Chapter 3
Synthesis and secretory phospholipase A$_2$

inhibitory studies of trimethoxyphenyl

isoazolidine derivatives
3.1 Introduction

Phospholipases are major class of enzymes to hydrolyze the ester group in glycerophospholipids. On the basis of hydrolyses at different position of ester group in phospholipid, they are classified as PLA$_1$, PLA$_2$, PLC, PLD (Figure 3.1). Among phospholipase enzymes, Phospholipase A$_2$ (PLA$_2$) has been studied extensively, this is corresponds to class of enzymes that catalyse the hydrolysis of membrane glycerophospholipid at Sn-2 position to release fatty acids and lysophospholipids. More than ten different forms of PLA$_2$ enzymes are identified. All these enzymes catalyze hydrolysis of 2-acyl ester of 3-sn phosphoglycerides to yield free fatty acid (mainly arachidonic acid) and lysophospholipid (Figure 3.2), which is a rate limiting step for the production of proinflammatory lipid mediators such as prostoglandins, leukotrienes, lipoxins and platelet activating factors. In many inflammatory diseases, high levels of PLA$_2$ enzymes are detected and are believed to be responsible for part of the inflammatory response. Group I PLA$_2$ is present in pancreatic juice and Old World snakes, whereas, group II PLA$_2$ is present in mammals and New World snakes. Increased level of group II PLA$_2$ is found in rheumatoid synovial fluid and in plasma after inflammatory challenge. Injection of purified PLA$_2$ from synovial fluid and from snake venom into animal joints confirmed the development of an acute inflammatory response with edema, swelling of synovial cells, hemorrhage and hyperplasia. Thus several findings point to the importance of PLA$_2$ in inflammatory reactions. Inhibition of such PLA$_2$ enzyme by endogenous inhibitors, xenobiotics and synthetic compounds is of potential therapeutic relevance in many inflammatory diseased states. The occurrence of different types of PLA$_2$ drew attention to the importance of finding selective and specific inhibitors for the group II PLA$_2$ enzyme.
Extensive research effort in our laboratory has been committed to find novel compounds for the treatment of inflammatory diseases.\textsuperscript{8,10-13} The search for potent nonsteroidal anti-inflammatory drugs (NSAIDs) for treatment of inflammation is still in progress. Literature survey revealed the high potency of isoxazolidine derivatives serving as anti-inflammatory,\textsuperscript{14,15} anti-HIV,\textsuperscript{16} analgesic,\textsuperscript{17} anti-fungal and anti-bacterial agents.\textsuperscript{18,19} The potent anti-inflammatory property of these compounds has been shown to be due to the inhibition of cyclo-oxygenase enzyme.\textsuperscript{17} However, some of the derivatives exhibited \textit{in-vivo} anti-inflammatory activity but failed to inhibit cyclo-oxygenase enzyme, suggesting a different mechanism.\textsuperscript{17} To establish the mechanism of anti-inflammatory
action of these compounds, its interaction with PLA2 enzyme has been studied.

Herein, we reported the synthesis of ten different derivatives of trimethoxyphenyl isoxazolidine with variable substitution at 4th position of N-phenyl ring and at 5th position of isoxazolidine ring leading to increased hydrophobicity and aromaticity. The comparison of PLA2 inhibitory activity of these derivatives with purified PLA2 from *Vipera Russelli* 20 (group II; VRV-PL-VIII) *Naja naja* 21 (group I; NN-PL-I) snake venoms and partially purified synovial fluid PLA2 (group II; S-PLA2) 8,22 is performed. This study reveals additional information on structure activity relationship of group II PLA2 for hydrocarbon chain length and unsaturated aromatic rings of isoxazolidine derivatives.

### 3.2 Chemistry

The synthetic method used to prepare the regioisomeric 5-substituted isoxazolidines 5 \( a(i-v) \) and 5b \( i-v \) is illustrated in Scheme 3.1. 1:1.5 molar equivalents of aldehyde 1, nitroarene 2, and 2 to 2.5 mL of ammonium buffer solution (pH~10.2) have taken in a round bottom flask containing 10 mL of 1:1 v/v methanol/methylenedichloride. To the stirred reaction solution 2.5 molar equivalents of zinc powder was added slowly (The reaction is exothermic) and continued the stirring for 4-6 hours at room temperature. Complete conversion of reactant to nitrone 3 was confirmed by TLC using n-hexane and ethyl acetate as eluent. The reaction mixture was filtered and the solvent was evaporated under vacuum. The residue was recrystallised with ethylacetate and n-hexane to yield corresponding nitrone 3. Subsequent cycloaddition of 3 with monosubstituted olefin 4 afforded two regioisomers, 5 and 5' in good yield as shown in Scheme 3.1.
Reagent conditions: a ammonium buffer solution (pH ~10.2)/ activated Zn dust at room temperature in Methanol and Methylene dichloride. 

The major isomer, 5 was separated either by recrystallization or column chromatography using appropriate combination of solvents (Table 3.1). The yield of 5 was in the range of 60-76% with high purity (> 96%). All novel isoxazolidines 5 were structurally characterized by 1H NMR, 13C NMR, IR and elemental analysis. Isoxazolidines 5 were showed a set of δ 2.38-2.81 (ddd), 2.51-3.33(ddd), 5.20-3.05(dd), and 6.94-4.15 (dd) for C- 4H, 4H, C-3H and C-5H respectively. The derivatives were classified into two series based on the substitution in the 4th position of the N-phenyl ring of isoxazolidines. The derivatives with –H in the 4th position of N-phenyl ring are classified as 5a series and derivatives were made electronegative by replacing –H with –Cl and are classified as 5b series (Scheme 3.1).
Table 3.1. Reaction conditions and physical data of 5-substituted 3,4,5-trimethoxy phenyl isoxazolidines 5a(i-v) and 5b(i-v).

