Synthesis and Evaluation of Benzophenone Oximes Derivatized with Sydnone as Inhibitors of Secretory Phospholipase A_2 with Anti-inflammatory Activity

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A series of benzophenone oximes appended with sydnone (3a—h) bearing different substituents on aroyl moiety were synthesized to evaluate *in vivo* and *in vitro* for their inhibitory activity against purified phospholipase A_2 (PLA_2) enzymes from snake venom and human inflammatory pleural and ascites fluid. *In vivo* and *in vitro* inhibition studies were carried out against PLA_2 with respect to the modification of the pharmacophore (substituent) to analyze the specificity for PLA_2. The substituent at the aroyl ring was responsible for enhancing the inhibition towards PLA_2 enzymes. Most of the newly synthesized compounds inhibit the purified PLA_2 enzyme, and the inhibition was more in hydrophobic and aromatic substituents and less when no such substituents were present. The inhibitory effect of the compounds appeared to be due to the direct interaction of compounds with the enzyme. Inhibition is substrate dependent, and the inhibition competes with the substrate for the same binding site of the enzyme. The most active interacting compound 3h from *in vitro* inhibition of PLA_2 activity showed similar potency in the *in vivo* neutralization of PLA_2 induced mouse paw edema and hemolytic activity. Thus, the *in vitro* inhibition correlated well with the *in vivo* inhibition and hence the reported derivatives are therapeutically important anti-inflammatory drugs.

Key words benzophenone oxime; nonsteroidal anti-inflammatory drug; phospholipase A_2; sydnone; synthesis

Phospholipases A_2 (PLA_2) are a family of key enzymes in the metabolism of phospholipids possessing various substrate specificities, co-factor requirements, sub cellular localization and cellular functions. All of these enzymes catalyze hydrolysis of the 2-acylester of 3-*sn* phosphoglycerides to yield arachidonic acid (AA) and lysophospholipid, which is a rate limiting step in the production of pro-inflammatory lipid mediators such as prostaglandins, leukotrienes, lipoxins and platelet activating factor (PAF). The AA is metabolized further to eicosanoids by cycloxygenase and lipoxygenase. These Lipid mediators in turn exert a wide range of potent physiological effects, with excess production linked to diseases such as inflammation, allergy, brain injury, cancer development and metastasis, and cardiovascular disorders. More than ten different forms of the PLA_2 enzymes have been identified.[1)] Group I PLA_2 is present in pancreatic juice and old world snakes, where as II PLA_2 are found in mammals and new world snakes.[2—5)] Increased levels of group II are found in rheumatoid synovial fluid[6)] and in plasma after an inflammatory challenge.[7)] Injection of purified PLA_2 enzyme into the animal joints developed an acute inflammatory response *viz.*, edema, swelling of synovial cells and hyperplasia.[8)] Thus, several findings point to the importance of PLA_2 in inflammatory reactions. Inhibition of such a PLA_2 enzyme by endogenous inhibitors, xenobiotics and synthetic compounds is of potential therapeutic relevance in many inflammatory diseased states. The existence of different types of PLA_2 drew attention to the importance of finding the selective and specific inhibitors for the group PLA_2 enzyme.

Extensive research in preliminary studies has been committed to finding novel compounds for the treatment of inflammation, haemostasis, and bacterial diseases in our laboratory.[9—11)] The search for potent nonsteroidal anti-inflammatory drugs (NSAIDs) to treat the inflammation is still in progress. A literature survey revealed the high potency of anti-inflammatory and other pharmacological activity by benzophenone oxime analogues. The potent anti-inflammatory activity of these compounds has been shown to be due to the inhibition of cycloxygenase enzyme. Inhibition of cycloxygenase and lipoxygenase also indicated the inhibition of PLA_2 enzyme and hence resulted in the reduction of lipid mediators. Hence, Such PLA_2 inhibitors can be used as anti-inflammatory drugs. Benzophenone oximes have structural resemblance with fenoprofen, which belongs to the class of aryl acetic acids. Introduction of hydrophobic alkyl groups on the aryloxy moiety of benzophenone oxime assists in binding with PLA_2 enzyme resulting in the increase of anti-inflammatory activity.[12,13)] A series of derivatives of 3-(2-phenylthio)-ethylsydnones have been synthesized which are more potent than hydrocortisone and phenylbutazone towards adjuvant arthritis.[14)] 3-[4-[3-(Substituted aryl)-1-oxo-2-propenyl]-phenyl]-sydnones were condensed with hydrazine to yield 3-[4-[4,5-dihydro-5-(substituted aryl)-1*H*-pyrazol-3-yl]-phenyl]-sydnones and have shown anti-inflammatory activity in carrageenan induced edema assay in rats.[15)] Upadhya *et al.* have reported 3-substituted 4-(4′-thiazolyl)-sydnones which have potent anti-inflammatory properties.[16)] It has been revealed that coupling of two or more biodynamic molecules resulted in the enhanced biological activity. Hence, in this study we report the synthesis of eight different derivatives of benzophenone oximes appended to sydnones along with variable substituents *viz.*, methyl, *n*-propyl and *n*-butyl groups at *para* position of aryl moiety and a phenyl ring at 4-position of sydnone ring leading to increased hydrophobicity and aromaticity respectively. A comparison of PLA_2 inhibitory, neutralization of edema inducing

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and neutralization of indirect hemolytic activity of these derivatives with purified PLA_2 from *Vipera russelli*, *Naja naja*, *Trimeresurus malabaricus*, synovial and ascites fluid was performed. This study revealed additional information on the extensive structure–activity relationship (SAR) for hydrocarbon chain length and unsaturated aromatic ring on aroyl and sydnone rings respectively.

