4.1. CHEMISTRY

4.1.1. Chemicals and Reagents

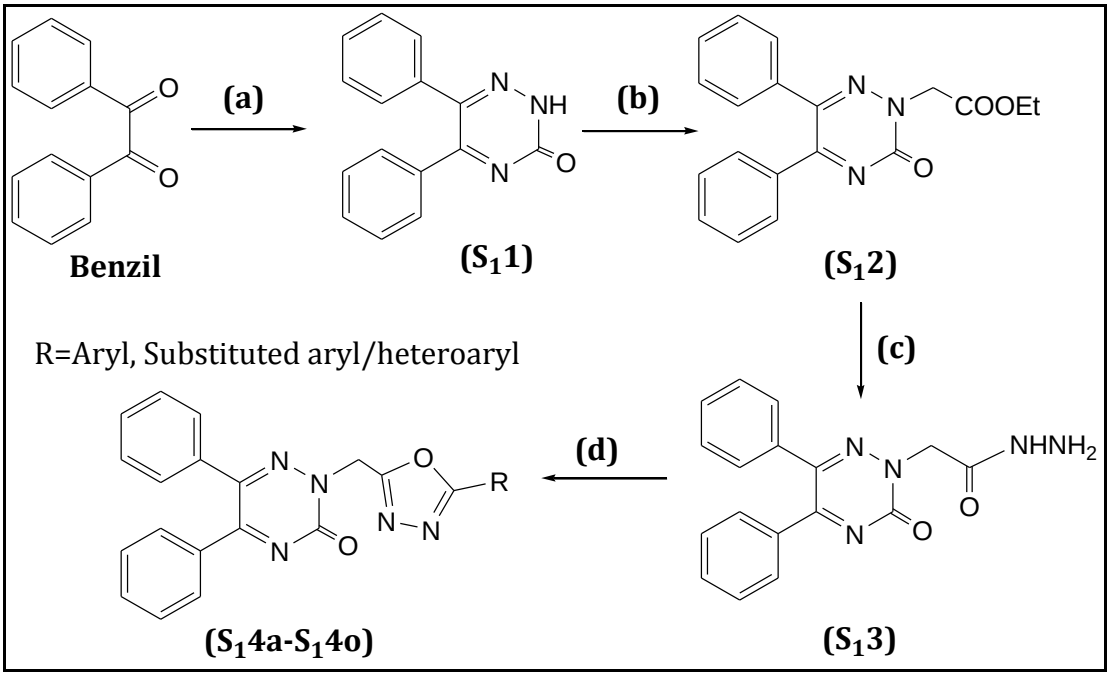
All the chemicals and reagents used in the study were of analytical grade and were procured from Sigma–Aldrich (India), Merck (Germany) and SD Fine Chemicals (India).

4.1.2. Series 1

Synthesis of 5,6–diphenyl–1,2,4–triazine–3(2*H*)–ones bearing 5–substituted 1,3,4–oxadiazole.

4.1.2.1. Method of Preparation

The reaction conditions necessary for the synthesis of a 5,6–diphenyl–1,2,4–triazin–3(2*H*)–one derivatives bearing 5–substituted 1,3,4–oxadiazoles is illustrated in **Scheme 4.1**.



Scheme 4.1. The general scheme for the synthesis of target compounds **($S_1$4a-$S_1$4o)**: **(a)** Semicarbazide, Glacial acetic acid, Reflux 6 h **(b)** $ClCH_2COOEt$, *N*, *N*-DMF, Reflux 6 h **(c)** $NH_2NH_2.H_2O$, ethanol, Reflux 6 h **(d)** $POCl_3$, substituted aryl/heteroaryl acid**,** Reflux 6-8 h.

- **5,6–Diphenyl–1,2,4–triazin–3(2*H*)–one ($S_1$1)**

5,6–Diphenyl–1,2,4–triazin–3(2*H*)–one **($S_1$1)** was prepared with slight modifications to the reported procedure in the literature (Mullick *et al.*, 2009) wherein 0.05 mol of benzil (I) (0.3 g, 26 mmol) (1 Mol. Eq.) and semicarbazide (0.56 g, 26 mmol) (1 Mol. Eq.) were dissolved in 30 mL of glacial acetic acid. The reaction mixture was refluxed for 6 h instead of reported 8-10 h, the progress being monitored by TLC using n-hexane/ethyl acetate (1:1) as the mobile phase. After completion, the reaction mixture was allowed to cool and poured onto the crushed ice. The solid mass precipitated was filtered, washed with water, and recrystallised from ethanol to yield the title compound **$S_1$1.**

- **Ethyl 2–(3–oxo–5,6–diphenyl–1,2,4–triazin–2(3*H*)–yl)acetate ($S_1$2)**

Intermediate **$S_1$2** was synthesised from 5,6–Diphenyl–1,2,4–triazin–3(2*H*)–one **($S_1$1)** with slight modifications to the procedure reported in the literature (Abdel Rahman *et al.*, 1994) wherein **($S_1$1)** (0.5 g, 2 mmol) (1 Mol. Eq.) and Chloroethyl acetate (0.25 g, 2 mmol) (1 Mol. Eq.) were dissolved in 25 ml of *N*, *N*-Dimethyl formamide (DMF) and refluxed for 6 h instead of reported 8 h. The progress of the reaction was monitored by TLC using n-hexane/ethyl acetate (1:1) as the mobile phase. After completion, the reaction mixture was allowed to cool and poured onto the crushed ice. The solid mass precipitated was filtered, washed with water, and recrystallised from ethanol to yield the title compound **$S_1$2.**

- **2–(3–Oxo–5,6–diphenyl–1,2,4–triazin–2(3*H*)–yl)acetohydrazide ($S_1$3)**

Intermediate **$S_1$3** was synthesised from Ethyl 2–(3–oxo–5,6–diphenyl–1,2,4–triazin–2(3*H*)–yl)acetate **($S_1$2)** with slight modifications to the reported procedure (Abdel Rahman *et al.*, 1994). Ethyl 2-(3-oxo-5,6-diphenyl-1,2,4-triazin-2(3*H*)-yl)acetate (0.6 g, 1.789 mmol) (1 Mol. Eq.) was dissolved in 10 ml methanol by heating at around 60°C. To this solution, hydrazine hydrate (4 Mol. Eq.) was added dropwise. The reaction mixture was refluxed for 6 h instead of reported 1 h with the progress being monitored by TLC using DCM/methanol (2:8) as the mobile phase. Post completion, the reaction mixture was allowed to

cool and poured onto crushed ice. The solid that separated was filtered dried and recrystallised from ethanol to yield the title compound **$S_1$3**.