<table>
<thead>
<tr>
<th>Isoxazolidines</th>
<th>Solvent&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time in h</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio&lt;sup&gt;c&lt;/sup&gt; of 5/5'</th>
<th>Eluent&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Yield of 5 in %</th>
<th>m.p in °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5a(i)</td>
<td>Toluene</td>
<td>38</td>
<td>0.20</td>
<td>79/21</td>
<td>H: EA (7:1)</td>
<td>62</td>
<td>Oil</td>
</tr>
<tr>
<td>5a(ii)</td>
<td>Toluene</td>
<td>42</td>
<td>0.22</td>
<td>73/27</td>
<td>H: EA (9:1)</td>
<td>60</td>
<td>Oil</td>
</tr>
<tr>
<td>5a(iii)</td>
<td>Toluene</td>
<td>48</td>
<td>0.23</td>
<td>73/27</td>
<td>H: EA (9:1)</td>
<td>61</td>
<td>Oil</td>
</tr>
<tr>
<td>5a(iv)</td>
<td>Toluene</td>
<td>50</td>
<td>0.35</td>
<td>90/10</td>
<td>H: EA (4:1)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>76</td>
<td>117</td>
</tr>
<tr>
<td>5a(v)</td>
<td>Toluene</td>
<td>52</td>
<td>0.36</td>
<td>89/11</td>
<td>H: EA (8:1)</td>
<td>72</td>
<td>127</td>
</tr>
<tr>
<td>5b(i)</td>
<td>Toluene</td>
<td>42</td>
<td>0.21</td>
<td>78/22</td>
<td>H: EA (9:1)</td>
<td>61</td>
<td>Oil</td>
</tr>
<tr>
<td>5b(ii)</td>
<td>Toluene</td>
<td>44</td>
<td>0.21</td>
<td>74/26</td>
<td>H: EA (9:1)</td>
<td>60</td>
<td>Oil</td>
</tr>
<tr>
<td>5b(iii)</td>
<td>Toluene</td>
<td>48</td>
<td>0.24</td>
<td>70/30</td>
<td>H: EA (9:1)</td>
<td>60</td>
<td>Oil</td>
</tr>
<tr>
<td>5b(iv)</td>
<td>Toluene</td>
<td>48</td>
<td>0.31</td>
<td>93/7</td>
<td>H: EA (4:1)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>72</td>
<td>121</td>
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<tr>
<td>5b(v)</td>
<td>Toluene</td>
<td>50</td>
<td>0.38</td>
<td>91/9</td>
<td>H: EA (8:1)</td>
<td>72</td>
<td>134</td>
</tr>
</tbody>
</table>

<sup>a</sup>Used for cycloaddition reaction.  
<sup>b</sup>Used H (hexane)/EA (ethylacetate) as eluent.  
<sup>c</sup>Regioisomeric ratio of crude ascertained by <sup>1</sup>H NMR (400 MHz) after passing through a short column using H/EA (4:1).  
<sup>d</sup>Used for purification of isoxazolidines.  
<sup>e</sup>Recrystallized in alcohol after passing through a short column.
3.3 Biology

**PLA₂ activity**

This activity was assayed according to the method of Vishwanath et al.⁴⁴ Purified VRV-PL-VIII, NN-PL-I and 40,000 fold purified synovial fluid PLA₂ were diluted in 30 μL of 50 mM Tris-HCl buffer pH-7.5. Enzymes were pre-incubated at 37 °C for 10 min with 10 μL of test compound solution or its vehicle in a final volume of 320 μL containing 5 mM CaCl₂ and 100 mM Tris-HCl buffer of pH-7.5. Incubation proceeded for 1h in the presence of 30 μL of C¹⁴oleate-labelled autoclaved E-coli membranes (equivalent to 60 nmoles of fatty acid) and was terminated by adding 100 μL of 0.2 N HCl followed by 100 μL of 10% fat free BSA in double distilled water. After thoroughly cyclo-mixing samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The radioactivity in the supernatant was determined by liquid scintillation counting.

Results are expressed as percentage of inhibition and IC₅₀ values are determined by plotting log concentration versus inhibition values in the range of 10 to 94 %.

**Spectrophotofluorimetric interaction studies**

The fluorescence was monitored using Shimadzu Spectrophotofluorimeter RF-5301PC. Reaction mixture of 2 mL was taken in 1 cm path length quartz cuvette, which contained 0.1 M Potassium phosphate buffer pH-7.4 and 30 μg of protein. A Fluorescence spectrum was measured between 200-400 nm, after excitation at 280 nm. The marked quenching of fluorescence intensity compared with native enzyme was noted by incubating the compounds with the enzyme for about 30 min at varying mole-to-mole ratio of enzyme and the inhibitor.

**Neutralization of Indirect hemolytic activity**

Indirect hemolytic activity was assayed by the method of Boman & Kalletta.⁴⁵ Briefly, packed human erythrocytes egg yolk and phosphate
buffered saline (1:1:8,V/V) were mixed. 1 mL of this suspension was incubated with enzyme (20 µg) for 10 min at 37 °C. After adding 9 mL of ice-cold phosphate buffered saline the reaction mixture was centrifuged at 4 °C for 10 min at 2000 rpm. The amount of hemoglobin released in the supernatant due to hemolysis was measured at 540 nm. The hydrolysis of erythrocytes caused due to addition of 9 mL distilled water was taken as 100 % hydrolysis. Values are presented as mean of four independent determinations.