Experimental

Materials The starting compounds 4-aroyl anilines (**1a**—**d**) were prepared according to the reported method.[17] All the reagents were purchased from the Merck Chemicals (Merck India) and used without further purifications.

Methods Melting points were determined using a Thomas–Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were as films with KBr plates on JASCO (Japan) FTIR-460 plus spectrometer. Nuclear Magnetic Resonance (^{1}H-NMR) spectra were recorded on a Bruker Varian–300 MHz FT-NMR spectrometer. *J* values are given in Hz. Male Albino mice weighing 20—25 g were obtained from the Central Animal Facility, Indian Institute of Science, Bangalore.

***N*-Nitroso-(4-benzoyl-phenyl)-amino Acetic Acid 2a: General Procedure** 4-Amino benzophenone (1.97 g, 10 mmol) **1a**, was mixed with neutralized monochloro acetic acid (0.94 g, 10 mmol) and refluxed on the sand bath for 5 h. The solid obtained was cooled, filtered dried, and subjected to nitrosation at 0—5 °C using sodium nitrite and hydrochloric acid. After completion of the reaction, the formed pale yellow solid nitroso derivative was washed thoroughly with water until the washings were neutral. Purification of this solid by recrystallization using absolute ethanol gave **2a** (2.27 g, 80%) as pale yellow needles; mp 128—130 °C; IR (film): 3471 (carboxylic OH), 1776 (carboxylic C=O), 1642 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 3.75 (2H, s, CH_2), 7.10—7.28 (5H, m, Ar-H), 7.35 (2H, d, Ar-H, J=6.85 Hz), 7.54 (2H, d, Ar-H, J=6.85 Hz), 9.2 (1H, s, OH).

Compounds **2b**—**h** were prepared using a similar sequence of reactions using different substituents on the aroyl moiety of 4-aminobenzophenone. For the preparation of compounds **2e**—**h** ethyl-α-chloro-α-phenyl acetate (1.98 g, 10 mmol) (followed by acid hydrolysis) was used in stead of monochloroacetic acid. The physical and spectral data for **2b**—**h** are listed below.

***N*-Nitroso-[4-{(4′-methyl)-benzoyl}-phenyl]-amino Acetic Acid 2b** Compound **2b** was obtained as pale yellow solid in 82% yield; mp 125—126 °C; IR (film): 3450 (carboxylic OH), 1763 (carboxylic CO), 1635 (C=O) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ: 2.70 (3H, s, CH_3), 3.70 (2H, s, CH_2), 6.85 (2H, J=6.5 Hz, d, Ar-H), 7.01 (2H, J=7.0 Hz, d, Ar-H), 7.10 (2H, J=6.0 Hz, d, Ar-H), 7.30 (2H, d, Ar-H, J=6.0 Hz), 9.0 (1H, s, OH).

***N*-Nitroso-[4-{(4′-propyl)-benzoyl}-phenyl]-amino Acetic Acid 2c** Compound **2c** was obtained as white solid in 65% yield; mp 187—189 °C; IR (film): 3501 (carboxylic OH), 1751 (carboxylic C=O), 1641 (C=O) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.0 (3H, t, CH_3), 1.57 (2H, m, CH_2), 2.40 (2H, t, CH_2), 3.78 (s, 2H, CH_2), 6.67 (d, J=5.8 Hz, Ar-H), 6.91 (2H, d, Ar-H, J=5.8 Hz), 7.22 (2H, d, Ar-H, J=6.0 Hz), 7.35 (2H, d, Ar-H, J=6.0 Hz), 9.0 (s, 1H, OH).

***N*-Nitroso-[4-{(4′-*n*-butyl)-benzoyl}-phenyl]-amino Acetic Acid 2d** Compound **2d** was obtained as pale yellow solid in 60% yield; mp 122—124 °C; IR (film): 3466 (carboxylic OH), 1760 (carboxylic C=O), 1625 (C=O) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.92 (t, 3H, CH_3), 1.34 (m, 2H, CH_2), 1.62 (m, 2H, CH_2), 2.55 (t, 2H, CH_2), 3.70 (s, 2H, CH_2), 6.60 (2H, d, Ar-H, J=6.2 Hz), 6.84 (2H, d, Ar-H, J=6.2 Hz), 7.05 (2H, d, Ar-H, J=5.4 Hz), 7.14 (2H, d, Ar-H, J=5.4 Hz), 9.2 (s, 1H, OH).

