

(E)-4- Aminostyryl Acetate a Novel Pancreatic Lipase Inhibitor Produced by a *Streptomyces* sp. MTCC 5219

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Abstract—A *Streptomyces* sp. MTCC 5219 isolated from a soil sample of a cow barnyard in India was found to produce a new lipase inhibitor. This bioactive compound was produced under optimum fermentation conditions and extracted by solvent extraction followed by chromatographic separation. The pure compound was found to have a molecular weight of 176 Daltons belonging to the class of enol acetate of p-amino phenyl acetaldehyde. It showed in vitro pancreatic lipase inhibition activity.

Index Terms—pancreatic lipase inhibitor, *Streptomyces* sp., structure, in-vitro assay.

I. INTRODUCTION

Pancreatic lipase (PL), the principal lipolytic enzyme synthesized and secreted by the pancreas plays a key role in the efficient digestion of triglycerides [1,2]. PL inhibition is one of the most widely studied mechanisms for the determination of the potential efficacy of natural products as anti-obesity agents [3,4]. Tetrahydrolipstatin (THL), a hydrogenated analogue of lipstatin isolated from actinomycetes (*Streptomyces toxytricini*), is a potent inhibitor of gastrointestinal lipases [5] and is commercially available as orlistat. In our course of screening for novel naturally occurring lipase inhibitor from actinomycetes, the ethyl acetate extract of the mycelium of a *Streptomyces* sp. MTCC 5219 was found to have potent in vitro bacterial lipase as well as porcine pancreatic mammalian lipase inhibition activity. The extract was subjected to bioactivity-guided fractionation to isolate a pure compound, which was found to be a novel compound.

II. MATERIALS AND METHODS

A. Organism and Fermentation

The organism used for the production of the inhibitor is a rifampicin resistant natural variant of *Streptomyces* sp. isolated from the soil of a cow barnyard and identified as *Streptomyces vayuensis* by Central Food Technological Research Institute (CFTRI), Mysore, India. The strain is deposited in Microbial Culture Collection Centre, India (MTCC no. 5219) as a new strain. The culture was obtained from CFTRI and the natural variant of this strain, which is resistant to 2.5 mcg/ml of rifampicin, was used for further studies.

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The producing culture was maintained in the form of working stock in a test tube slant having a composition of L-asparagine (anhydrous) 1g, glycerol 10g, potassium hydrogen phosphate (anhydrous) 1g, trace salt solution (TSS) (copper sulphate 7g, ferrous sulphate 1g, manganese chloride 8g, zinc sulphate 2g and demineralized water 1L) 1ml/L, agar 20g and demineralized water 1L (pH 7.0-7.4). The seed medium contained glucose 15g, corn steep liquor 5g, soybean meal 15g, calcium carbonate (CaCO₃) 2g, sodium chloride (NaCl) 5g and demineralized water 1L. The pH of the medium was adjusted to 7.0-7.4 with 0.5(N) NaOH before sterilization. Three different production medium were tested for optimum production of this molecule in the shake flask. Those include 135SS (soluble starch 25g, glucose 10g, soybean meal 20g, yeast extract 2g, CaCO₃ 3g, NaCl 5g, magnesium sulphate 1g and demineralized water 1L), 134 (soluble starch 10g, glucose 10g, peptone 7.5g, malt extract 7.5g, NaCl 3g, magnesium sulphate 1g and demineralized water 1L) and 5189M (malt extract 20g, yeast extract 2g, glucose 10g, (NH₄)₂HPO₄ and demineralized water 1L). The pH of the medium was adjusted to 7.0-7.4 with 0.5(N) NaOH before sterilization. The mycelial culture of streptomyces from the slant was inoculated into a 1L of Erlenmeyer flask containing 200ml of seed medium. The inoculated flask was incubated on an orbital pilot shaker at 30 °C for 72h at 220rpm. A 3% of this seed was transferred to 1L of Erlenmeyer flask containing 200ml of each production media, followed by incubation on an orbital pilot shaker at 30 °C for 72h at 220rpm. Periodic sampling was carried out from each flask to carry out PMV (Packed Mycelial Volume) and lipase inhibition Assay as described below.

Production of the active molecule was carried out in a 25L fermentor (Emenvee, McClins India, Pune) containing 18L optimized production medium (135SS). The inoculum was prepared in three 1L Erlenmeyer flasks containing 200ml of seed medium cultivated for 72h as described above. A 3% seed was used for inoculating the fermentor. The fermentation was carried out at 30 °C, 180rpm, and 0.625vvm with a backpressure of 0.5bar. The batch was terminated at 72h and processed for purification of active compound.