- **General procedure for the synthesis of compounds ($S_1$4a–$S_1$4o):**

Compound **$S_1$3** (0.3 g, 0.93 mmol) (1 Mol. Eq.) and different substituted aryl/heteroaryl acids (1 Mol. Eq.) were placed in a round–bottom flask and cooled in an ice bath. 10–12 ml of phosphorous oxychloride ($POCl_3$) was slowly poured into the above reaction mixture. After the complete addition, the contents were refluxed for 6–8 h. The progress of the reaction was monitored by TLC using n-hexane/ethyl acetate (1:1) as the mobile phase. After completion, the reaction mixture was allowed to cool and poured onto crushed ice and kept overnight. The contents of the beaker were filtered to yield a crude product that was recrystallised from ethanol to yield compounds **$S_1$4a–$S_1$4o**.

4.1.2.2. Reaction Mechanism for Series 1

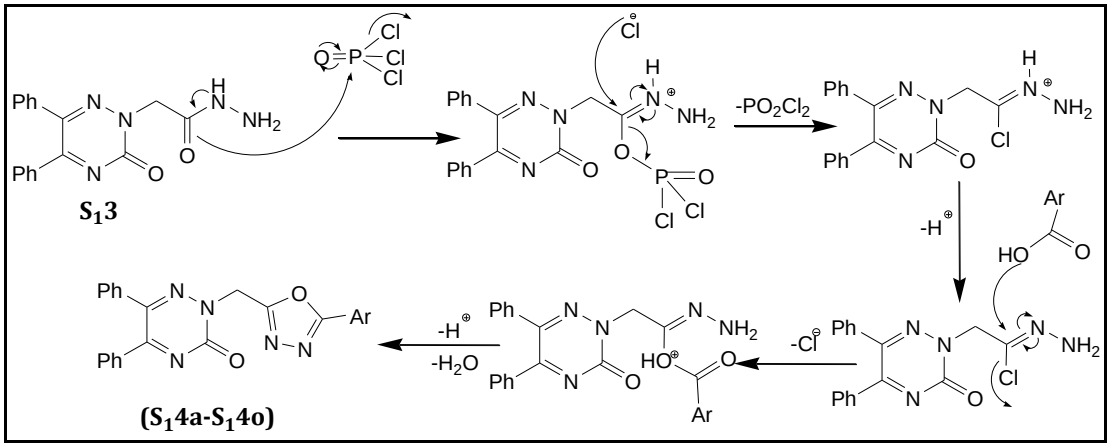


Figure 4.1. Possible mechanism of reaction for the synthesis of compounds **$S_1$4a–$S_1$4o.**

The amide portion of the acetohydrazide **$S_1$3** reacts with phosphorus oxychloride wherein the aceto group departs and is replaced by chlorine. The intermediate iminium cation reacts with the carboxylic acid group of the respective aryl/substituted aryl/heteroaryl acid. Thus, a nucleophilic substitution reaction takes place at the imine intermediate, in which the chloride group is substituted with the -OH group of aryl/substituted aryl/heteroaryl acid. The –OH moiety from the acid acts as a nucleophile with the chloride atom as the

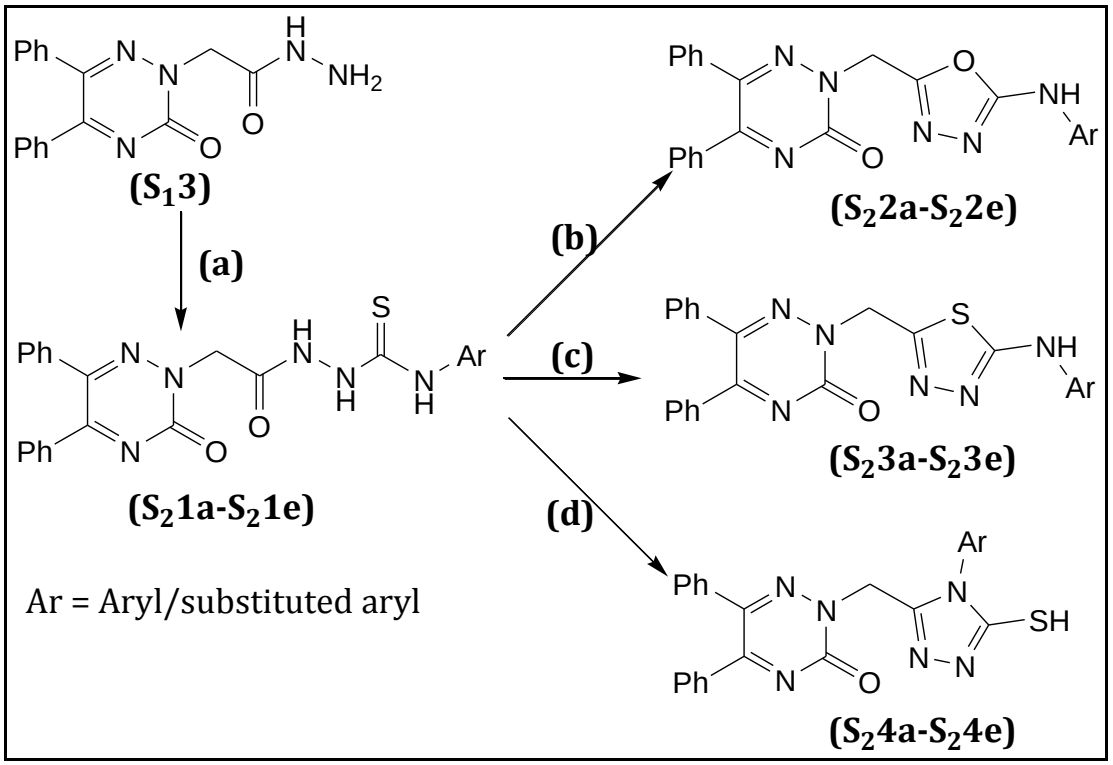
leaving group. This intermediate further undergoes deprotonation followed by cyclic dehydration to afford the 1,3,4-oxadiazole nucleus **(Fig. 4.1)** as seen in compounds **$S_1$4a– $S_1$4o**.