***N*-Nitroso-[4-(benzoyl)-phenyl]-phenyl-amino Acetic Acid 2e** Compound **2e** was obtained as white solid in 80% yield; mp 127—130 °C; IR (film): 3480 (carboxylic OH) 1739 (carboxylic C=O), 1615 (C=O) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.77 (1H, s, CH), 7.10—7.35 (14H, bm, Ar-H), 9.80 (s, 1H, OH).

***N*-Nitroso-[4-{(4′-methyl)-benzoyl)}-phenyl]-phenyl-amino Acetic Acid 2f** Compound **2f** was obtained as pale yellow solid in 87% yield, mp 133—135 °C; IR (film): 3492 (carboxylic OH), 1742 (carboxylic C=O), 1609 (C=O) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.95 (3H, s, CH_3), 3.75 (1H, s, CH), 6.95 (2H, d, Ar-H, J=7.15 Hz), 7.05 (2H, d, Ar-H, J=7.15 Hz), 7.10 (2H, d, Ar-H, J=7.0 Hz), 7.19 (2H, d, Ar-H, J=7.0 Hz), 7.26—7.7 (5H, m, Ar-H), 9.25 (1H, s, OH).

***N*-Nitroso-[4-{(4′-*n*-propyl)-benzoyl)}-phenyl]-phenyl-amino Acetic Acid 2g** Compound **2g** was obtained as white solid in 72% yield, mp 165—167 °C; IR (film): 3457 (carboxylic OH), 1750 (carboxylic C=O), 1614 (C=O) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.04 (3H, t, CH_3), 1.54 (2H, m, CH_2), 2.44 (2H, t, CH_2), 3.80 (1H, s, CH), 6.74 (2H, d, Ar-H, J=5.8 Hz), 6.97 (2H, d, Ar-H, J=5.8 Hz), 7.06 (2H, d, Ar-H, J=7.0 Hz), 7.12 (2H, d, Ar-H, J=7.0 Hz), 7.20—7.54 (5H, m, Ar-H), 9.2 (1H, s, OH).

***N*-Nitroso-[4-{(4′-*n*-butyl)-benzoyl)}-phenyl]-phenyl-amino Acetic Acid 2h** Compound **2h** was obtained as pale yellow solid in 65% yield, mp 173—175 °C; IR (film): 3400 (carboxylic OH), 1762 (carboxylic C=O), 1620 (C=O) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.00 (3H, t, CH_3), 1.33 (2H, m, CH_2), 1.57 (2H, m, CH_2), 2.04 (2H, t, CH_2), 3.72 (1H, s, CH), 6.72 (2H, d, Ar-H, J=6.0 Hz), 6.83 (2H, d, Ar-H, J=6.0 Hz), 6.95 (2H, d, Ar-H, J=6.8 Hz), 7.08 (2H, d, Ar-H, J=6.8 Hz), 7.14—7.49 (5H, m, Ar-H), 8.95 (1H, s, OH).

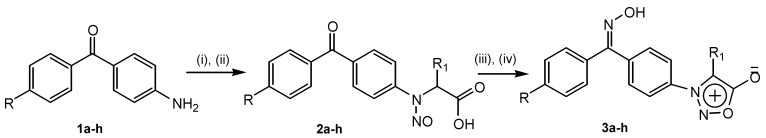
3-[4′-(Hydroxyimino-phenyl-methyl)-phenyl]-sydnone 3a *N*-Nitroso-(4-benzoyl-phenyl)-amino acetic acid **2a** (2.84 g, 10 mmol) was added to acetic anhydride (5.0 ml), and the mixture was refluxed for 3 h. After cooling to 25 °C, the solution was poured into water. The solid obtained was filtered, dried and further refluxed with hydroxyl amine hydrochloride (0.69 g, 10 mmol) for 2 h in 20 ml of absolute ethanol. The reaction mixture was concentrated and the solid obtained was filtered, dried and recrystallized from ethanol to get colorless needles of **3a** in 90% yield; mp 184—186 °C; IR (film): 3400 (OH), 3100 (sydnone C_4–H), 1732 (sydnone C=O), 1600 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.70 (1H, s, sydnone C–H), 6.75 (2H, d, Ar-H, J=7.35 Hz), 6.91 (2H, d, Ar-H, J=6.85 Hz), 7.14 (2H, d, Ar-H, J=6.2 Hz), 7.26 (2H, J=6.2 Hz, d, Ar-H), 8.85 (1H, s, OH).

Compounds **3b**—**h** were prepared using similar cyclization reaction using acetic anhydride followed by ammoximation using hydroxylamine hydrochloride. The stereochemistry of the oximes prepared was not established. The physical and spectral data for the same are listed below.

3-[4′-(Hydroxyimino-*p*-tolyl-methyl)-phenyl]-sydnone 3b Compound **3b** was obtained as pale yellow solid in 61% yield, mp 146—145 °C; IR (film): 3403 (OH), 3110 (sydnone C_4–H), 1728 (sydnone C=O), 1613 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.17 (3H, s, CH_3), 6.65 (1H, s, sydnone C–H), 6.90 (2H, d, J=6.0 Hz, Ar-H), 7.0 (2H, d, Ar-H, J=6.0 Hz), 7.15 (2H, d, Ar-H, J=5.4 Hz), 7.22 (2H, d, Ar-H, J=5.4 Hz), 8.64 (1H, s, OH).

