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Purification and Characterization of an Alkaline Lipase from a Newly Isolated *Pseudomonas mendocina* PK-12CS and Chemoselective Hydrolysis of Fatty Acid Ester[†]

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Abstract—Lipase isolated from a soil isolate, *Pseudomonas mendocina* (PK-12CS) chemoselectively hydrolyzed the fatty ester group in presence of arbamate of compound 5-amino-2,4-dihydro-3H-1,2,4-triazole-3 ones, a class of compounds which are attractive starting materials for the synthesis of triazole annealed heterocycles. The enzymatic method provides an easy access to the synthesis of *N*-substituted glycine. Under optimized fermentation conditions the culture produced 3510 Lipolytic Units/mL of cell free fermentation broth in 20 h of fermentation. The purified lipase exhibited molecular mass of 80 kDa on SDS polyacrylamide gel electrophoresis. The enzyme was stable at room temperature for more than a month and expressed maximum activity at 37 °C and pH 8.

Introduction

Lipase in preparative biotransformation has drawn attention since long.[1–3] In the course of the years, the priority in organic synthesis has shifted from classical chemical methods to chemico-enzymatic methodology for the preparation of substances in optically pure forms. One of the most exciting aspects of this fast growing field is the possibility of the stereoselective/enantioselective reaction, which is often rather difficult, to introduce centers of chirality or perform regiospecific transformations by conventional chemical methods.[4–10]

However, most of the isolated enzymes in pure form are expensive and to make the field of biocatalysis cost-effective, methods other than those which employ pure enzymes are desired. Crude-enzyme has emerged in recent years as an efficient tool and has displayed excellent synthesis of optically active substances from achiral or racemic compounds.[11–21]

Pseudomonas mendocina PK-12CS, which was isolated in this laboratory, harboured lipase/esterase gene. The gene coding for lipase bears Gen Bank accession number AY091666 and partial nucleotide sequence of esterase gene (Gen BankAF 395200-AF 395202). An extensive search made on databases (NCBI, EMBI, ExPASy) revealed that the *P. mendocina* PK-12CS lipase examined by us is different from a detergent lipase from *P. mendocina*.[22,23] The PK-12CS lipase was resistant to non-ionic surfactants, exhibited higher activities towards natural oils and triglycerides of long chain fatty acids than that toward triglycerides of short-chain fatty acids and showed good stability in water-miscible and immiscible solvents. This is the property of the enzyme which is uniquely suited for chemico-enzymatic methodology in organic synthesis. Although a number of lipases and esterases capable of exhibiting chemoselective reactions have been reported earlier, the chemoselective preference of

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lipases for fatty acid esters over carbamates have not been reported. The present observations clearly demonstrates that lipase from genus *Pseudomonas* possess this property. This fact and the ground reality concerning the chemical hydrolysis of not being able to differentiate these two functionalities during basic hydrolysis make the lipases from *Pseudomonas* attractive for bio-organic use.

Results and Discussion

Mineral medium supplemented with glycerol and DAP favoured maximum yield of extracellular pNPC16 activity (3510 units/mL fermentation broth) in 20 h of fermentation period. The isolate *P. mendocina* PK-12CS was able to grow on olive oil, soyabean oil, tributyrin and trioline as a sole source of carbon and energy. However the presence of these oils and triglycerides were found to be ineffective in enhancing the extracellular level of lipase in fermentation broth. Different optimization approaches for lipase production by micro-organisms have revealed that high activity levels are achieved when lipids are present in the growing medium.[24–27] However, in some cases lipids have also been found to inhibit lipase production.[28] Since PK-12CS can produce extracellular lipase without lipids, it is assumed that the micro-organism synthesizes lipase constitutively. This property of the micro-organism in lipase synthesis has also been reported by Kunimoto et al.[29]