4.1.3. Series 2

Synthesis of a series of 5,6-diphenyl-1,2,4-triazine-3(2*H*)-ones bearing five-member (1,3,4-oxadiazole/thiadiazole, 1,2,4-triazole) heterocyclic moieties.

4.1.3.1. Method of Preparation

The reaction conditions necessary for the synthesis of 5,6-diphenyl-1,2,4-triazine-3(2*H*)-ones bearing five-member (1,3,4-oxadiazole/thiadiazole, 1,2,4-triazole) heterocyclic moieties is illustrated in **Scheme 4.2**.



Scheme 4.2. The general scheme for the synthesis of target compounds: **(a)** Phenyl/substituted phenyl isothiocyanate, Ethanol (75% v/v), Reflux 6 h **(b)** KI/I_2, aq. NaOH (5N), Reflux 1 h **(c)** cold H_2SO_4**,** Stirring 4-6 h **(d)** aq. NaOH (4N), Reflux 2 h.

- **General procedure for the synthesis of compounds ($S_2$1a-$S_2$1e):**

2-(3-Oxo-5,6-diphenyl-1,2,4-triazin-2(3*H*)-yl)acetohydrazide (0.5 g, 1.55 mmol) (1 Mol. Eq.) **($S_1$3)** was dissolved in aq. ethanol (75% v/v) with gentle heating at around 70°C. Various phenyl/substituted phenyl isothiocyanate derivatives (1.5 Mol. Eq.) were added to the above mixture in small portions with stirring. The reaction mixture was refluxed for 6 h. The progress of the reaction was monitored by TLC with DCM/methanol (9.0:1.0) as the mobile phase. After completion, the reaction mixture was filtered, and the precipitate was washed with a small amount (3x5 ml) of ethyl acetate (EtOAc) to remove away any unreacted isothiocyanate. The residual EtOAc layer was evaporated in a rotary vacuum evaporator to yield the title compounds **($S_2$1a-$S_2$1e)**.

- **General procedure for the synthesis of compounds ($S_2$2a-$S_2$2e):**

A suspension of intermediate **($S_2$1a-$S_2$1e)** (0.4g, 1 Mol. Eq.) in 25 ml of ethanol was dissolved in 1 ml of 5N aq. NaOH with cooling at around 30°C with stirring resulted in a clear solution. To this, iodine in potassium iodide solution (5%) was added gradually with stirring till the colour of the iodine persisted at room temperature. The reaction mixture was then refluxed for 1 h. The progress of the reaction was monitored by TLC with DCM/methanol (9.5:0.5) as the mobile phase. After completion, the contents of the beaker were poured over crushed ice. The solid that separated was filtered, dried and recrystallised from ethanol to yield the title compounds **($S_2$2a-$S_2$2e)**.

- **General procedure for the synthesis of compounds ($S_2$3a-$S_2$3e):**

Intermediates (**$S_2$1a-$S_2$1e**) (0.4g, 1 Mol. Eq.) were added gradually with stirring to a cold solution of concentrated sulphuric acid (10 ml). The reaction mixture was stirred for 4-6 h in an ice bath. The progress of the reaction was monitored by TLC with DCM/methanol (9.5:0.5) as the mobile phase. After completion, the contents of the beaker were poured over crushed ice and the solid thus separated was filtered, washed with water, and recrystallised from ethanol to yield the title compounds **($S_2$3a-$S_2$3e)**.

- **General procedure for the synthesis of compounds ($S_2$4a-$S_2$4e).**

A suspension of intermediates **($S_2$1a-$S_2$1e)** (0.4g, 1 Mol. Eq.) in 25 ml of absolute ethanol was dissolved in aq. NaOH (4N, 2 ml) and gently refluxed for 2 h. The progress of the reaction was monitored by TLC with DCM/methanol (9.5:0.5) as the mobile phase. After completion, the resulting solution was cooled, concentrated and filtered. The pH of the filtrate was adjusted between 5-6 with dilute acetic acid (40% v/v) and kept aside for 1 h to afford the compounds **($S_2$4a-$S_2$4e)**.The crude product was filtered, washed with water, dried and recrystallised from ethanol.

4.1.3.2. Reaction Mechanism for Series 2

The amine group of the acetohydrazide derivative **$S_1$3** acts as a nucleophile which attacks the isothiocyanate group of respective phenyl/substituted phenyl isothiocyanate to yield the derivatives **$S_2$1a-$S_2$1e** *via* a nucleophilic addition reaction **(Fig. 4.2)**.

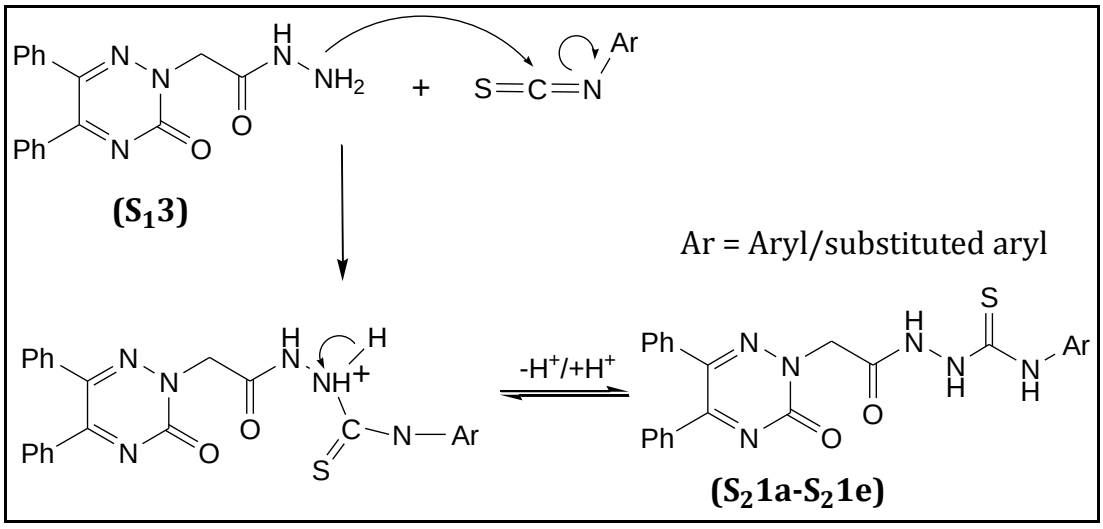


Figure 4.2. Possible mechanism of reaction for the synthesis of compounds **$S_2$1a–$S_2$1e**.