Neutralization of edema inducing activity

The edema induced by VRV-PL-VIII, NN-PL-I and S-PLA2 was assayed according to the method of Yamakawa et al.46 Mice weighing between 20-25 g were used for all experiments. Right footpad of hind limb of each mouse was injected with the indicated amount of enzyme in a total volume of 30 µL saline. Control animals were injected with 30 µL saline. After 45 min the mice were sacrificed under ether anesthesia and both hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as percent of edema compared with control (117 ± 4 % of un-injected limb). The edema ratio is calculated by the weight of the edematous limb to the weight of the saline or vehicle-injected limb X 100. The effect of the inhibitor on edema inducing activity of enzymes was studied by mixing the indicated concentration of the mixture and enzyme and was allowed to stand for 30 min at room temperature prior to co-injection. The data is reported taking the mean of four different experiments conducted at similar conditions.

3.4 Results

The parent compound is known to have anti-inflammatory properties. The derivatives of 5a and 5b series of isoxazolidines were tested for anti-inflammatory properties by studying their interaction with different PLA2 enzymes. Inflammatory PLA2 from synovial fluid (S-PLA2) toxic and inflammatory PLA2 from V. russelli (VRV-PL-VIII) and N. naja
(NN-PL-I) were used to study the interaction with isoxazolidine derived compounds. **Figure 3.3** shows the interaction of all the ten isoxazolidine-derived compounds (75 µM concentration) with *V. russelli*, *N. naja* and synovial PLA$_2$ enzyme activity. All of them inhibit PLA$_2$ enzyme activity. These derivatives inhibit group II PLA$_2$ activity of *V. russelli* and synovial PLA$_2$ more, compared to the group I PLA$_2$ from *N. naja* snake venom. This difference is higher than 3 fold in both the series with methyl ester-derived isoxazolidine compounds [5a(i) and 5b(i)]. As the hydrophobicity increased the difference between the inhibition of group II PLA$_2$ and group I PLA$_2$ has been decrease by 2 fold, which is the phenyl-derived isoxazolidine compounds [5a(iv) and 5b(iv)]. There is 25% significant increase in inhibition of VRV-PL-VIII when phenyl group is replaced by phenyl carboxylate even though the later is less hydrophobic. Overall increase in inhibition of group II and I PLA$_2$ by methyl ester to phenyl derivative is doubled. The inhibition of all the PLA$_2$ enzymes is least with isoxazolidine compounds where the substitution is methyl ester [5a(i) and 5b(i)]. The inhibition gradually increases as the hydrophobicity in the isoxazolidine ring at 5$^{th}$ position increases. The inhibition of VRV-PL-VIII by 5a(v) and 5b(v) is increased by 2.5 fold compared to their respective methyl ester isoxazolidine compounds [5a(i) and 5b(i)]. Between the two series of 5a and 5b isoxazolidine compounds, where the electronegativity has been increased in 5b series by replacing –H with –Cl, there is no significant difference in the PLA$_2$ inhibition of all the three enzymes. The data correlating the increased inhibition, with that of increased hydrophobicity was further strengthened by *in-vitro* fluorescent interaction studies of VRV-PL-VIII with all the ten compounds as shown in **Figure 3.4**. The intrinsic fluorescent spectrum of *V. russelli* PLA$_2$ was measured both in presence and in absence of these isoxazolidine compounds. **Figure 3.4 (Panel A** and **Panel B)** shows the fluorescent spectra with first compound of 5a series (5a(i)) and last compound of 5b series (5b(v)) respectively. Both the series of compounds quenches the fluorescent spectra of *V. russelli* PLA$_2$ indicating the direct interaction of these compounds with the
enzyme. The quenching of the fluorescent spectra increases in both the series of compounds as the hydrophobicity in the 5th position of the isoxazolidine increases. This correlates well with increased inhibition property of the highly hydrophobic and aromatic derivatives. From each series highly potent compound 5a(v) and 5b(v) with substitution of benzyl ester were used for further characterization with V. russelli enzyme.

**Figure 3.5** shows the dose dependent inhibition of V. russelli PLA2 with increasing concentration of 5a(v) and 5b(v) compounds. More than 94% inhibition was achieved with both the series of compound at 105 µM concentration. The IC50 calculated for these compounds were 54.8 µM for 5b(v) and 55 µM for 5a(v).

Since 5a(v) and 5b(v) compounds inhibit all the PLA2 enzymes effectively, the nature of inhibition was studied with these inhibitors. The effect of different concentration of substrate and Ca2+ was determined on the inhibition of 5a(v) and 5b(v) compounds. With increasing substrate concentration the inhibition was completely relieved with both the series of compounds indicating the inhibitory activity of 5a(v) and 5b(v) is substrate dependent (**Figure 3.6**). The Ca2+ concentration (5 mM - 50 mM) did not affect the PLA2 inhibition (data not shown). These compounds interact with the enzyme and probably compete for the substrate-binding region of the enzyme.

All the three PLA2 enzyme exhibited indirect hemolytic activity, which is an indirect way of measuring PLA2 activity using egg yolk, and washed erythrocytes as the substrates. The effect of 5b(v) compound on these three indirect hemolytic activities is given in **Figure 3.7**. 5b(v) compound inhibits the indirect hemolytic activity in a dose dependent manner. The inhibition is more with V. russelli PLA2 than N. naja PLA2, which correlates with the inhibitory activity of PLA2 enzyme.