Attempts to purify the lipase by classical protein purification techniques were not effective. Similar problems in lipase purification has been experienced by several investigators.[30–33] To refine the purification step, as it has been reported for *Alternaria brassicicola* Lipase[33] the PK-12CS lipase was first fractionated on DE-52 anion-exchanger and the most active fractions were pooled and re-chromatographed on DE-52. Lipase thus purified was homogeneous on the SDS-PAGE gel (Fig. 1) and its molecular mass was estimated to be 80 kDa based on the position of the marker proteins. The specific activity of the purified enzyme was 275×10^3 U/mg, the purification being 241-fold starting from the culture filtrate with activity yield of 14.78% (Table 1). The activity assay of the purified protein after SDS-PAGE was carried out by a zymogram technique,[34] but the enzyme activity in the gel could not be detected. Possibly the renaturation of the protein which is essential for the expression of the lipase activity in zymogram technique, was not successful due to the sensitivity of the enzyme which was severely inhibited by 1% SDS. However, when performed native-PAGE, the location of purified band in unstained gel corresponding to the protein band in the gel stained with Coomasie Brilliant Blue R-250 or Silver staining showed lipase activity against the substrate pNPC16 (data not shown). The purified enzyme was smeared significantly on the separating gel when performed native-PAGE and was visible after staining the gel. This suggest that the enzyme had a tendency to aggregate under the experimental conditions and resembled other lipase preparations described in the literature.[35,36] The enzyme exhibited higher activities towards triglycerides of long-chain fatty acids, (tripalmitin, triolein, trilinolin) and natural oils like olive oil, soyabean oil, mustard oil, coconut oil and almond oil than that towards short chain triglyceride at pH 8.0 (Table 2).

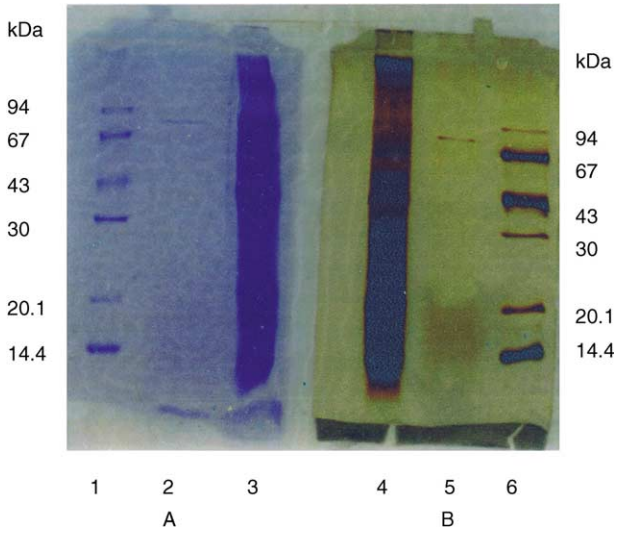


Figure 1. SDS-PAGE of purified PK-12CS lipase: (A) gel stained with Coomasie Brilliant Blue R-250; lane 1, molecular weight marker; lane 2, purified lipase; lane 3, acetone precipitate; (B) gel stained with silver-nitrate; lane 4, acetone precipitate; lane 5, purified lipase; lane 6, molecular weight marker.

Biochemical properties of the PK-12CS lipase

The lipase showed optimum activity at pH 8 and activity decreased sharply above pH 8.6 or below pH 7.6. The enzyme was stable at broad pH range (5.6–9.0), with highest in the pH range 7.6–8.6 at 37 °C. At pH 2 it was almost instantaneously inactivated (complete inactivation after 5 min). At pH 12, a half-life of 90 min was calculated (Table 1). The enzyme can be regarded as alkaline lipase on the basis of its optimum pH value for lipolytic activity.

The enzyme proved to be temperature sensitive. Maximum activity was expressed at 37 °C and above 37 °C the activity declined sharply. Approximately, 50% of the enzyme activity was lost during 1 h incubation at 45 and at 57 °C the enzyme retained 25% of its initial activity. The half-life of the enzyme at 60 and 65 °C was 10 and 4 min, respectively (Table 1). At room temperature (28–30 °C), no loss of activity could be detected at pH 8 after a month.

Various compounds were examined for their effect on the activity of the enzyme (Table 3). The lipase activity was not influenced by SH-inhibitor such as phenyl methyl sulfonyl fluoride (PMSF), suggesting that the thiol groups do not play an important role in the active site of PK-12CS lipase.[37] But the involvement of histidine residue in the enzyme activity was apparent by its strong inhibition by diethylpyrocarbonate (DEPC) at 1 mM and 65% at 0.1 mM.[38] Like many Lipases from *Pseudomonas*,[38,39] the enzyme was strongly inhibited by Hg^{2+}. However, the inhibition by other metal ions like Fe^{3+}, Ca^{2+}, Cu^{2+}, Ba^{2+} Zn^{2+} and Mn^{2+} was negligible.