The synthesis of 1,3,4-oxadiazoles derivatives **($S_2$2a-$S_2$2e)** occurs *via* activation of the sulphur atom through an iodine-mediated oxidation followed by an intra-molecular C–O/C–N bond formation in the presence of a base (aq. NaOH) **(Fig. 4.3)**.

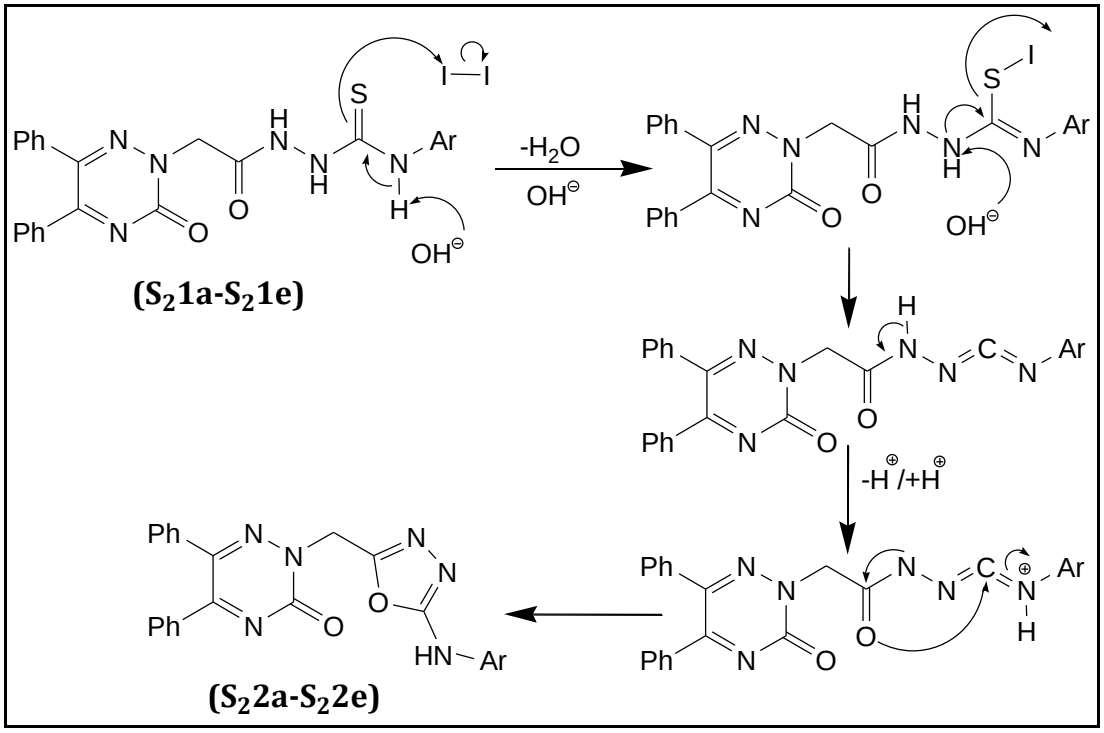


Figure 4.3. Possible mechanism of reaction for the synthesis of compounds **$S_2$2a–$S_2$2e.**

A nucleophilic addition reaction takes place at the carbonyl of thiosemicarbazide derivative in which the thiol group acts as a nucleophile to afford the thiadiazole derivative **($S_2$3a-$S_2$3e)** *via* an intra-molecular cyclisation followed by rearrangement and dehydration in the presence of cold sulphuric acid **(Fig. 4.4)**.

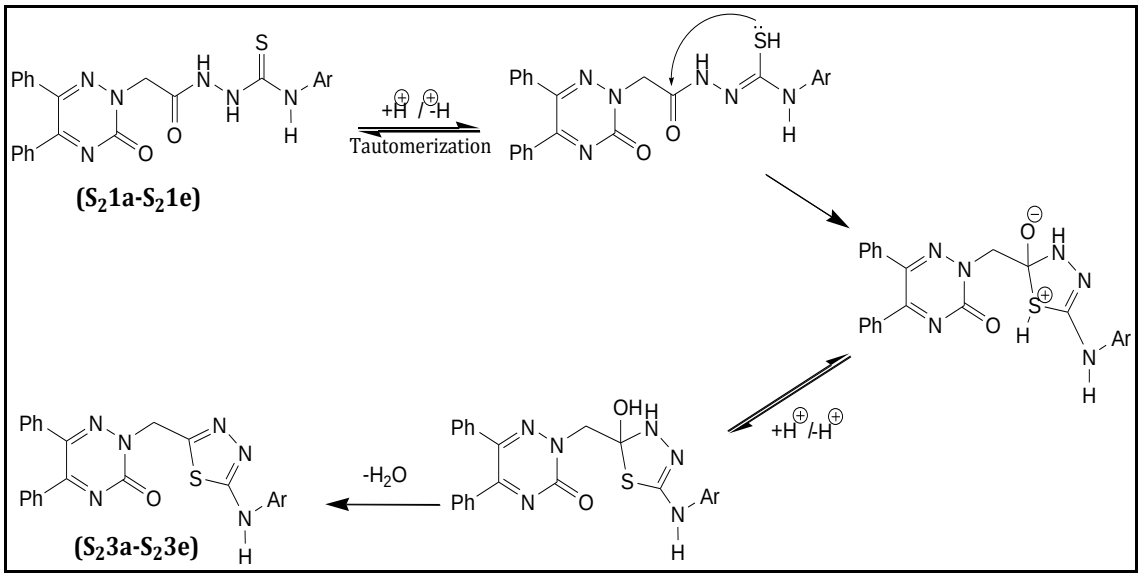


Figure 4.4. Possible mechanism of reaction for the synthesis of compounds **$S_2$3a–$S_2$3e.**

The thiosemicarbazide **($S_2$1a-$S_2$1e)** yields the 1,2,4-triazole derivatives **($S_2$4a-$S_2$4e)** *via* a nucleophilic addition reaction followed by intra-molecular cyclisation and dehydration wherein an intra-molecular C–N bond is formed in the presence of a base (4N NaOH) **(Fig. 4.5)**.