Since 5b(v) compound effectively inhibits the PLA2 enzyme activity and indirect hemolytic activity its effect on the edema inducing activity is shown in the **Figure 3.8**. All the three PLA2 enzymes when injected into the mouse hind paw induce edema. 5b(v) inhibits the edema inducing
activity of all the PLA$_2$ enzyme in a dose dependent manner. Complete neutralization was observed with the _V. russelli_ PLA$_2$ and synovial PLA$_2$ and not with the _N. naja_ PLA$_2$. This inhibition correlates well with the _in-vitro_ enzymatic inhibition by 5b(v) compound.

![Graph showing inhibition of NN-PL-I, VRV-PL-VIII and S-PLA$_2$ by compounds at 75 µM concentration.](image)

**Figure 3.3.** Inhibition of NN-PL-I, VRV-PL-VIII and S-PLA$_2$ by compounds at 75 µM concentration.

_Each compound (75 µM) is pre-incubated with enzyme for 30 min. The reaction mixture 350 µL contained 30 µL of substrate (added after pre-incubation), 35 µL of 1M Tris-HCl buffer pH-7.5 and 5 mM CaCl$_2$, made up to 350 µL with distilled water and incubated for 1h at 37 °C. Data represent mean ±SEM for n=4._
Figure 3.4. Intrinsic fluorescence spectra of interaction of VRV-PL-VIII in the presence of different concentration of 5a(i) and 5b(v). Panel-A: The reaction mixture contained 30 µg of VRV-PL-VIII in 100 mM Tris-HCl buffer pH-7.5, 5 mM CaCl₂ with varied mole: mole ration of enzyme to 5a(i)-1: 0 (—), 1: 10 (- - -), 1: 15 (······), 1: 20 (———).
Figure 3.5. Does dependent inhibition of VRV-PL-VIII by 5a(v) and 5b(v).

The reaction mixture 350 µL containing 2 µg of VRV-PL-VIII in 100 mM Tris-HCl buffer pH-7.5; 5 mM CaCl₂ and indicated concentration of 5a(v) (♦) and 5b(v) (■) is preincubated for 30 min at 37 °C. The PLA₂ reaction is initiated by adding substrate (60 nmoles of E-coli phospholipid) and continued incubation at 37 °C for 1 h.
Figure 3.6. Effect of Substrate concentration on inhibition of VRV-PL-VIII by 5a(v) and 5b(v).

The reaction mixture 350 µL contains 2 µg of VRV-PL-VIII in 100 mM Tri-HCl buffer pH-7.5; 5 mM Ca²⁺ and 55 µM concentration of 5a(v) (▲) and 54.8 µM 5b(v) (■) is preincubated at 37 °C. The PLA₂ reaction is initiated by adding indicated concentration of substrate and continued incubation at 37 °C for 1 h.
Figure 3.7. Neutralization of Indirect hemolytic activity by 5b(v).

20 µg enzyme in 100 µL of phosphate buffer saline (PBS) was pre-incubated with 0 to 100 µg of 5b(v). The reaction is started by adding erythrocytes, egg yolk and PBS (1: 1: 8 V/V) incubated for 10 min at 37 °C. The released hemoglobin in the supernatant was measured by taking absorbance at 540 nm. The result shown is mean ± SEM for n=4.
Figure 3.8. Neutralization of edema inducing activity of VRV-PL-VIII, NN-PL-I and S-PLA_2 by 5b(v).

The reaction mixture 30 µL contained 5 µg of each, VRV-PL-VIII, NN-PL-I and S-PLA_2 enzymes incubated for 30 min with increasing concentration of 5b(v). 30 µL of saline injected to mice footpad served as control. Data represent mean ± SEM for n=4.