Table 1. Purification of the extracellular alkaline lipase from *P. mendocina* PK-12CS and its characteristics

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Cell free extract	21.04×10^6	18,375.00	1.14×10^3	100	1
Acetone precipitation	13.53×10^6	3135.50	4.32×10^3	64.30	3.79
DE-52 anion exchange chromatography	3.11×10^6	11.32	274.73×10^3	14.78	240.99

The enzyme was purified from 6000 mL of culture filtrate. The activity assay was performed using *p*-NPC16 as substrate. Characteristics: Optimum pH range, 7.6–8.2; optimum temperature 37 °C; stability: $t_{\frac{1}{2}}$ at 37 °C, 168 h; $t_{\frac{1}{2}}$ at 45 °C, 60 min; $t_{\frac{1}{2}}$ at 60 °C, 10 min; $t_{\frac{1}{2}}$ at 65 °C, 4 min; pH 5.6–9, 14 h; $t_{\frac{1}{2}}$ at pH 4, 2 h; $t_{\frac{1}{2}}$ at pH 11, 5 h; $t_{\frac{1}{2}}$ at pH 12, 1.5 h.

Table 2. Activity of PK-12CS lipase towards various triglycerides and oils

Natural oils/triglycerides	Relative activity (%)[a]
Olive oil	100.00
Soyabean oil	109.50
Mustard oil	70.75
Coconut oil	106.50
Almond oil	116.73
Triolein	87.85
Tripalmitin	89.00
Trilinolin	93.70
Tributyrin	44.50

Data are mean of triplicate determinations.
[a]Results are the relative activity as the percentage of that of olive oil.

About 20% activation in the activity was observed with 1 mM Mg^{2+}. A reduction in the activity up to 38% was observed with 1 mM EDTA. The effect of EDTA could be due to influence on the interfacial area between substrate and enzyme.[40] The lipase showed appreciably good stability in water-miscible and immiscible solvents (Table 4).

Chemoselective hydrolysis

The compounds **1** and **2** synthesized in this laboratory[41] possess both ester and carbamate group and is interesting substrate for the enzymatic reaction because of the significant difference in the chemical reactivities of these groups[42] and it has been observed that even controlled base or acid catalyzed hydrolysis will invariably lead to the hydrolysis of carbamates with simultaneous decarboxylation.[43–45] Reaction of compounds **1** and **2** with crude lipase resulted in compounds **3** and **4** respectively, in which selectively *N*-methylethoxycarbonyl (CH_2CO_2Et) was hydrolyzed and *N*-methoxycarbonyl (CO_2Me) group remained unchanged. By conventional chemical methods the compounds **3** and **4** in refluxing DMF (130 °C, 10 h) yield the *N*-substituted glycine **5** and **6**, respectively. Chemically compound **5** and **6** were also obtained by first refluxing compounds **1** and **2** in DMF (130 °C, 8 h) to yield the decarboxylated products **7** and **8**, which on acid hydrolysis (85 °C, 3 h) resulted in free acids **5** and **6** (Scheme 1). An example of the utilization of mild reaction conditions is further demonstrated by enzymatic hydrolysis of compounds **7** and **8** to yield *N*-substituted glycine **5** and **6**. The characterization data for various product is given in Table 5. It is quite likely that the carbamate group in compound **1** and **2** in the present investigation is hydrogen bonded and may effect the hydrolysis via the lipase, however, our experiments with the *N*-methyl derivative (Me instead of CO_2Me) gave the free acid while in a separate experiment, the derivative with Me instead of CH_2CO_2Et in compounds **1** and **2** did not show hydrolysis of the carbamate group. This demonstrates that the selectivity of the biocatalyst depends on the nature of the ester that is to be hydrolyzed. Attempts were also made to effect the aforesaid chemoselective reaction with commercially available lipase CCL (*Candida cylinderacea*, Sigma). The lipase CCL was sluggish towards compounds **1** and **2**.