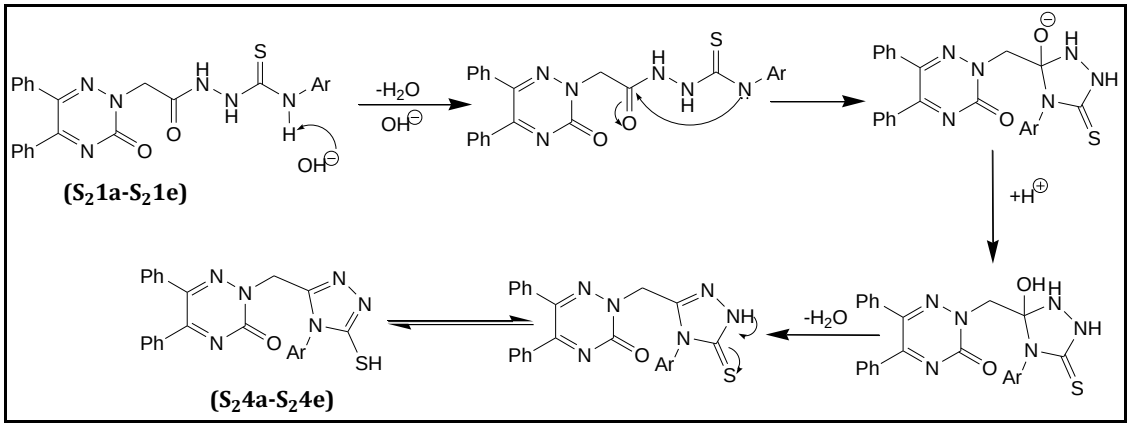


Figure 4.5. Possible mechanism of reaction for the synthesis of compounds **$S_2$4a–$S_2$4e.**

4.1.4. Characterisation of Synthesised Compounds of Series 1 and Series 2

The following procedures were employed to ascertain the structures of the synthesised compounds.

4.1.4.1. Physicochemical Characterisation

Physicochemical characterisation of synthesised compounds includes

- **Thin layer chromatography (TLC) analysis (R_f value):**

The TLC is an important technique used to qualitatively monitor the progress of a reaction and it also ascertains the purity of the substance. The R_f value is a characteristic property of a given compound in a particular solvent composition and was calculated by using the following equation:

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

The reactions were monitored by TLC on a pre-coated Merck silica gel 60 F254 aluminium sheets (Merck, Germany) measuring 7.5 cm x 2.5 cm. The

chromatograms were developed by ascending technique, and the locations of spots were inspected by using the ultraviolet cabinet and iodine chamber.

- **Melting point determination:**

Melting point implies the relationship between structure and properties. Hence different compounds tend to have different melting points. It is one of the important criteria which gives an indication of purity. The melting points were determined in open capillaries using Stuart SMP10® (Barloworld Scientific Ltd., UK), Electrothermal melting point apparatus and were uncorrected.

- **Log P value determination:**

Partition coefficient quantifies the partitioning of a neutral (unionised) substance between water and *n*-octanol. The logarithm of the concentration of the unionised solute in the solvents is called Log P. The Log P value is also known as a measure of lipophilicity. It is an important parameter used in the assessment of environmental fate and transport of organic chemicals because the n-octanol phase is a surrogate for the lipid phase or organic carbon content of the environmental compartments. Partition coefficient (K_{ow}) was calculated by shake flask method (Takacs-Novak and Avdeef, 1996) using the equation:

$$\mathbf{K_{ow} = C_o / C_w}$$

C_o = Concentration in octanol phases

C_w = Concentration in water phases

4.1.4.2. Spectral Characterisation and Elemental Analysis

Spectral characterisation and elemental analysis were performed by using following instruments:

- **FT-IR Spectroscopy**

FT-IR spectra were recorded on a Shimadzu FTIR 8400S spectrophotometer at the scanning range of 4000-400 cm^{-1} at the Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi.

- **Nuclear Magnetic Resonance Spectroscopy**

^{1}H NMR (300 MHz and 500 MHz) and ^{13}C NMR (75 MHz and 100 MHz) were recorded on a JEOL FT–NMR in DMSO–d_6 using tetramethylsilane (TMS) as an internal standard at the Department of Chemistry, Institute of Science, Banaras Hindu University, Varanasi.

- **Elemental analysis**

Elemental analysis was performed using Exeter CE-440 elemental analyser at the Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi. The elemental analysis results were within ±0.4% of the theoretical values.

4.2. BIOLOGICAL ACTIVITY

4.2.1. Animals

Swiss albino mice (20–30 g) and rats (180–200 g) of either sex were procured from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University. Animals were housed at a room temperature of 22±3°C and 45% relative humidity under 12 h light/dark ambience. Animals had free access to commercial rodent feed and water *ad libitum.*

M/s Kekule Pharma Ltd., Hyderabad; M/s GenPharma International Pvt. Ltd., Pune; M/s Emcure Pharmaceuticals Pvt. Ltd., Pune and M/s Ind-Swift Laboratories Ltd., Mumbai generously provided Celecoxib, Indomethacin, Zileuton and Pentazocine as gift samples.

Based on the outcome of the initial *in vitro* screening, selected derivatives were further evaluated *in vivo* for anti–inflammatory and analgesic activity, followed by the assessment of their gastric, hepatic, renal and cardiotoxicity liabilities. The test compounds and standard drugs (indomethacin/ celecoxib/ zileuton/ pentazocine) were administered as oral suspensions at a dose equivalent to 10 mg/kg indomethacin in a vehicle consisting of 0.3% w/v carboxymethylcellulose sodium (CMC) in distilled water. The *in vivo*

experimental protocol was approved by the Central Animal Ethical Committee of BHU, Varanasi, India (Protocol No. Dean/13–14/CAEC/320).

4.2.2. Evaluation of Anti-inflammatory Activity

4.2.2.1. Albumin Denaturation Assay (Ramesh *et al.*, 2011)

The albumin denaturation assay is a quick and inexpensive method, which seeks to limit the use of animals in the initial drug discovery process (Williams *et al.*, 2008). Standard drugs (indomethacin, celecoxib) and synthesised derivatives (Series 1 & Series 2) were dissolved in a minimum amount of dimethylformamide (DMF) and diluted with phosphate-buffered saline (PBS) (pH 7.4) maintaining the concentration of DMF in all solutions less than 2.5%. Test solutions (1 ml, 100 mg/ml) were mixed with 1 ml of 1% w/v albumin in PBS and incubated at 27±1°C for 15 min. Denaturation was induced by heating the mixture at 60±1°C for 10 min. After cooling, the turbidity was measured at 660 nm. The percentage inhibition of denaturation was calculated from the control, where no drug was added. Each experiment was performed in triplicate, and an average was taken.