3-[4-{Hydroxyimino-(4″-*n*-propyl-phenyl)-methyl}-phenyl]-sydnone 3c Compound **3c** was obtained as pale yellow solid in 56% yield, mp 164—166 °C; IR (film): 3397 (OH), 3106 (sydnone C_4–H), 1745 (sydnone C=O), 1612 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.00 (3H, t, CH_3), 1.39 (2H, m, CH_2), 2.50 (2H, t, CH_2), 6.72 (1H, s, sydnone C–H), 7.05 (2H, d, Ar-H, J=5.0 Hz), 7.13 (2H, d, Ar-H, J=5.0 Hz), 7.20 (2H, d, Ar-H, J=6.2 Hz), 7.26 (2H, d, Ar-H, J=6.2 Hz), 8.85 (1H, s, OH).

3-[4-{Hydroxyimino-(4″-*n*-butyl-phenyl)-methyl}-phenyl]-sydnone 3d Compound **3d** was obtained as yellow solid in 50% yield, mp 112—114 °C; IR (film): 3421 (OH), 3096 (sydnone C_4–H), 1744 (sydnone C=O), 1607 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.04 (3H, t, CH_3), 1.42 (2H, m, CH_2), 1.50 (2H, m, CH_2), 2.10 (2H, t, CH_2), 6.80 (1H, s, sydnone C–H), 6.98 (2H, d, Ar-H, J=6.4 Hz), 7.09 (2H, J=6.4 Hz, d, Ar-H), 7.14 (2H, d, Ar-H, J=6.0 Hz), 7.20 (2H, d, Ar-H, J=6.0 Hz), 8.9 (1H, s, OH).



a; R = H, R_1 = H, **b**; R = CH_3, R_1 = H, **c**; R = n- Propyl, R_1 = H, **d**; R = n-Butyl, R_1 = H,
e; R = H, R_1 = Ph, **f**; R = CH_3, R_1 = Ph, **g**; R = n- Propyl, R_1 = Ph, **h**; R = n-Butyl, R_1 = Ph,

Reagents and conditions: i) $ClCHR_1COOR_2$ (**4i**—**j**)/heat, ii) $NaNO_2$/HCl, 0—-5 °C, iii) Ac_2O/reflux, iv) $NH_2OH \cdot HCl$/reflux.

Chart 1

3-[4′-(Hydroxyimino-phenyl-methyl)-phenyl]-4-phenyl-sydnone 3e Compound **3e** was obtained as yellow solid in 66% yield, mp 203—205 °C; IR (film): 3401 (OH), 1722 (sydnone C=O), 1610 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 6.89 (2H, d, Ar-H, J=6.0 Hz), 7.09 (2H, d, Ar-H, J=6.0 Hz), 7.14—7.40 (10H, m, Ar-H), 8.8 (1H, s, OH).

3-[4′-(Hydroxyimino-*p*-tolyl-methyl)-phenyl]-4-phenyl-sydnone 3f Compound **3f** was obtained as yellow solid in 58% yield, mp 147—149 °C; IR (film): 3421 (OH), 1701 (sydnone C=O), 1608 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 2.00 (3H, s, CH_3), 6.95 (2H, d, Ar-H, J=5.3 Hz), 7.02 (2H, d, Ar-H, J=5.3 Hz), 7.09—7.36 (9H, m, Ar-H), 9.0 (1H, s, OH).

3-[4-{Hydroxyimino-(4″-*n*-propyl-phenyl)-methyl}-phenyl]-4-phenyl-sydnone 3g Compound **3g** was obtained as yellow solid in 62% yield, mp 221—223 °C; IR (film): 3412 (OH), 1706 (sydnone C=O), 1597 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.14 (3H, t, CH_3), 1.40 (2H, m, CH_2), 2.42 (2H, t, CH_2), 6.87 (2H, d, Ar-H, J=6.8 Hz), 6.93 (2H, d, Ar-H, J=6.8 Hz), 7.00—7.30 (9H, m, Ar-H), 8.67 (1H, s, OH).

3-[4-{Hydroxyimino-(4″-*n*-butyl-phenyl)-methyl}-phenyl]-4-phenyl-sydnone 3h Compound **3h** was obtained as yellow solid in 57% yield, mp 160—162 °C; IR (film): 3400 (OH), 1713 (sydnone C=O), 1611 (C=N) cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.97 (3H, t, CH_3), 1.35 (2H, m, CH_2), 1.58 (2H, m, CH_2), 2.44 (2H, t, CH_2), 6.94 (2H, d, Ar-H, J=5.6 Hz), 7.0 (2H, d, Ar-H, J=5.6 Hz), 7.14—7.35 (9H, m, Ar-H), 8.95 (1H, s, OH).