Table 3. Effect of various reagents on the activity of PK-12CS lipase

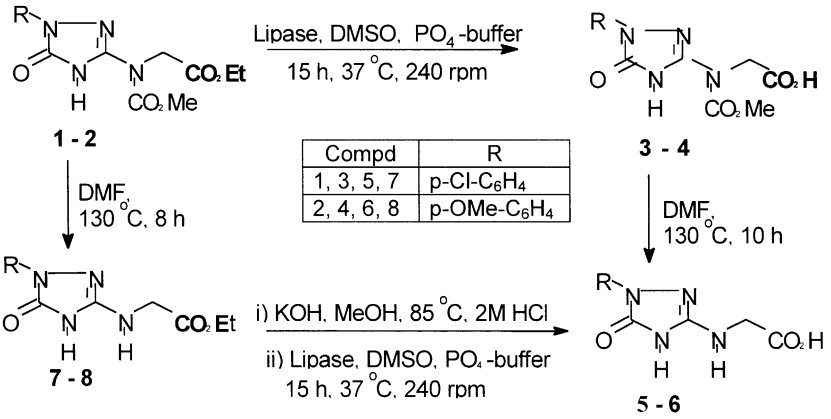
Reagents	Concentration (mM)	Relative activity (%)[a]
$BaCl_2$	1	72.40
$CuCl_2$	1	48.27
$FeCl_3$	1	2.76
KCl	1	100.00
NaCl	1	103.00
$MnCl_2$	1	82.76
$MgCl_2$	1	120.69
$ZnSO_4$	1	93.10
$HgCl_2$	1	No activity
$CaCl_2$	1	103.44
EDTA	1	62.07
PMSF	0.1	100.00
DEPC	0.1	34.48
SDS	0.4%	5.10
Triton X-100	4%	100.00
Tween-80	4%	100.00
Tween-20	4%	100.00
No addition	—	100.00

Data are mean of triplicate determinations.
PK-12CS Lipase was incubated with different reagents at 37 °C for 2 h before the hydrolytic activity was measured by the pNPC16 substrate.
[a]Results are the relative activity as the percentage of the maximum activity recorded without any addition.

Table 4. Stability of PK-12CS lipase in various organic solvents

Solvents	Relative activity (%)[a]
No addition	100.00
Methanol	76.92
Ethyl alcohol	82.69
DMF	69.23
Heptane	97.25
Hexane	99.23
Toluene	99.00

Data are mean of triplicate determination. PK-12CS lipase was incubated in various solvents at room temperature (28–30 °C) for $2\frac{1}{2}$ h before the hydrolytic activity was measured by pNPC16 substrate.
[a]Results are the relative activity as the percentage of the maximum activity recorded without any addition.



Scheme 1.

Table 5. Physical and spectroscopic data of compounds **3–8**

Reaction products	IR, ^{1}H NMR, MS	Yield (%)	Mp (°C)
3	*m/z* 326 (M^+); IR (KBr) 1620 (CO_2H), 1674 (CO); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.37 (s, 3H, OMe), 4.14 (s, 2H, CH_2), 7.42 (d, 2H, *J* = 7 Hz, ArH), 7.76 (d, 2H, *J* = 7 Hz, ArH), 10.40 (brs, 1H, NH).	31.34	161
4	*m/z* 322 (M^+); IR (KBr) 1629 (CO_2H), 1678 (CO); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.76 (s, 3H, OMe) , 3.82 (s, 3H, OMe), 4.16 (s, 2H, CH_2), 7.41 (d, 2H, *J* = 7 Hz, ArH), 7.75 (d, 2H, *J* = 7 Hz, ArH), 10.41 (brs, 1H, NH).	32.75	156
5	*m/z* 269 (M^+); IR (KBr) 1636 (CO_2H), 1685 (CO); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.94 (d, 2H, *J* = 8 Hz, CH_2), 5.78 (brs, 1H, NH), 7.44 (d, 2H, *J* = 7 Hz, ArH), 7.77 (d, 2H, *J* = 7 Hz, ArH), 10.56 (brs, 1H, NH); analysis calcd for: $C_{10}H_9ClN_4O_3$: C, 44.70; H, 3.37; N, 20.85; found: C, 44.51; H, 3.49; N, 21.93.	45.60	218
6	*m/z* 264 (M^+); IR (KBr) 1630 (CO_2H), 1691 (CO); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.85 (s, 3H, OMe), 4.01 (d, 2H, *J* = 8 Hz, CH_2), 5.81 (brs, 2H, NH), 7.48 (d, 2H, *J* = 7 Hz, ArH), 7.82 (d, 2H, *J* = 7 Hz, ArH), 10.50 (brs, 1H, NH); analysis calcd for: $C_{11}H_{12}N_4O_4$: C, 50.00; H, 4.57; N, 21.20; found: C, 50.35; H, 4.58; N, 21.63.	51.30	198
7	*m/z* 297 (M^+); IR (KBr) 1732 (C=O ester) 1691 (CO); 3365 (NH); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.28 (t, 3H, *J* = 8 Hz, CH_3), 4.25 (d, 2H, *J* = 8 Hz, CH_2), 5.10 (s, 2H, NCH_2), 7.40 (t, 2H, *J* = 7 Hz, ArH), 7.77 (d, 2H, *J* = 7 Hz, ArH).	65.00	132
8	*m/z* 292 (M^+); IR (KBr) 1732 (C=O ester) 1690 (CO); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.29 (t, 3H, *J* = 8 Hz, CH_3), 3.83 (s, 3H, OMe), 4.31 (d, 2H, *J* = 8 Hz, CH_2), 5.18 (s, 2H, NCH_2), 7.50 (d, 2H, *J* = 7 Hz, ArH).	64.56	128