The percentage inhibition was calculated using the formula

$$\textbf{\% Inhibition of denaturation} = [(V_t/V_c) - 1] \times 100$$

Where V_t = absorption of test compound

V_c = absorption of control

4.2.2.2. Acute Oral Toxicity Studies

Based on the outcome of preliminary *in vitro* assessment, acute oral toxicity studies of selected compounds from Series 1 and Series 2 were performed on nulliparous, non-pregnant, healthy female albino rats as per OECD-425, 2001 guidelines. Compounds were administered in graded doses ranging from 100-500 mg/kg, *p.o.* The animals were monitored continuously for 24 h for any changes in their autonomic or behavioural responses and also for tremors, convulsion, salivation, diarrhoea, lethargy, sleep and coma and then monitored for mortality, if any, for the following 14 days.

4.2.2.3. Carrageenan-Induced Rat Paw Oedema (Winter *et al*., 1962)

Acute inflammation was produced by the subplantar administration of 0.1 ml of a 1% w/v solution of lambda carrageenan in normal saline on the left hind paw of the rats. Different groups were pre-treated orally with standard drugs (indomethacin, celecoxib) or selected compounds from Series 1 and Series 2, 1 h before the administration of carrageenan. The total increase in the paw volume was measured at 1 h intervals till 6 h post carrageenan injection by digital Vernier callipers (*Mitutoyo*). The increase in the oedema volume was calculated at each interval and evaluated statistically. The percentage inhibition of inflammation was calculated using the following formula:

$$\% \text{ Inhibition} = E_c - E_t / E_c \times 100$$

E_c = Mean oedema volume of the control group

E_t = Mean oedema volume of the test group

4.2.2.4. Arachidonic Acid-Induced Rat Paw Oedema (Di Martino *et al.,* 1987)

Oedema was induced by a single injection of 0.1 ml of 0.5% w/v arachidonic acid (AA) in 0.2 M carbonate buffer (pH 8.4) into the right hind paw (sub–plantar) of rats 30 min post drug treatment. Group I served as negative control, group II received zileuton (10 mg/kg, *p.o.*) **[ZIL 10]**, group III received indomethacin (10 mg/kg, *p.o.*) **[Indo 10]** and group IV were administered the selected compounds from Series 1 and Series 2 at an equimolar oral dose relative to 10 mg/kg indomethacin. The swelling thickness (mm) was measured 1 hour after the arachidonic acid injection using digital Vernier callipers.

4.2.2.5. Cotton Pellet-Induced Granuloma in Rats (Chattopadhyay *et al.*, 2004)

Autoclaved cotton pellets weighing 35±1 mg each were implanted subcutaneously through a small incision made along the axillar region of the anaesthetised rats. Different groups of rats of either sex received standard drugs (indomethacin, celecoxib) or selected compounds from Series 1 and Series 2 at an equimolar oral dose relative to 10 mg/kg indomethacin once daily for seven consecutive days from the day of the cotton pellet insertion. The control group received the vehicle (10 ml/kg, *p.o.*).

On the eighth day, the cotton pellets covered by the granulomatous tissue were excised and dried in a hot air oven at 60°C until a constant weight was achieved. The granuloma weight was calculated by subtracting the weight of the cotton pellet on day zero (before the start of the experiment) from the weight of the cotton pellet on the eighth day.

4.2.2.6. Freund's Adjuvant-Induced Arthritis in Rats (Latha *et al.*, 1998)

Arthritis was induced by the subplantar injection of 0.1 ml of a freshly prepared suspension of Freund's complete adjuvant (FCA) consisting of 1.0 mg dry heat-killed *Mycobacterium tuberculosis* per millilitre sterile paraffin oil into the left hind paw of rats. Treatment was initiated after 14 days from the day of adjuvant injection. Rats divided into different groups of six animals each received standard medication (indomethacin, celecoxib) and selected compounds from Series 1 and Series 2.

Swelling of the right hind paw was quantified on 3rd, 6th, 9th, 12th, 15th, 18th and 21st day after the treatment using digital Vernier callipers. Percentage inhibition was calculated, and the difference in the severity of arthritis between the experimental and control groups was statistically analysed.

4.2.2.7. Evaluation of Ulcerogenic Liability

Ulcerogenic liability of the potential derivatives from Series 1 and Series 2 was evaluated on the 21st day of the adjuvant-induced arthritis bioassay. Rats under anaesthesia were sacrificed, and the stomach was removed, opened along the curvature, washed with distilled water and cleaned gently in normal saline. After washing, the stomach mucosa was examined for ulcers using a handheld lens. The lesions were counted, and an ulcer index (UI) for each animal was calculated (Szelenyl *et al.*, 1978). It was followed by a histopathological evaluation wherein the stomachs were fixed in 10% v/v formalin and embedded in paraffin blocks for sectioning. The sections (1-3 mm) were stained with hematoxylin and eosin and photographed using a Nikon digital microscope (Eclipse 200) at 10x magnification (Laloo *et al.*, 2013).

UI= (*n* lesion I) + (*n* lesion II)2 + (*n* lesion III)3

Where, n = number of ulcers

I = ulcer area covering less than 1 mm^2

II = ulcer area covering area from 1 to 3 mm^2 and

III = ulcer area covering more than 3 mm^2

4.2.2.8. Lipid Peroxidation Study

The extent of lipid peroxidation of selected compounds from Series 2 was determined as per the protocol reported by Ohkawa (Ohkawa *et al.*, 1979). After the calculation of UI, the gastric mucosa (100 mg) was scraped with two glass slides and homogenised in 1.8 ml of a 1.15% w/v of ice-cold potassium chloride (KCl) solution. The homogenate was supplemented with 0.2 ml of 8.1% w/v sodium dodecyl sulphate (SDS), 1.5 ml of acetate buffer (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid (TBA). The mixture was then heated at 95°C for 60 min.

After cooling to room temperature, the reactants were extracted with 5 ml of a mixture comprising of n-butanol and pyridine (15:1 v/v), shaken vigorously for 1 min and centrifuged for 10 min at 4000 rpm. The absorbance of the organic supernatant layer was measured at 532 nm, and the results were expressed as nmol malondialdehyde (MDA) 100/mg tissue.

4.2.2.9. Assessment of Hepatic and Renal Toxicity (Reitman *et al.*, 1956; King *et al.*, 1934)

On the 21st day of the adjuvant-induced arthritis bioassay model, blood was obtained from all groups of rats by puncturing the retro-orbital plexus. Blood samples were allowed to clot at room temperature, and the serum was separated by centrifugation at 2500 rpm for 15 min.