B. Extraction and Isolation

As the product is intracellular, the 18L harvested broth was subjected to filter press to get 1.831kg mycelial cake that was further extracted with 18L of ethyl acetate. The concentrated ethyl acetate extract (23g) was further fractionated with 1.5L petroleum ether. The insoluble portion (13.5g) was treated with 1.5L of CHCl₃. The CHCl₃ fraction (5g) was further purified by means of silica gel column (50g, 100-200 mesh, 100 x 2.5cm i.d.) and eluted

with mixture of CHCl_3 and CH_3OH with increasing polarity. The eluted fractions were analyzed for bacterial lipase inhibition assay and TLC. The bioactive fraction was concentrated to dryness to get 23mg pure compound and subjected to ^1H NMR, ^{13}C NMR, MS and IR in order to elucidate its molecular structure.

C. Structural Elucidation Of Isolated Compound

NMR spectra were recorded on a 300 MHz using CD_3OD as solvent and TMS as internal standard. Mass spectrum was measured on a Bruker Daltronics analytical spectrometer. IR spectrum was taken on a Perkin Elmer Paragon 100 spectro- photometer. HPLC analysis was performed on a LC-2010HT Liquid Chromatograph; Shimadzu, Japan, 8A model equipped with a quaternary pump system, a PDA detector and computerized data system. The mobile phase consists of CH_3CN and H_2O , 98:2 (v/v), with a flow rate of 1ml/min. The column used was 125 \times 4m, Lichrosphere100 RP-18C (5 μm). Analytical TLC was carried out on precoated silica gel plates (10 \times 2cm, 0.2mm thickness) from Merck (Silica gel GF₂₅₄) using CHCl_3 and CH_3OH , 9:1 (v/v) and the detection by visualization under UV light. (λ_{254} and λ_{366}).

D. Biological Activity in Vitro

Bacterial lipase (AMANO pancreatic lipase) assay was carried out using Secomam auto analyzer. The crude extract (20mg/ml) was dissolved in DMSO (50 μl) and added to sodium-deoxycholate (80 μl of a stock solution of 1mg/ml of distilled water). The buffer used for the assay was 100mM-monobasic sodium di-hydrogen phosphate containing 150mM-NaCl. The 0.5% triton-X100 made to pH 7.4 was added to the assay mixture along with 10 μl of enzyme solution from stock solution of 10mg/ml. Finally the substrate, 50mM of p-nitro phenyl butyrate (p-NPB) was added to start the reaction. The increase in optical density (OD) was recorded at 400nm for 3mins using auto analyzer.

Mammalian lipase (Sigma Porcine pancreatic lipase) inhibition assay was carried out using triolein as a substrate. The hydrolysis was carried out at pH 8.0 and 37 $^\circ\text{C}$ over the period of 10min using a pH stat. The substrate emulsion (1.5ml/assay) was prepared by ultrasonication of triolein (30mg/ml) in a solution containing 1mM-taurochenodeoxycholate, 9mM-taurocholate, 0.1mM-cholesterol, 2mM-tris/ hydrochloric acid, 1mM-phosphatidylcholine, 15mg/ml bovine serum albumin (BSA), 100mM-NaCl and 1mM-calcium chloride. After the addition of the test compound, which was dissolved in 150 μl of DMSO, the pH was adjusted to 8.0 and the reaction was started within 1min by the addition of 15-20 μl of lipase (dissolved in saline/ 4% BSA at a concentration of 70 $\mu\text{g}/\text{ml}$). A drop in pH was monitored over the period of 10mins. A blank DMSO without the test compound was a solvent control for substrate inhibition [6]. The amount of lipolytic activity was adjusted to result in the generation of 0.2 to 0.3 μmol fatty acid/ml per min.

III. RESULTS AND DISCUSSION

In our present study, a new lipase inhibitor was produced from a *Streptomyces* sp. MTCC 5219 using optimized medium and fermentation conditions. As the active

molecule is intracellular in nature and growth associated, the higher biomass buildup is desirable to get a higher product yield. During the initial shake flask studies, we screened few production media containing different sources of carbon and nitrogen. The shake flask kinetics of different media is shown in figure 1. figure 1a shows the biomass trend obtained with three media in terms of PMV. The bacterial lipase inhibition activity of the mycelial extract obtained from three different mediums at different log hours is shown in figure 1b. Since media 135SS was found to produce higher biomass and active metabolite in shorter period of time, it was selected as a production medium for large scale fermentation.

The fermentation process was scaled up from shake flask to 25L fermentor and harvested broth processed to get 23mg of pure bioactive compound. The active compound could be easily extracted from the mycelia using ethyl acetate. During bioactivity-guided fractionation of ethyl acetate crude extract, petroleum ether took away inactive impurities whereas CHCl_3 soluble portion retained the maximum activity. Table I showed the in vitro bacterial lipase inhibition activity of the different fractions.