Conclusions

Thus we have described isolation and characterization of a lipase from a soil isolate, *P. mendocina* PK-12CS. This lipase was found to affect chemoselective hydrolysis of ester in the presence of carbamate. The useful characteristics of PK-12CS lipase are that it resist most metal ions, surfactants like Triton X-100, Tween-80, and organic solvents, besides chemoselectivity. Apparently, the lipase has great potential in fat/oil modification, organic synthesis.

Materials and Methods

Micro-organism and culture conditions

P. mendocina (PK-12CS), a lipase producing bacteria was isolated from soil samples collected from oil crusher site where mustard oil is extracted from mustard seeds. A 1-g soil sample was suspended in 50 mL of medium containing (g/L): $(NH_4)_2HPO_4$, 5.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.3; Peptone, 4.0; mustard oil 5.0 mL and incubated at 37 °C for 120 h on a rotary shaker (240 rpm). Single colonies were isolated by

plating on Luria-Agar (pH 7.0; sterilization, 121 °C for 15 min) containing (g/L): Tryptone, 10; Yeast extract, 5.0; NaCl 10.0; Agar, 20.0; Tributyrin (emulsified by sonication with gum acacia) 10.0. Colonies exhibiting clear zone were regarded as lipase producers and were maintained at 4 °C on Luria-Agar slants. For enzyme production the isolate PK-12CS was cultivated in 500-mL Erlenmeyer flask containing 100 mL of basal medium (g/L): Soyabean meal, 10.0; $NaNO_3$, 10.0; Na_2CO_3, 2.5; $MgSO_4.7H_2O$, 0.3; GlyceroL 10 and Diammonium hydrogen phosphate-DAP, 15 on a rotary shaker (240 rpm) at 37 °C for 20 h. The culture medium was centrifuged to remove the cell mass and other solids. The supernatant was used for lipase purification.

Purification of the enzyme

For enzyme purification, the crude supernatant was precipitated by acetone.[46] All steps in the purification after acetone precipitation were carried out at room temperature (28–30 °C). Proteins recovered from the culture fluid (35–60% acetone fraction) were resuspended in 10 mM phosphate buffer pH 7.6 and dialyzed. This is referred as crude enzyme. To this material Triton X-100 was added to a final concentration of 0.1% and was applied on DE-52 column (1.5×9.0 cm). The elution of the adsorbed proteins was performed with linear increase of NaCl concentration from 0 to 200 mM. The most active fractions were pooled and dialyzed. To refine the purification step the active lipase fraction was recycled on same DE-52 column. The fractions containing lipase were pooled, lyophilized and the resulting material was used for the determination of homogeneity, molecular weight by SDS-PAGE (Laemmli, 1970)[47] and biochemical properties. The molecular mass of the lipase was assessed by its electrophoretic mobility with reference to the mobility of Phosphorylase b (94 kDa), Albumin (67 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa) contained in the low molecular weight calibration kit of Amersham Pharmacia Biotech.