Determination of Edema Inducing Activity All protocols of animal experiments have been approved by the Institutional Animal Ethics Committee (IAEC). Groups of six mice (22—24 g) were injected in the right footpads of hind limbs with 3 mM dose of benzophenone analogues in 20 μl saline. The left footpads received 20 μl of saline, which served as control. After 45 min mice were sacrificed by cervical dislocation and both legs were removed at the ankle joint and weighed individually. The increase in weight due to edema was calculated as the edema ratio, which equals the weight of edematous leg×100/weight of the normal leg. Minimum edema dose is defined as the microgram of protein causing an edema ratio of 120%. Injecting a fixed dose protein into mice footpads and sacrificing them at regular period of time obtained time course curve of edema inducing activity. Edema ratio was calculated as defined.[12)]

Determination of PLA_2 Activity Assayed with [^{14}C] oleate-labelled autoclaved *Escherichia coli* as the substrate.[19)] The reaction mixture, 350 μl contained 100 mM Tris/HCl, pH 8.0, 5 mM Ca^{2+} and 3.15×10^9 autoclaved *E. coli* cells (corresponding to 10000 cpm and 60 nmol of lipid phosphorous). The amount of enzyme protein was chosen such that 10—15% hydrolysis of substrate was obtained when incubated at 37 °C for 60 min. The reaction components were mixed in the following order: buffer, calcium, water and benzophenone analogue. Adding labelled *E. coli* substrate started the reaction. The reaction was terminated by adding 100 μl 2.0 M HCl and 100 μl of fatty acid free BSA (100 mg/ml). The tubes were vortex mixed and centrifuged at 20000 mg for 5 min. Aliquot (140 μl) of the supernatant containing released [^{14}C] oleic acid was mixed with scintillation cocktail and counted in a Hewlett Packard liquid Scintillation Analyzer TRICARB 2100 TR.

Neutralization of Indirect Hemolytic Activity Indirect hemolytic activity was assayed using the method of Boman and Kaletta.[18)] Briefly, packed human erythrocyte egg yolk and phosphate-buffered saline (1 : 1 : 8, v/v) were mixed. This suspension (1 ml) was incubated with the 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 erythrocyte caused by the addition of 9 ml distilled water taken as 100%. Values are presented as the mean of 4 independent determinations.

Results

The synthetic route of compounds **3a**—**h** is as depicted in Chart 1. The starting compounds 4-aroyl aniline (**1a**—**c**) was refluxed with **4i**—**j** in alkaline medium to get the corresponding esters of substituted amino acids. The esters of amino acids were hydrolyzed with 10% NaOH to get substituted amino acid which was nitrosated at 0—5 °C using $NaNO_2$ in 25% HCl to give the *N*-nitroso derivative (**2a**—**h**). The nitroso derivative (**2a**—**h**) was cyclized to get the sydnone derivative on refluxing with acetic anhydride. This was followed by ammoximation using hydroxylamine hydrochloride in absolute alcohol to get the final product (**3a**—**h**). The structures of nitroso derivative (**2a**—**h**) and the final products (**3a**—**h**) were confirmed by their IR, NMR data which are in complete agreement.

Derivatives were of two series based on the substitution in the 4th position of sydnone ring. Derivatives with –H in the 4th position of the sydnone **3a**—**d** along with the hydrophobic groups on *para* position of the benzoyl moiety and those with phenyl ring at 4th position of and hydrophobic substituents on the *para* position of benzoyl moiety **3e**—**h**. In both series, **3a**—**d** have only the alkyl substituents which are hydrophobic in nature. Whereas, the compounds **3e**—**h** have both hydrophobic alkyl groups and phenyl group which adds up the aromatic character.

The parent compound benzophenone is known to have anti-inflammatory properties. The compounds **3a**—**h** were tested for anti-inflammatory properties by studying their interaction with different PLA_2 enzymes. Inflammatory PLA_2 from *V. russelli*, *N. naja*, *T. malabaricus*, synovial and ascites fluid were used to study interaction with benzophenones derivatized with sydnone ring. Figure 1 shows the interaction of all 8 benzophenone oxime analogues (75 μM concentration) with different types of enzymes. All of them inhibited PLA_2 enzyme activity. These derivatives especially **3e**—**h** have inhibited all the types of PLA_2 enzymes. The compounds **3g**—**h** have shown the inhibition activity almost equal to the standard Luffariellin B due to the presence of hydrophobic group on the aroyl moiety and the aromatic phenyl ring at the 4th position of the sydnone moiety. The compounds **3b**—**d** have shown moderate activity due to the presence of the only the presence of hydrophobic alkyl groups. The compound **3a** has shown very weak activity due to the absence of both type of groups *viz.*, hydrophobic and aromatic. The similar trend is observed also in case of the edema inducing activity.