3.5 Discussion

Understanding the molecular interaction between the enzyme PLA_2 and the agents inhibiting the activity is very crucial in designing powerful inhibitors. Several endogenous and exogenous agents such as lipocortin, cis-unsaturated fatty acids, gangliosides, manoalide, cacospongionolide, petrosaspongionolide, surfactin, retinoids, flavonoids, aristolochic acid, natural plant lipids, and synthetic lipids have been shown to inhibit PLA_2 enzyme. From the inhibition studies with different inhibitors, it is generalized that most of the PLA_2 inhibitors are hydrophobic compounds containing unsaturated long hydrocarbon chain with one to several aromatic rings. More the
hydrophobic nature of the molecule better is the interaction with PLA₂ enzyme and its inhibitory activity. The parent compound isoxazolidine derivatives have shown to be an anti-inflammatory properties by selective inhibition of cyclooxygenase-2 (COX-2) enzyme. Clinical results with cyclooxygenase and lipoxygenase inhibitors demonstrated that inhibition of PLA₂ enzyme results in reduction of both lipid mediators, as a result these PLA₂ inhibitors can be used as anti-inflammatory drugs which are as powerful as anti-inflammatory steroidal compounds. Some of the derivatives of isoxazolines exhibited potent anti-inflammatory activity but failed to inhibit COX-2 suggesting its action by a different mechanism.¹⁷ This observation lead to the inference that these compound might interact with PLA₂ enzyme so that they exhibited stronger anti-inflammatory activity. In this study several isoxazolidine derivatives were synthesized with increase in hydrophobicity and the aromaticity at the 5th position of the parent compound and their effect on in-vitro PLA₂ inhibition and edema inhibiting activity in mouse model were examined. The hydrophobic substitution in the 4th position of isoxazolidine ring is not possible because of steric hindrance. In 5a series hydrophobic substitution in the 5th position included increase in aliphatic chain length and aromatic substitution. In the entire substituted compound, another series of derivative were synthesized with increased electro negativity by replacing –H with –Cl at the 4th position of the N- phenyl ring, which are classified as 5b series. All the ten derivatives of isoxazolidine compounds inhibited group I and group II PLA₂ enzymes. The inhibition is stronger with inflammatory PLA₂ enzyme and other group II PLA₂ over snake venom group I PLA₂ enzyme. Though group I and group II PLA₂ enzymes exhibit more than 70% homology many inhibitors inhibit these enzymes differentially.⁸ The variable extent to which group I and group II PLA₂ enzymes are inhibited may be due to differential binding affinities with these enzymes. These differential affinities with isoxazolidine compounds varied as the hydrophobicity and aromaticity was increased at the 5th position of the ring.
The inhibition is dose dependent and the inhibition increases with increase in hydrophobicity and aromaticity at the 5\textsuperscript{th} position of the isoxazolidine ring. The increased inhibition is somewhat linear with increased hydrophobicity in the aliphatic chain length of methyl, ethyl and butyl esters. When the aliphatic side chains are replaced with phenyl ring, the inhibition was much stronger and it was further enhanced with phenylcarboxylate substitution indicating the enzymes preference for hydrophobic molecules with unsaturated aromatic rings as better inhibitors. Similar hydrophobic compounds, which are inhibitors of PLA\textsubscript{2}, are group of chemically modified tetracyclines,\textsuperscript{36} derivatives of isoxazolines,\textsuperscript{12,17} indole analogues,\textsuperscript{13} benzophenone oxime analogues\textsuperscript{18} and flavanoids\textsuperscript{31} all exhibit stronger PLA\textsubscript{2} inhibition with increased hydrophobicity and unsaturated rings in their structure. The substitution of electronegative group in the 4\textsuperscript{th} position of the N-phenyl ring of the isoxazolidine ring did not show any difference in the PLA\textsubscript{2} inhibition with both group I and group II PLA\textsubscript{2} enzymes. These studies clearly indicate that the hydrophobicity and aromatic nature in the 5\textsuperscript{th} position of isoxazolidine ring is very critical for the inhibition of the enzyme.

The fluorescence interaction data suggests that the nature of inhibition by these derivatives is by direct interaction with the \textit{V. russelli} PLA\textsubscript{2} enzyme. All the derivatives quench the fluorescence property of the enzyme. The intensity of quenching is related to the hydrophobic and aromatic group at the 5\textsuperscript{th} position of the isoxazolidine compounds. Stronger quenching was observed with the highly hydrophobic and aromatic derivatives. The electro negativity differences at the 4\textsuperscript{th} position of the N-phenyl ring do not influence the fluorescence quenching with the two series of compounds. These derivatives interact with the enzyme at the substrate-binding site, as the inhibition by these compounds is substrate dependent and probably both the substrate and isoxazolidine compounds compete to the same site for binding. Oxazolidinone, which is very much similar to isoxazolidine, were used in synthesizing phospholipid analogues as PLA\textsubscript{2} inhibitors.\textsuperscript{37} Computer modeling
analysis of the interaction between phospholipid analogues and PLA\textsubscript{2} enzyme showed complex formation similar to those formed by the genuine substrate\textsuperscript{38}. These studies further strengthen the present data that isoxazolidine derivative bind with PLA\textsubscript{2} enzyme at the active site where the substrate binds. For effective binding at the active site by isoxazolidine derivative the type of hydrophobic and aromatic group in the 5\textsuperscript{th} position is very critical.

The inhibition of PLA\textsubscript{2} enzyme by isoxazolidine derivative reflected in the inhibition of indirect hemolytic activity where it is assayed with crude egg phospholipids mixture dispersed in the buffer as micelles, rather than an intact \textit{E. coli} membrane. Since the isoxazolidine derivatives bind at the substrate binding regions of the enzyme, the enzyme activity is inhibited irrespective of the substrate nature provided for its activity.

Injection of purified PLA\textsubscript{2} enzyme from snake venom and inflammatory fluids into animal joints resulted in the acute inflammatory responses with edema swelling of synovial cells and hyperplasia. This may be due to their combined effect to hydrolyze membrane phospholipid resulting in the loss of membrane integrity, as well as the generation and metabolism of products such as eicosanoids, which then function to amplify inflammatory events\textsuperscript{39}. Several studies with specific PLA\textsubscript{2} enzyme modifying agents such as p-BPB resulted in the loss of enzyme activity and edema-inducing activity directly indicate that the edema produced by these PLA\textsubscript{2} enzymes mediated by the catalytic domain of the enzyme\textsuperscript{40}. The isoxazolidine derivatives on co-injection with snake venom PLA\textsubscript{2} enzyme decreased the edema inducing activity in a dose-dependent manner. The concomitant inhibition of PLA\textsubscript{2} enzyme activity and \textit{in-vivo} edema inducing activity by these isoxazolidine derivatives suggests a strong correlation between lipolytic activity and pro-inflammatory activities. Since the isoxazolidine derivative binds at the catalytic site of the enzymes, these are very effective in preventing the enzyme from inducing edema.
3.6 Experimental

General procedure for the synthesis of novel isoxazolines 5a (i-v) and 5b (i-v)

To a solution of the corresponding nitrone (3a and 3b) in 20 ml toluene was added monosubstituted alkenes 4 (2 equivalent), the solution was refluxed under nitrogen atmosphere. The progress of the reaction was monitored by the TLC and on completion, solvent was removed under reduced pressure to offer crude product, which was purified by column chromatography over silica gel using appropriate solvents (Table 1) as eluent to give major product 5 with above 96% purity analyzed by \(^1\)H NMR.