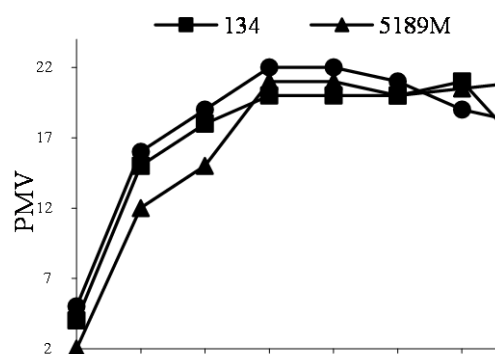


Figure 1a. Comparison of biomass in different production media. (PMV is determined by centrifuging 10ml of whole broth sample at 5000rpm for 15mins.)

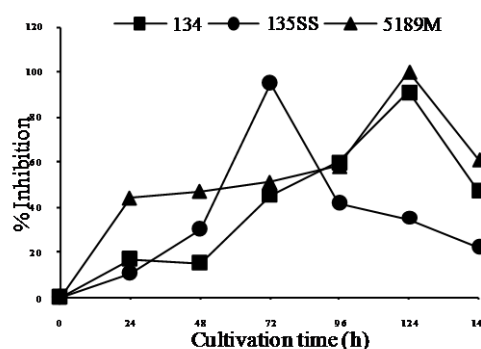


Figure 1b. Bacterial Lipase Inhibition activity of three different media at different log hours.

TABLE I. BIOACTIVITY GUIDED FRACTIONATION.

Fraction name	%Inhibition (Bacterial lipase)
Petroleum ether soluble fraction	29
Chloroform soluble fraction	100
Ethyl acetate soluble fraction	34
Water soluble fraction	10

The CHCl_3 soluble active fraction was further purified by silica gel column chromatography using CHCl_3 and CH_3OH mixture. Table II showed the different fractions obtained with silica gel column chromatography along with their in vitro bacterial lipase inhibition activity.

The fraction D showed the presence of bioactive compound and hence it was subjected to HPLC, which showed major peak at RT 12.9min in HPLC with 95.5% purity as shown in figure 2.

This fraction was concentrated to dryness to get 23mg compound. The molecular weight of the compound was determined to be 177 as the mass spectrum showed M-1 as 176 as shown in figure 3.

IR spectrum showed absorptions at 1669 cm^{-1} and 3300 cm^{-1} showing the presence of a carbonyl group and an aromatic amine respectively as shown in figure 4.

TABLE II. BIOACTIVITY GUIDED COLUMN PURIFICATION

Column fractions	% Inhibition (Bacterial lipase)
Fraction A (99% CHCl_3 -1% CH_3OH)	No inhibition
Fraction B (98% CHCl_3 -2% CH_3OH)	6
Fraction C (97% CHCl_3 -3% CH_3OH)	27
Fraction D (96% CHCl_3 -4% CH_3OH)	95
Fraction E (95% CHCl_3 -5% CH_3OH)	11
Fraction F (94% CHCl_3 -6% CH_3OH)	9
Fraction G (93% CHCl_3 -7% CH_3OH)	5
Fraction H (92% CHCl_3 -8% CH_3OH)	2
Fraction I (90% CHCl_3 -10% CH_3OH)	No inhibition

The ^1H NMR signals at δ 7.124 (2 H, d, $J = 29, 8.7\text{ Hz}$, H-2), δ 6.682 (2H,d, $J = 29, 8.7\text{ Hz}$, H-3), δ 7.241 (1 H, d, $J = 49, 14.7\text{ Hz}$, H-1), δ 6.099 (1 H, $J = 49, 14.7\text{ Hz}$, H-4), δ 1.986 (3 H, s, H-5), indicated the presence of para-disubstituted phenyl ring attached to a *trans* double bond. The singlet at δ 1.986 clearly indicated an acetyl methyl group. Analysis of the ^{13}C NMR spectrum showed one carbonyl signal (d 169.09), two olefinic signals (d 156.119, 127.724), four aromatic signals (126.304, 120.083, 115.088, 113.241) and one alkyl signal (21.121) as shown in Table III.