Discussion

Understanding the molecular interaction between the PLA_2 enzyme and agents inhibiting its activity is crucial in designing the powerful inhibitors. Several endogenous and exogenous agents such as lipocortin,[19)] *cis*-unsaturated fatty acids,[20)] gangliosides,[21)] manoalide,[22)] cacosponginolide, petrosasponginolide,[23)] surfactin,[24)] retinoids,[10,25)] flavonoids,[26)] aristolochic acid,[27)] natural plant lipids,[28)] and synthetic lipids,[29)] have been shown to inhibit the PLA_2 enzyme. From studies with the different inhibitors, it is generally understood that most PLA_2 inhibitors are hydrophobic compounds containing long hydrocarbon chain with one to several aromatic rings.[19—30)] Molecules with a more hydrophobic nature have a better interaction with the PLA_2 enzyme and greater inhibitory activity. The parent compounds benzophenone derivatives have been shown to have anti-inflammatory properties by selective inhibition of cycloxygenase-2 (COX-2) enzyme.[31,32)] The clinical results with COX and lipoxygenase inhibitors demonstrated that inhibition of the PLA_2 enzyme results in a reduction of both lipid mediators. As a result, these PLA_2 inhibitors can be used as anti-inflammatory drugs that are as powerful as anti-inflammatory steroidal compounds. Some derivatives of benzophenone oximes exhibited potent anti-inflammatory activity but failed to exhibit COX-2, thus suggesting the action by a different mechanism. This observation led to the inference that these compounds might

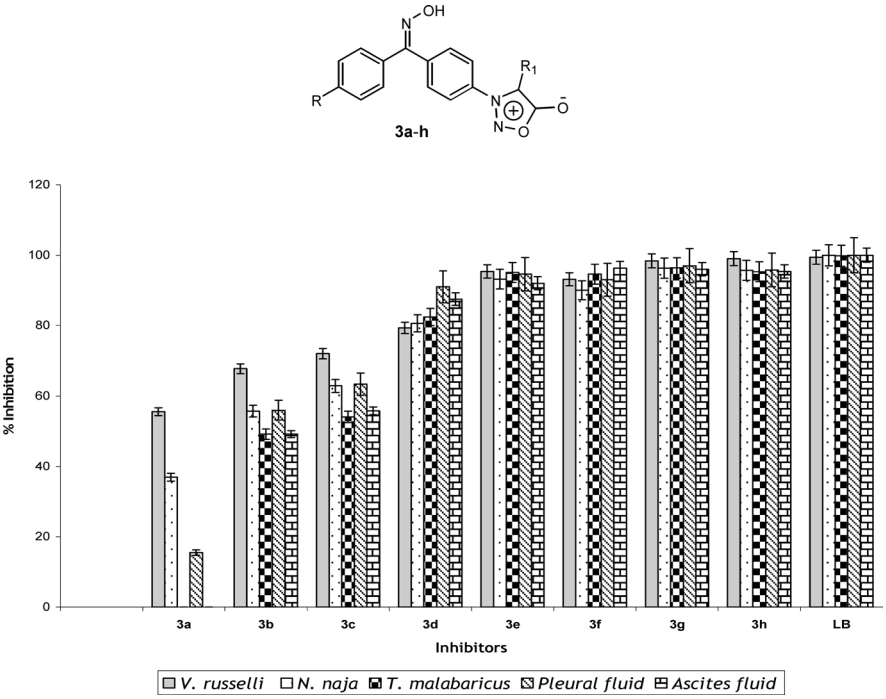


Fig. 1. *In Vitro* Inhibition of PLA_2 Enzyme Activity Data for 3-[4-{Hydroxyimino-(aryl)-methyl}-phenyl]-4-(un)-substituted-sydnones **3a**—**h**

Inhibition of PLA_2 enzymes by compounds at 75 μM concentration (LB is Luffariellin B, standard). Each compound is preincubated with enzyme for 30 min. The 350 μl reaction mixture contained 30 μl of substrate (added after preincubation), 35 μl of 1 M Tris–HCl buffer pH 7.5, 5 mM $CaCl_2$ and distilled water, and was incubated for 1 h at 37 °C. Data represent mean±S.E.M. for $n=4$.

interact with the PLA_2 enzyme and exhibit stronger anti-inflammatory activity. In this study, several benzophenone derivatives were synthesized with increased hydrophobicity at the aroyl moiety and 4th position of sydnone ring of the parent compound **3a**, and their effect on *in vitro* PLA_2 inhibition and edema inhibiting activity in a mouse model was examined. The variable extent to which different PLA_2 enzymes are inhibited may be due to differential binding affinities. These differential affinities with benzophenones derivatized with sydnone varied as the hydrophobicity and aromaticity were increased in the ring (Fig. 1). The percentage inhibition was increased from **3a** to **3h** as the chain length of alkyl group was increased. The inhibition was minimum when no alkyl substituent was present (*e.g.*, the inhibition of **3a** against *V. russeli* was 55.6% and that of **3h** with *n*-butyl substituent was 79.3%).

The inhibition is dose dependent and increases with the increase in hydrophobicity. The increased inhibition is somewhat linear with increased hydrophobicity in the aliphatic chain length of methyl, *n*-propyl and *n*-butyl groups. When the aromatic phenyl ring is introduced along with the aliphatic side chains, the inhibition was much stronger and further enhanced indicating a preference by the enzyme for hydrophobic molecules along with unsaturated aromatic rings as better inhibitors (Fig. 2). Similar hydrophobic compounds, which are inhibitors of PLA_2, are chemically modified tetracyclins,[33] derivatives of isoxazolines,[11,13] indole analogues,[12] and flavonoids,[26] all exhibiting stronger PLA_2 inhibition with increased hydrophobicity and unsaturated rings in their structure. These studies clearly indicate that the hydrophobicity and aromatic nature is critical for inhibition of the enzyme.