Synthesis of methyl 3-(3,4,5-trimethoxyphenyl)-2-phenyl isoxazolidine-5-carboxylate 5a(i). This compound was obtained from 3a (2 g, 6.96 mmol) and methacrylate (1.26 ml, 13.9 mmol). The obtained yield is 1.61 g (62%).

\(^1\)H NMR: \(\delta\) 2.32 (ddd, 1H, \(J = 12.8, 5.8, 4.3\) Hz, \(H_{4a}\)); 2.51 (ddd, 1H, \(J = 12.8, 9.0, 7.8\) Hz, \(H_{4b}\)); 3.35 (s, 3H, CH\(_3\)); 3.92 (s, 6H, OCH\(_3\)); 3.91 (s, 3H, OCH\(_3\)); 4.32 (dd, 1H, \(J = 8.4, 6.1\) Hz, \(H_{3}\)); 5.85 (dd, 1H, \(J = 9.0, 4.95\) Hz, \(H_{5}\)); 6.9-7.4 (m, 7H, Ar-H).

\(^13\)C NMR: \(\delta\) 171.6, 153, 152.6, 137.2, 133.2, 128.2, 122.5, 115.6, 103.0, 81.4, 74.7, 56.5, 56.4, 53.1 40.1.

IR (KBr): 2970 (m), 2892 (w), 1730 (s), 1590 (m), 1506 (m), 1120 (s), 1263 (s), 904 (m), 860 (m), 750 (m), 630 (m) cm\(^{-1}\).

Anal. calcd for C\(_{20}\)H\(_{22}\)O\(_6\)N: C, 64.33; H, 6.21; N, 3.75. Found: C, 64.36; H, 6.19; N, 3.72.

Synthesis of ethyl 3-(3,4,5-trimethoxyphenyl)-2-phenylisoxazolidine-5-carboxylate 5a(ii). This compound was obtained from 3a (2 g, 6.96 mmol) and ethyl acrylate (1.5 ml, 3.9 mmol). The obtained yield is 1.63 g (60%).
$^1$H NMR: $\delta$ 1.69 (t, 3H, CH$_3$), 4.12 (q 2H CH$_2$); 2.78 (ddd, 1H, $J = 13.1, 5.8, 7.1$ Hz, H$_{4a}$); 3.28 (ddd, 1H, $J = 13.1, 8.8, 7.1$ Hz, H$_{4b}$); 3.82 (s, 6H, OCH$_3$); 3.86 (s, 3H, OCH$_3$); 4.53 (dd, 1H, $J = 8.4, 6.1$ Hz, H$_3$); 6.8 (dd, 1H, $J = 4.96, 7.54$ Hz, H$_5$); 6.9-6.7 (m, 3H Ar-H); 7.21 (m, 2H, Ar-H); 7.39 (m, 2H Ar-H).

$^{13}$C NMR: $\delta$ 171, 153.6, 150.7, 136.1, 134.2, 128.9, 128.5, 115.8, 103.3, 76.2, 69.4, 60.8, 57.1, 56.2, 42.6, 14.1.

IR (KBr): 3070, 2963 (m), 1742 (s), 1590 (m), 1493 (m), 1371 (m), 1215 (m), 1246 (m), 1095 (m), 904 (s), 750 (m), 705 (m) cm$^{-1}$.


**Synthesis of butyl 3-(3,4,5-trimethoxyphenyl)-2-phenylisoxazolidine-5-carboxylate 5a(iii).** This compound was obtained from 3a (2g, 6.96 mmol) and butyl acrylate (1.5 ml, 13.9 mmol). The obtained yield is 1.77 g (61%).

$^1$H NMR: $\delta$ 0.92 (t, 3H, CH$_3$); 1.34 (m, 2H, CH$_2$); 1.57 (m, 2H, CH$_2$); 2.67 (ddd, 1H, $J = 13.5, 8.8, 7.4$ Hz, H$_{4a}$); 2.92 (ddd, 1H, $J = 13.5, 6.1, 4.6$ Hz, H$_{4b}$); 3.83 (s, 6H, OCH$_3$); 3.88 (s, 3H, OCH$_3$); 3.05 (dd, 1H, $J = 8.5, 7.4$ Hz, H$_3$); 4.15 (dd, 1H, $J = 9.3, 5.0$ Hz, H$_5$); 4.65 (t, 2H, CH$_2$); 6.73 (d, 2H, Ar-H); 6.79 (s, 2H, Ar-H); 7.04 (m, 1H, Ar-H); 7.25 (m, 2H, Ar-H).

$^{13}$C NMR: $\delta$ 171.0, 152.2, 150.6, 136.5, 135.8, 125.2, 124.6, 116.2, 106.0, 77.0, 73.8, 66.1, 58.4, 58.6, 39.7, 30.2, 18.6, 13.6.

IR (KBr): 2940 (m), 2926 (m), 2820 (m), 1738 (s), 1582 (s), 1510 (m), 1456 (m), 1415 (m), 1226 (s), 1115 (m), 986 (m), 860 (m), 740 (m), 609 (s) cm$^{-1}$.

Anal. calcd for: C$_{23}$H$_{25}$NO$_6$: C, 66.49; H, 6.74; N, 3.37. Found: C, 66.55; H, 7.0; N, 3.74.