Serum was analysed for various biochemical parameters such as Serum Glutamic Oxaloacetate Transaminase (SGOT), Serum Glutamic Pyruvic Transaminase (SGPT), Alkaline phosphatase (ALP) reflecting hepatic functions; total protein, total albumin collectively reflecting both hepatic as well as renal functions and creatinine and blood urea nitrogen (BUN) indicating renal

functions. Serum analysis was performed using commercial enzyme assay kits (Merck, Germany) as per the manufacturer's instructions.

4.2.2.10. Histopathological Assessment of Liver and Kidney

On the 21st day of the adjuvant-induced arthritis bioassay, rats under anaesthesia were sacrificed, and the liver and kidneys were excised, washed with normal saline and processed through graded alcohol and xylene and embedded in paraffin wax (Galigher and Kozloff, 1971). Sections of 5-6 μm in thickness were cut stained with hematoxylin and eosin (Luna, 1968). Mounted slides were examined and then photographed using a Nikon digital microscope (Eclipse 200) at 10x magnification.

4.2.3. Evaluation of Analgesic Activity

4.2.3.1. Acetic Acid-Induced Writhing in Mice (Koster *et al.*, 1959)

The numbers of writhing episodes were counted for 30 min following (0.6% v/v) the acetic acid injection into the peritoneal cavity. The control group received 0.3% w/v sodium CMC (10 ml/kg, *p.o.)*, the standard group received indomethacin/celecoxib and the remaining groups received selected compounds from Series 1 and Series 2 at an equimolar oral dose relative to 10 mg/kg indomethacin.

A significant reduction in the number of writhing episodes by the standard or test compounds compared to the amount of writhing in the control animals was considered a positive analgesic response. The analgesic activity was expressed as a percentage change from the writhing controls.

4.2.3.2. Formalin-Induced Paw Licking in Mice (Tjolsen *et al.*, 1992)

Post-administration of formalin 0.02 ml (1% v/v) by the subplantar route, the time in seconds that each mouse spent licking was recorded. Mice within the control group were administered 0.3% w/v sodium CMC (10 ml/kg, *p.o.*). Pentazocine (10 mg/kg, *p.o.*) **[PTZ 10]** indomethacin and celecoxib were used as

reference standards. Selected compounds from Series 1 and Series 2 were administered at an equimolar oral dose relative to 10 mg/kg indomethacin.

The time spent on licking the injected paw was determined. Mice were pre-treated with the reference standards and test compounds 1 h before being challenged with buffered formalin, and the responses were observed for 30 min.

4.2.4. *In Vitro* COX Enzymatic Studies

The ability of the potential compounds from Series 1 and Series 2 to inhibit ovine COX-1 and COX-2 was evaluated using a colorimetric COX (ovine) inhibitor screening assay kit (catalogue number 760111, Cayman Chemical, Ann Arbor, MI, USA), which utilises the peroxidase component of COX, as per the manufacturer's instructions.

The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidised *N,N,N′,N′*–tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm. The concentration of the test compounds producing 50% inhibition (IC_{50}, μM) was calculated from the concentration-inhibition response curve (duplicate determinations).

4.2.4.1. Enzyme Kinetics Study (Lineweaver and Burk, 1934)

Enzyme kinetics study was performed to assess the nature of inhibition by the potential derivatives from Series 1 and Series 2 on the COX-2 enzyme. The enzyme kinetics were determined, wherein the AA substrate either in the absence or presence of selected derivatives were evaluated at different concentrations between 20-100 μM.

The mode of inhibition was determined by following the Lineweaver-Burk double reciprocal plot analysis of the data and calculated as per the Michaelis-Menten kinetics. To understand the possible mode of action, K_m and V_{max} were also calculated. The inhibition constant (K_i) values in the presence of selected derivatives were determined by applying the Cheng-Prusoff equation.

4.2.5. Evaluation of Cardiotoxic Liability (Banerjee *et al.*, 2016)

Cardiotoxic liability studies of selected compounds from Series 2 were performed on adult male albino Wistar rats.

Experimental Design:

Group 1: [Normal Control rats] Treated with 0.3% w/v sodium CMC solution in distilled water (10 ml/kg, *p.o.*) for 15 days. Initial basal measurement of cardiac troponin I (cTnI) and Creatine kinase-MB (CK-MB) in serum was evaluated before treatment.

Group 2: [Isoproterenol (ISO) control rats] Administered with ISO (100 mg/kg, *s.c.*) followed by treatment with 0.3% w/v CMC solution in distilled water (10 ml/kg, *p.o.*) for 15 days.

Group 3: [ISO + Celecoxib treated rats] Administered with ISO (100 mg/kg, *s.c.*) followed by oral treatment with celecoxib in 0.3% w/v sodium CMC solution for 15 days.

Group 4: [ISO + Comp $S_2$2e treated rats] Administered with ISO (100 mg/kg, *s.c.*) followed by oral treatment with **$S_2$2e** in 0.3% w/v sodium CMC solution for 15 days.

Group 5: [ISO + Comp $S_2$3c rats] Administered with ISO (100 mg/kg, *s.c.*) followed by oral treatment with **$S_2$3c** in 0.3% w/v sodium CMC solution for 15 days.

Initial basal measurement of cTnI and CK-MB in serum was evaluated before the start of the experiment. ISO (100 mg/kg, *s.c.*) was dissolved in saline and injected into rats at an interval of 24 h on day 1 and day 2. ISO-induced MI was confirmed by elevated levels of serum cTnI and CK-MB in rat post 24 h of second ISO injection after which the treatment was initiated from the 3rd day and continued until the 18th day.

Blood samples were collected from the retro-orbital plexus. Serum was separated by centrifugation at 4000 rpm for 10 min, and stored at -80°C until assayed. The level of serum cTnI was estimated for both the sets of animals on days 3, 4, 7, 11 and 19 by Electro Chemiluminescence Immunoassay using a

standard kit (Roche Diagnostics, Switzerland). Serum CK-MB levels were assayed by commercial kit purchased from Agappe Diagnostics as per the manufacturer's instructions.

4.2.6. Statistical Analysis

The experimental results were expressed as the Mean ± S.D (n = 6) followed by a One–way analysis of variance (ANOVA). Tukey's multiple comparison test was applied for determining the statistical significance between different groups.