Assay of lipase activity

Method A. Activity measured spectrophotometrically by using *p*-nitrophenylpalmitate (pNPC16, Sigma) as substrate.[48] One unit is defined as the amount of enzyme which liberates 1 nmol of *p*-nitrophenol per min at 37 °C under the conditions specified in the assay procedure.

Method B. The assay was based on the titration of fatty-acids released from the substrate (triolein, tripalmitin, trilinolin, tributyrin, olive oil, soyabean oil, mustard oil, coconut oil, almond oil). Five millilters of each substrate was emulsified in 20 mL of 10 mM phosphate buffer (pH 7.6), containing gum acacia (20 mg/mL), with a homogeniser (ultraturrax T25, Janke and Kunkel) three times for 20 s at maximum speed. Twenty milliliters of substrate emulsion was equilibrated at 37 °C for 10 min, followed by addition of 100 μL enzyme solution, and stirring the reaction mixture at 37 °C for 20 min with a magnetic stirrer. The reaction was stopped by the addition of 10 mL of ethanol/acetone mixture (1:1, v/v). The whole mixture was titrated with 50 mM KOH solution using phenolphthalein solution as an indicator. The titer is V_s. For blank, bovine serum albumin was used in place of the enzyme. The titer is V_b. The difference $\Delta V = V_s - V_b$ was used for activity calculations. The value of ΔV was maintained below 2.5 mL by dilution of the enzyme with buffer. One unit is defined as the amount of enzyme which liberates 1 μmol of fatty acid per min at 37 °C under the assay conditions.

Determination of temperature, pH, solvent and inhibitor effects on lipase

The optimum temperature for enzyme activity was determined photometrically with pNPC16 as substrate. The activity assays were performed by incubation of reaction mixtures at various temperatures and pH 8. In order to determine the thermal stability, the enzyme (in 10 mM phosphate buffer, pH 8.0) was exposed to different temperatures for 1 h, prior to the assay of residual activity using pNPC16 as substrate. For the determination of the effect of pH on enzyme, lipase activity was measured at different pH by titration of fatty acids released from the substrate olive oil. The effect of pH on enzyme stability was analyzed by spectrophotometric assay with pNPC16 as substrate after preincubation of enzyme at different pH for 14 h at 37 °C.

The influence of various chemicals on the enzyme activity was determined by the incubation of the enzyme for 2 h at 37 °C in 10 mM phosphate buffer, pH 8.0, containing the reagent at a final concentration of 0.1–1 mM. The residual activity was measured using pNPC16 as substrate. For the determination of the effect of organic solvents on enzyme activity, the crude enzyme (acetone powder) was suspended in organic solvents and incubated at room temperature for $2\frac{1}{2}$ h. Then the solvents were taken off vaccum dried and suspended in 10 mM phosphate buffer, pH 8.0. The residual activity was measured using pNPC16 as substrate.

Chemoselective hydrolysis

To a solution of compound **l** or **2** (0.005 mol) in DMSO (0.25 mL) and l0 mM sodium phosphate buffer (pH 7.6; 25 mL) was added 50 mg of crude lipase and the mixture incubated for 15 h at 37 °C with shaking at 240 rpm. The reaction mixture was extracted with EtOAc (5×20 mL), the organic layer was dried (Na_2SO_4) and concentrated in vacuum to give an oily residue which was purified by column chromatography on Merck silica gel (70–230 mesh) using $CHCl_3$/MeOH (95:5 v/v) solvent system. Maintaining the above reaction conditions the hydrolysis of ester group of compound **7** and **8** to yield compounds **5** and **6** were carried out. The evidence for the structural assignment were obtained by IR spectra recorded on Perkin-Elmer 881 spectrophotometer, ^{1}H NMR spectra were measured in Bruker 400 FTNMR and Bruker Avance RX300 spectrometers while EI mass spectra were recorded on a Jeol-D-300

spectrometer. Elemental analysis were carried out on a Carlo-Erba EA ll08 elemental analyzer. Melting points were determined in a hot-stage apparatus and are uncorrected. The progress of reactions were monitored by TLC on silica gel 60 (E. Merck) of 0.25 mm thickness.

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