**Synthesis of 3-(3,4,5-trimethoxyphenyl)-2,5-diphenylisoxazolidine 5a(iv).** This was obtained from 3a (2g, 6.96 mmol) and styrene (1.65 ml, 13.9 mmol). The obtained yield is 2.07 g (76%).
**Synthesis of phenyl 3-(3,4,5-trimethoxyphenyl)-2-phenylisoxazolidine-5-carboxylate 5a(v).** This compound was obtained from 3a (2g, 6.96 mmol) and vinyl benzoate (1.9 ml, 13.9 mmol). The obtained yield is 2.18 g (72%).

\[
\begin{align*}
\text{H NMR: } & \delta 2.66 \text{ (ddd, } 1H, J = 13.58, 4.8, 0.9 \text{ Hz, } H_{4a}); \delta 3.22 \text{ (ddd, } 1H, J = 14.6, 8.85, 0.9 \text{ Hz, } H_{4b}); \delta 3.88 \text{ (s, } 6H, \text{ OCH}_3); \delta 4.54 \text{ (dd, } 1H, J = 9.6, 4.8 \text{ Hz, } H_5); \delta 6.85 \text{ (dd, } 1H, J = 8.6, 5.1 \text{ Hz, } H_6); \delta 7.20-7.60 \text{ (m, } 12H, \text{ Ar-H)}. \\
\text{13C NMR: } & \delta 152.2, 150.0, 138.3, 139.0, 130.0, 128.2(x2), 125.6, 124.1, 120.6, 118.1, 107.4, 79.7, 74.8, 58.5, 56.0, 46.3. \\
\text{IR (KBr): } & 2936 \text{ (m), } 1587 \text{ (s), } 1503 \text{ (m), } 1488 \text{ (m), } 1415 \text{ (m), } 1230 \text{ (m), } 1128 \text{ (s), } 995 \text{ (m), } 899 \text{ (w), } 696 \text{ (m), } 757 \text{ (m) cm}^{-1}. \\
\text{Anal. calcd for } C_{24}H_{25}NO_4: & \text{ C, 73.64; H, 6.44; N, 3.58. Found: C, 73.71; H, 6.51; N, 3.52.}
\end{align*}
\]

**Synthesis of methyl 2-(4-chlorophenyl)-3-(3,4,5-trimethoxyphenyl)isoxazolidine-5-carboxylate 5b(i).** This compound was obtained from mixture...
of 3b (2g, 6.23 mmol) and methyl acrylate (1.1 ml, 12.43 mmol). The obtained yield is 1.55g (61%).

\[
\text{H NMR: } \delta 2.38 \text{ (ddd, 1H, J = 12.7,5.9,4.5Hz, H}_{\text{4a}}); 2.60 \text{ (ddd, 1H, J = 12.9,8.8,7.3Hz, H}_{\text{4b}}); 4.02 \text{ (s, 3H, OCH}_{3}); 3.88 \text{ (s, 6H, OCH}_{3}); 4.54 \text{ (dd, 1H, J = 7.9,6.0 Hz, H}_{3}); 6.23 \text{ (dd, 1H, J = 9.25, 5.3 Hz, H}_{5}); 7.05 \text{ (d, 2H Ar-H J = 8.0 Hz); 7.39 (d, 2H Ar-H J = 8.3Hz); 7.21 (s, 2H Ar-H).}
\]

\[
\text{13C NMR: } \delta 170.6, 154.0, 154.9, 146.1, 138.5, 135.2, 130.8, 119.0, 108.5, 79.2, 70.2, 57.5, 57.3, 46.3, 38.4.
\]

IR (KBr): 3022 (m), 2880 (w), 1741(s), 1573 (m), 1510 (s), 1112 (s), 1280 (s), 920 (m), 845 (m), 721(m), 642 (m) cm\(^{-1}\).

Anal. calcd for C\(_{20}\)H\(_{22}\)ClNO\(_6\): C, 58.90; H, 5.44; N, 3.43; Found: C, 58.98; H, 5.48; N, 3.41.

**Synthesis of ethyl 2-(4-chlorophenyl)-3-(3,4,5-trimethoxyphenyl) isoxazolidine-5-carboxylate 5b(ii).** This compound was obtained from 3b (2 g, 6.23 mmol) and ethyl acrylate (1.4ml, 12.5 mmol). The obtained yield is 1.58 g (60%).

\[
\text{H NMR: } \delta 1.7 \text{ (t, 3H CH}_{3}); 2.79 \text{ (ddd, 1H, J = 12.7, 6.2, 5.1 Hz, H}_{\text{4a}}); 3.28 \text{ (ddd, 1H, J = 12.7, 7.3, 6.2 Hz, H}_{\text{4b}}); 3.91 \text{ (s, 6H, OCH}_{3}); 3.93 \text{ (s, 3H, OCH}_{3}); 4.21 \text{ (q, 2H, CH}_{2}); 4.55 \text{ (dd, 1H, J = 5.3, 6.7 Hz, H}_{3}); 6.88 \text{ (dd, 1H, J = 9.3, 4.4 Hz, H}_{5}); 7.24 \text{ (d, 2H, Ar-H J = 8.3 Hz); 7.89 (d, 2H, Ar-H J = 8.3 Hz); 7.41 (s, 2H Ar-H).}
\]

\[
\text{13C NMR: } \delta 175.8, 152.2, 148.6, 137.0, 135.1, 130.3, 128.7, 117.0, 104.2, 79.3, 71.3, 63.7, 57.2, 57.5, 43.2, 9.8.
\]

IR (KBr): 3060, 2923 (m), 1732 (s), 1560 (m), 1453 (m), 1361 (m), 1221 (m), 1256 (m), 1105 (m), 924 (m), 840 (s), 730 (m), 715 (m) cm\(^{-1}\).
Anal. calcd for C_{21}H_{24}Cl N O_{6}: C, 59.79; H, 5.73; N, 3.32; Found: C, 59.80; H, 5.71; N, 3.33.