Two–way ANOVA followed by a Bonferroni post-test was applied for the carrageenan-induced rat paw oedema and adjuvant-induced arthritis bioassays. GraphPad Prism (version 5) was used for all statistical analyses, and a *p*-value <0.05 was considered significant.

4.3. COMPUTATIONAL STUDIES

4.3.1. *In Silico* Docking

4.3.1.1. In Silico Docking Protocol for Series 1

Docking studies were performed using the Glide 5.8 module in the Schrödinger Maestro 9.3 program. The X–ray crystallographic structure of COX–2 complexed with 1–Phenylsulfonamide–3–trifluoromethyl–5–(4–bromophenyl) pyrazole (SC–558) (PDB ID: 1CX2) with a resolution of 3.0 Å was retrieved from the Protein Data Bank (Kurumbail *et al.*, 1996). Errors in the protein were corrected using the protein preparation wizard. The binding site was generated by keeping the co–crystallised ligand at the centre of a rectangular box within the receptor.

A 20 Å grid space was defined for the co–crystallised ligand using the Glide grid module of the software. The LigPrep module was used to produce low–energy conformers of all of the ligands. Ligands were kept flexible by producing the ring conformations and by penalising non–polar amide bond conformations, whereas the receptor was kept rigid throughout the docking studies.

All other parameters of the Glide 5.8 module were maintained at their default values. The lowest energy conformation was selected, and the ligand interactions (hydrogen bonding and hydrophobic interaction) with the amino acid residues constituting the active site of COX–2 were observed.

4.3.1.2. In Silico Docking Protocol for Series 2

The docking studies were performed using Schrödinger Glide module in Schrödinger Suite 10.5.014 MM Share Version 3.3.014 Release 2016-1 with workstation 4x Intel(R) Xeon(R) CPU E5-1607 v3 @ 3.10 GHz on Kernel Linux operating environment. The X-ray crystallographic structure of COX-2 complexed with celecoxib (PDB ID: 3LN1) with a resolution of 2.40 Å was retrieved from the Protein Data Bank (Wang *et al.*, 2010). GLIDE extra precision (XP) mode was applied to generate favourable ligand poses which were further screened through the filters to examine the spatial fit of the ligand into the COX-2 active site. Protein was prepared using the protein preparation wizard to achieve its lowest energy conformation.

This structurally corrected protein was further used for docking analysis. The binding site was generated by keeping the co-crystallised ligand (celecoxib) at the centre of a rectangular box within the protein. A 20 Å grid space was defined for the co-crystallised ligand using the Glide grid module of the software. The LigPrep module was used to generate low-energy conformers for all the ligands. Ligands were kept flexible by generating the ring conformations and by penalising non-polar amide bond structures, whereas the protein was kept rigid throughout the docking studies.

All other parameters of the Glide module were maintained at their default values. The lowest energy conformer was selected, and the ligand interactions with the active amino acid residues constituting the COX-2 active site were observed.

4.3.2. Molecular Dynamics (MD)

4.3.2.1. MD Simulation Protocol for Compound S_14d (Banerjee *et al.*, 2015)

The docked complex of compound **S_14d** with COX–2 (PDB ID: 1CX2) was further optimised by MD simulation using the Desmond module in the Schrödinger Maestro 9.3 program with an OPLS–AA force field in an explicit solvent with the TIP3P water model. The docked complex was soaked adequately in TIP3P water molecules, and the system was neutralised by adding chloride counter-ions to balance the net charge of the system. The generated solvent model of the docked complex consisted of 65391 atoms. Before the MD simulation, the system was minimised and pre-equilibrated using a default relaxation routine implemented in Desmond.

Minimisation was performed for 7000 steps, of which the first 2000 were steepest descents and the last 5000 were limited–memory Broyden–Fletcher–Goldfarb–Shanno (L–BFGS). MD simulations were performed using the minimised structures, and the Particle Mesh Ewald (PME) method was used to access long range electrostatic interactions. The time step of the simulation was 1.20 ns, and a 10 Å cut–off was used for non–bonded interactions. The Shake algorithm was employed to keep all bonds involving hydrogen atoms rigid. A constant number of atoms, N, volume, V, and temperature, T (NVT) molecular dynamics was performed for the first 100 ps, during which the temperature of the system was raised from 0–300 K.

For further simulations, the temperature was maintained at 300 K. Subsequently; the system was equilibrated at a constant number of atoms, N, pressure, P, and temperature, T (NPT), which consisted of minimisation and a short MD simulation (12–24 ps) to relax the model system. Then, a long equilibration MD simulation was performed for 2 ns, and a long production MD simulation was performed for 5 ns.

The production run was carried out using Langevin dynamics to maintain the temperature at 300 K and the pressure at 1.0132 bars. Data were collected every 1 ps during the MD runs.

4.3.2.2. MD Simulation Protocol for Compound $S_2$2e (Banerjee *et al.*, 2016)

The docked complex of compound **$S_2$2e** with COX-2 (PDB ID: 3LN1) was further optimised by MD simulation using the Desmond module of Schrödinger Maestro 10.5.014 programme with an OPLS-AA force field in an explicit solvent with the TIP3P water model. The docked complex was soaked adequately in 15727 TIP3P water molecules, and the system was neutralised by adding an appropriate number of chloride counter ions to balance the net charge of the system. The generated solvent model of the docked complex consisted of 56164 atoms.

The available system of solvated docked complex was further subjected to energy minimisation to maximum 7000 steps of which the first 2000 were steepest descents and the last 5000 were limited-memory Broyden-Fletcher-Goldfarb-Shanno (L-BFGS). The time step of the simulation was 1.20 ns, and a 10Å cut-off was used for non-bonded interactions. The SHAKE algorithm was employed to constrain the bonds involving hydrogen atoms to their equilibrium values (Ryckaert *et al.*, 1977).

A constant number of atoms, N, volume, V, and temperature, T (NVT) molecular dynamics was performed for the first 100 ps, during which the temperature of the system was raised from 0-300 K. For further simulations, the system was maintained at constant temperature (300 K) and pressure (1.0132 bars).

Subsequently, the system was equilibrated at a constant number of atoms, N, pressure, P, and temperature, T (NPT), which consisted of minimisation and a short MD simulation (12-24 ps) to relax the model system. Then, a long equilibration MD simulation was performed for 2 ns, and a long production MD simulation was performed for 5 ns. Long-range electrostatic interactions were accessed using Particle Mesh Ewald (PME) method.