Injection of purified PLA_2 enzyme from snake venom and

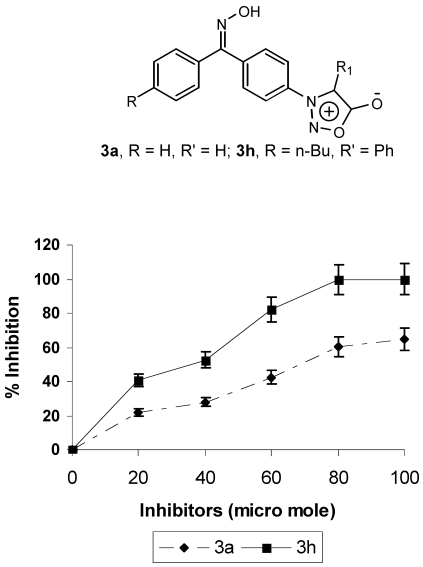


Fig. 2. Dose Dependent Inhibition of PLA_2 Enzymes by 3-[4′-(Hydroxyimino-phenyl-methyl)-phenyl]-sydnone **3a** and 3-[4-{Hydroxyimino-(4″-*n*-butyl-phenyl)-methyl}-phenyl]-4-phenyl-sydnone **3h**

The 350 μl reaction mixture containing 2 μg of *V. russelli* in 100 mM Tris–HCl buffer pH 7.5, 5 mM $CaCl_2$ and indicated concentrations of **3a** and **3h** was preincubated for 30 min at 37 °C. The PLA_2 activity was initiated by adding substrate (60 nmol *E. coli* phospholipid), then continued incubation at 37 °C for 1 h.

inflammatory fluids into animal joints resulted in acute inflammatory response with edema, swelling of synovial cells and hyperplasia. This may be due to their combined effect to hydrolyze the membrane integrity, as well as the generation and metabolism of products such as eicosanoids, which then function to amplify inflammatory events.[34,35] Several studies with the specific PLA_2 enzyme-modifying agents, such as *p*-

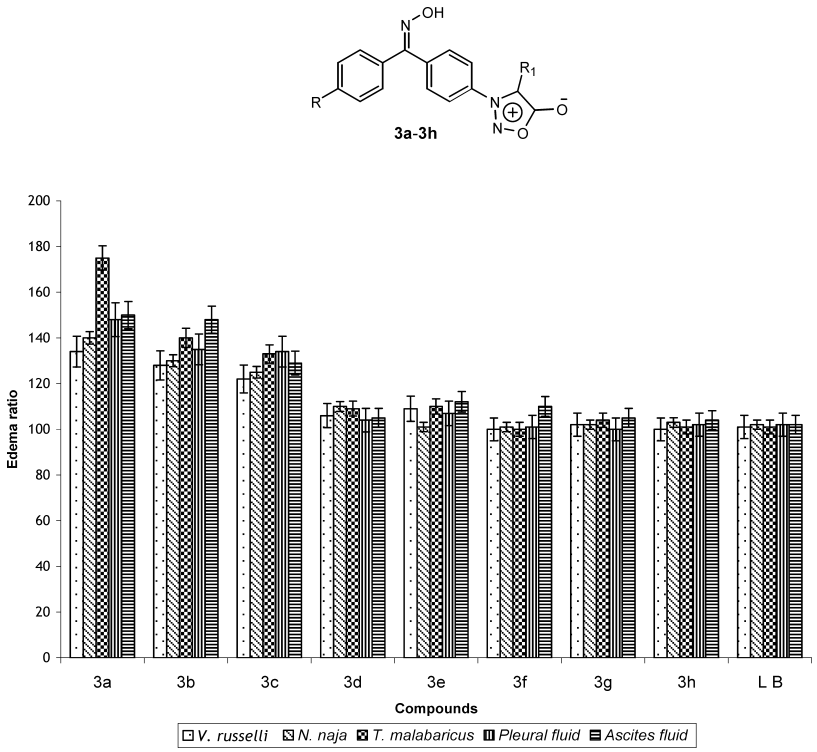


Fig. 3. *In Vivo* Edema Inducing Activity Data for 3-[4-{Hydroxyimino-(aryl)-methyl}-phenyl]-4-(un)-substituted-sydnones **3a**—**h**

Percent inhibition of sample concentration of 2.67 mM. Edema ratio=weight of edematous leg×100/weight of normal leg. Amount of venom taken for each assay was 1 μg. The PLA_2 enzyme (1 μg): benzophenone analogues/Luffariellin B (3 mM) mixture was preincubated at 37 °C for 1 h prior to injection into the mice footpads. Prolonged preincubation time up to 12 h, at 37 °C did not have any additional effect on edema ratio. Values of edema ratios are expressed as mean±S.D. (n=6), p values <0.05 were considered significant when compared to the control by student's t-test. Inhibitory rate (%)=nanomoles of labelled substrate released as compared to the control.