**Synthesis of butyl 2-(4-chlorophenyl)-3-(3,4,5-trimethoxyphenyl) isoxazolidine-5-carboxylate 5b(iii).** This compound was obtained from 3b (2 g, 6.23 mmol) and butyl acrylate (1.8 ml, 12.43 mmol). The obtained yield is 1.68 g (60%).

\[
\text{H NMR: } \delta 1.06 (t, 3H, CH}_3; 1.35 (m, 2H, CH}_2; 1.37 (m, 2H, CH}_2); 2.81 (ddd, 1H, J = 12.7, 5.9, 4.5 Hz, H\text{a}; 3.33 (ddd, 1H, J = 12.7, 8.8, 7.3 Hz, H\text{b}; 3.83 (s, 6H, OCH}_3); 3.88 (s, 3H, OCH}_3); 4.08 (t, 2H, CH}_2); 4.58 (dd, 1H, J = 7.9, 6.0 Hz, H}_3); 6.89 (dd, 1H, J = 9.25, 5.3 Hz, H}_3); 7.23 (s, 2H, Ar-H); 7.32 (d, 2H, J = 8.4 Hz, Ar-H); 7.56 (d, 2H, J = 8.9 Hz, Ar-H).
\]

**Synthesis of 2-(4-chlorophenyl)-3-(3,4,5-trimethoxyphenyl)-5-phenyl isoxazolidine 5b(iv).** This compound was obtained from 3b (2g, 6.23 mmol) and styrene (1.4 ml, 12.5 mmol). The obtained yield is 1.91g (72%).

\[
\text{H NMR: } \delta 2.73 (ddd, 1H, J = 15.3, 4.62, 1.35 Hz, H\text{a}; 3.27 (ddd, 1H, J = 15.3, 8.2, 4.92 Hz, H\text{b}); 3.85 (s, 6H, OCH}_3); 3.91 (s, 3H, OCH}_3); 4.58 (dd, 1H, J = 8.22, 4.4 Hz, H}_3); 6.94 (dd, 1H, J = 5.02, 7.9 Hz, H}_3); 7.26 (d, 2H, J = 8.8 Hz, Ar-H); 7.41 (d, 2H, J = 8.0 Hz, Ar-H); 7.67-7.90 (m, 7H, Ar-H).
\]
**Chapter 3**

\[ ^{13}C \text{ NMR: } \delta 152.6, 148.9, 140.2, 138.5, 134.2, 128.9, 128.2(x2), 126.6, 126.1, 118.5, 108.2, 74.2, 69.8, 56.5, 56.2, 46.3. \]

IR (KBr): 2942 (m), 1560 (s), 1512 (m), 1480 (m), 1420 (m), 1136 (s), 990 (m), 870(w), 685 (m), 760 (m) cm\(^{-1}\).


**Synthesis of phenyl 2-(4-chlorophenyl)-3-(3,4,5-trimethoxyphenyl) isoxazolidine-5-carboxylate 5b(v).** This compound was obtained from \(3b\) (2 g, 6.23 mmol) and vinyl benzoate (1.7 ml, 12.5 mmol). The obtained yield is 2.14 g (72%).

\[ \text{H NMR: } \delta 2.65 \text{(ddd, 1H, J = 13.6, 5.1, 1.9 Hz, H}_4\text{a}); 3.22 \text{(ddd, 1H, J = 13.6, 8.3, 5.5 Hz, H}_4\text{b}); 3.83 \text{(s, 6H, OCH}_3\text{); 3.87 \text{(s, 3H, OCH}_3\text{); 5.2 \text{(dd, 1H, J = 8.8, 3.9 Hz, H}_3\text{); 6.9 \text{(dd, 1H, J = 7.8, 4.96 Hz, H}_5\text{); 6.94 \text{(d, 2H, J = 8.2 Hz, Ar-H); 7.27 \text{(m, 2H, J = 8.4 Hz, Ar-H); 7.6-7.8 \text{(m, 7H, Ar-H).}}}}\]

\[ ^{13}C \text{ NMR: } \delta 167.0, 152.1, 151.4, 146.3, 138.5, 136.2, 128.7, 127.1, 126.7, 125.6, 119.0, 106.2, 71.2, 56.2, 56.8, 55.8, 42.1. \]

IR (KBr): 2980 (m), 2832 (m), 1742 (s), 1485 (m), 1470 (m), 1410 (m), 1280 (s), 1210 (m), 1138 (s), 936 (m), 910 (w), 709 (m) cm\(^{-1}\).

Analytical values calcd for C\(_{25}\)H\(_{24}\)O\(_6\)NCl: C, 63.90; H, 5.15; N, 2.98. Found: C, 63.93; H, 5.18; N, 3.10.
3.7 References


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$^{1}H$ NMR spectra of 3-(3,4,5-trimethoxyphenyl)-2,5-diphenylisoxazolidine

5a(iv)
$^{13}$C NMR spectra of 3-(3,4,5-trimethoxyphenyl)-2,5-diphenylisoxazolidine

5a(iv)