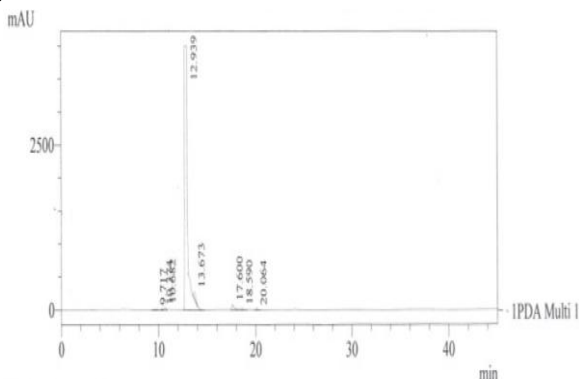


Figure 2. HPLC purity of isolated compound

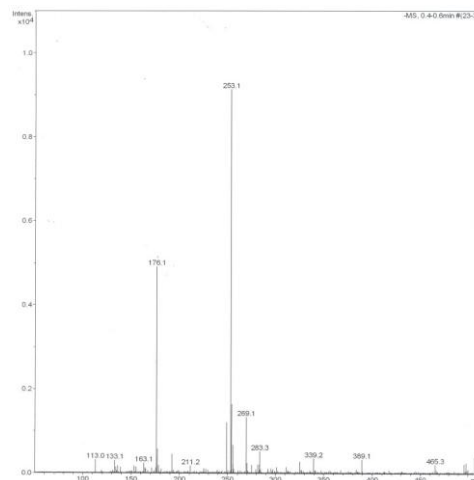


Figure 3. Mass Spectrum of isolated compound

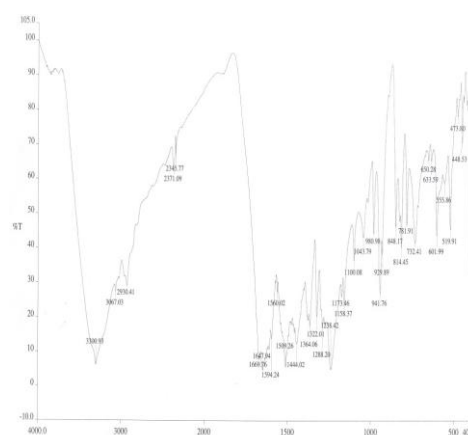


Figure 4. IR Spectrum of isolated compound

From the spectral data, the structure of the isolated compound is elucidated as shown in figure 5. Based on the above studies, we propose that the active molecule is (E)-4-Aminostyryl acetate belonging to the class of enol acetate of p-amino phenyl acetaldehyde.

TABLE III. NMR DATA OF ISOLATED COMPOUND IN CD_3OD , TMS (BRUKER DALTRONICS).

No.	$\delta^{13}\text{C}$	$\delta^1\text{H}$ (mult, J)
1	169.09	
2	156.119	7.241 (d, 49, 14.7 Hz)
3	127.724	6.099 (d, 49, 14.7 Hz)
4	126.304	7.124 (d, 29, 8.7 Hz)
5	120.083	
6	115.088	6.682 (d, 29, 8.7 Hz)
7	113.241	
8	21.121	1.986 (s)

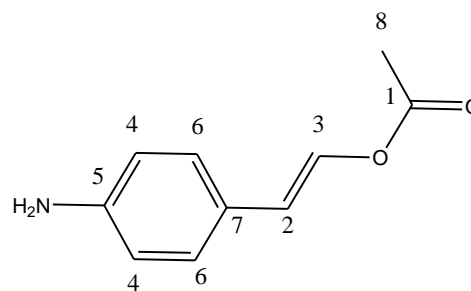


Figure 5. Structure of isolated pure compound.

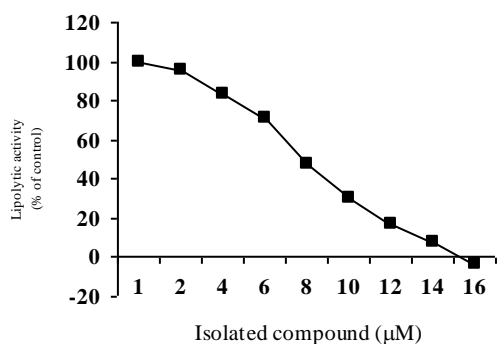


Figure 6. Inhibition of porcine pancreatic mammalian lipase by isolated pure compound.

The *in vitro* mammalian lipase inhibition activity of the isolated compound is shown in figure 6. The hydrolysis of triolein is measured in terms of drop in pH under assay condition and presence of enzyme inhibitor affects hydrolysis.

The compound inhibited the hydrolysis of trioleate by porcine pancreatic lipase dose dependently with IC₅₀ of 7.46µM. Even though the IC₅₀ value of this molecule is high compared to commercial pancreatic lipase inhibitor orlistat, the novelty and possibility of derivatization might make this molecule a good candidate for further investigation

IV. CONCLUSION

A low molecular weight molecule isolated from *Streptomyces sp.* MTCC5219 showed *in-vitro* lipid

lowering activity in mammalian lipase inhibition assay. Further *in-vivo* activity evaluation and chemical modification studies along with toxicity and pharmacodynamic studies are underway. We believe that the lower size of the molecule along with a good solubility property would give this molecule a better druggability character.

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