bromophenacylbromide (p-BPB) resulting in the loss of enzyme activity and edema inducing activity, directly indicates that the edema produced by these PLA_2 enzymes is mediated by the catalytic domain of the enzyme.[36] Benzophenones derivatized with sydnones **3a**—**h** with a co-injection of snake venom PLA_2 enzyme activity decreased the edema-inducing activity in dose dependent manner. The concomitant inhibition of PLA_2 enzyme activity and *in vivo* edema-inducing activity by these derivatives suggests a strong correlation between lipolytic activity and pro-inflammatory activities. Since the benzophenone derivative binds at the catalytic site of the enzymes, it is effective in preventing the enzyme from inducing edema (Figs. 3, 4). In this case also the compounds with hydrophobic alkyl groups and presence of an aromatic ring on the 4th position of sydnone ring inhibit the enzyme thus reducing the edema inducing activity.

Inhibition of the PLA_2 enzyme by benzophenone derivatives is reflected in the inhibition of indirect hemolytic activity (Fig. 5) where it was assayed with crude egg phospholipids mixture dispersed in the buffer as micelles, rather than an intact *E. coli* membrane. Since the benzophenone derivative binds at the substrate-binding regions of the enzymes, enzyme activity is inhibited irrespective of the substrate nature provided for its activity. It has been observed that increase in the dose has increased the inhibition of indirect hemolytic activity.

In summary, the above-synthesized compounds are good inhibitors of the PLA_2 enzyme. The inhibition by benzophenone derivatives was further enhanced by substituting hydrophobic and aromatic groups on the *para* position of aroyl moiety and 4th position of the sydnone ring which is essential for binding at the catalytic domain of the enzyme. As the *in vitro* inhibition of the enzyme by these derivatives corre-

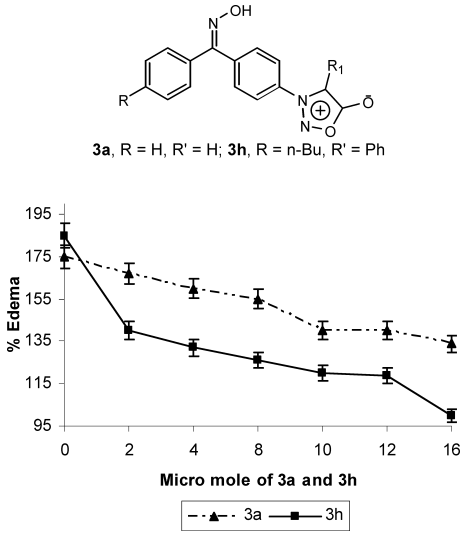


Fig. 4. Dose Dependent Neutralization of Edema Inducing Activity of *V. russelli* by 3-[4′-(Hydroxyimino-phenyl-methyl)-phenyl]-sydnone **3a** and 3-[4-{Hydroxyimino-(4″-*n*-butyl-phenyl)-methyl}-phenyl]-4-phenyl-sydnone **3h**

The reaction mixture 30 μl contains 5 μg PLA_2 enzyme was incubated for 30 min with increasing concentration of **3a** and **3h**. Saline (30 μl) injected into the mouse footpad served as control. Data represent ±S.E.M. for n=4.

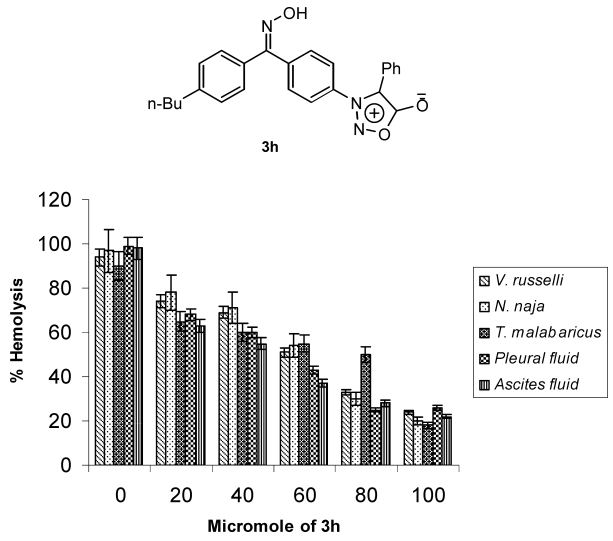


Fig. 5. Dose Dependent Neutralization of Indirect Hemolytic Activity of 3-[4-{Hydroxyimino-(4″-*n*-butyl-phenyl)-methyl}-phenyl]-4-phenyl-sydnone **3h**

Enzyme (20 μg) in 100 μl of phosphate-buffered saline (PBS) was preincubated with 0 to 100 μg of **3h**. The reaction was 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 shows ±S.E.M. for n=4.

lated well with the *in vivo* inhibition of the edema-inducing activity, these derivatives are therapeutically important as anti-inflammatory drugs.

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