11}H_{23}$	$R=C_{13}H_{25}$	$R=C_{13}H_{27}$
R′=Rhamnose	**1** 530 nM	**2** 280 nM	**3** 96 nM
R′=H	**4** 170 nM	**5** 740 nM	**6** 35 nM

in 10% methanolic buffer (pH 4.8, 48°C) with 3 U of enzyme per mg of **1**. This system afforded 63% conversion after 16 hours. In an attempt to drive the reaction equilibrium it was found that the addition of bisulphite (provided as $Na_2S_2O_5$) to complex the liberated rhamnose increased levels of conversion to 83%. Further increasing the enzyme levels to 4 U/mg substrate afforded conversions of >98%.

Larger scale reactions were carried out using this system to prepare material for crystallisation and biological testing. The reactions were quenched after 16 hours by adding an equal volume of methanol to dissolve the product and precipitate the enzyme. Following centrifugation, the product was recovered from this solution by passage through a Biotage C18 Flash-40™ cartridge. The bound product was eluted with methanol and concentrated to yield a white powder. Residual substrate was removed by preparative C18 HPLC. The biotransformation of **2** and **3** was similarly effected on 10 mg scale.

Deglycosylation appeared to increase the potency of the saturated side-chain species (**1**, **3**) but reduced the potency of **2** which incorporates an unsaturated sidechain (Table 1). SB-311009, like its parent SB-253514, exhibited time dependent inhibition of $LpPLA_2$ and did not inhibit any of the cytochrome P450 isozymes tested (data not shown). The bioavailability of SB-311009 (**4**) was tested by oral dosing to WHHL rabbits and gratifyingly the deglycosylated compound showed significant and prolonged inhibition of $LpPLA_2$ (data to be published elsewhere).

Crystallisation of **4** was successfully achieved by slow evaporation from methanol at 7°C, and yielded clusters of colourless plates. These crystals were used to determine the stereochemistry of these species by X-ray crystallography.[7]

Experimental

Naringinase from *Penicillin decumbens* (320 U/g) was obtained from Sigma. Samples were assayed for inhibitory activity as previously described[6] but without pre-incubation of enzyme and compound. The reaction was monitored by HPLC as described for analysis of the parent compounds.[6] 410 mg of **3** was dissolved in MeOH (50 ml) and added to 450 ml of 50 mM CaOAc/AcOH buffer (pH 4.8) at 45°C, containing $Na_2S_2O_5$ (160 mg, 2.0 eq. $^-HSO_3$) and naringinase (4.8 g, 1500 U). The resulting suspension was stirred at 45°C for 14 hours when HPLC analysis indicated that the reaction was >98% complete. The mixture was cooled to 16°C, stirred with an equal volume of MeOH then centrifuged. The clarified solution was passed through a Biotage C18 Flash-40™ cartridge (4×15 cm) at 25 ml/minute. The column was washed with 50% MeOH (250 ml) then eluted with 100% MeOH. The product was collected and evaporated to yield a white powder (500 mg). Residual substrate and other impurities were removed by chromatography on a 44.6×250 mm C18 HPLC column eluting at 30 ml/minute with 80% MeCN for 30 minutes, then 90% MeCN. The product containing fractions (27~32 minutes) were collected and evaporated to yield a white powder (203 mg, 67% yield).

Acknowledgements

The author is indebted to HELEN BOYD, COLIN MACPHEE and KEVIN MILLINER for the measurement of *in vitro* and *in vivo* activity data.

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