CHAPTER-II

Experimental

2.1. Introduction: All chemicals used were of the highest grade commonly available. The solvents used were purified as per the standard procedures. Column chromatographic separations were carried out on silica gel 60-120 mesh size. Melting points of all synthesized compounds were determined by using open capillary method and are uncorrected. Elemental analyses (CHN analyses) of all synthesized compounds were determined by using Vario EL analyzer. FT-IR spectra of all the final products were recorded on a Perkin-Elmer-100 instrument by averaging 50 scans with a resolution of 2 cm\(^{-1}\) measuring in absorbance mode by using the KBr self-supported pellet technique. The \(^1\)H NMR spectra of samples were recorded on a JEOL 300-MHz NMR spectrometer using Tetramethylsilane (TMS) as an internal standard in DMSO-d\(_6\)/CDCl\(_3\). Mass spectra for all synthesized compounds were recorded on a MALDI-MS using 1,8,9-trihydroxyanthracene (mw = 226, cas no: 1143-38-0) as matrix.

Preparation and characterization of the silica gel supported sulfuric acid, mesoporous aluminosilicate (AlKIT-5) catalysts, standard procedures for biological activity were presented in this chapter.

**Procedure for the preparation of silica gel supported sulfuric acid (SiO\(_2\)-H\(_2\)SO\(_4\)) catalyst\(^1\):** The slurry of 400 mesh size silica gel (10g) in
dry diethyl ether (50 ml), sulfuric acid (3 ml) was added with shaking for 5 min. The solvent was evaporated under reduced pressure resulting in free flowing silica gel supported sulfuric acid (SiO$_2$-H$_2$SO$_4$) was dried at 110°C for 3 h.

**Procedure for the preparation of the mesoporous aluminosilicate (AlKIT-5) catalyst:**

**Materials:** Aluminium isopropoxide purchased from Aldrich whereas triblock copolymer (Pluronic F127, EO$_{97}$PO$_{69}$EO$_{97}$, molecular weight = 12500) was used as the structure directing agent and obtained from Sigma Co.

**Preparation of the Materials:** AlKIT-5 samples with different $n_{Si}/n_{Al}$ ratios were synthesized using Pluronic F127 as the template in an acidic medium. In a typical synthesis, 5.0 g of F127 is dissolved in the required amount of HCl (35 wt %) and 240 g of distilled water. To this mixture, 24.0 g of tetraethyl orthosilicate (TEOS) and the required amount of the desired ‘Al’ source were added, and the resulting mixture was stirred for 24 h at 100°C. Subsequently, the reaction mixture was heated for 24 h at 100°C under static condition for hydrothermal treatment. The solid product was filtered off and then dried at 100°C without washing. The product was calcined at 540°C for 10 h. A first set of samples was prepared by changing the molar water to hydrochloric acid ratio using Aluminium isopropoxide as the ‘Al’ source and denoted as AlKIT-5(xH) where $x$ denotes the molar water to hydrochloric acid ratio ($n_{H_2O}/n_{HCl}$). For this set of samples,
the $n_{Si}/n_{Al}$ ratio in the gel was fixed as 12. A second set of AlKIT-5 samples was prepared by varying the $n_{Si}/n_{Al}$ ratio in the synthesis gel using Al isopropoxide as the Al source and denoted as AlKIT-5($x$) where $x$ denotes the $n_{Si}/n_{Al}$ ratio in the final product. The molar gel composition of the reaction mixture was 1.0:0.025–0.071: 0.0035: 0.25–0.88:116.6–119 SiO$_2$:Al$_2$O$_3$: F127: HCl: H$_2$O. Pure siliceous KIT-5 was prepared using the same procedure with the $n_{H2O}/n_{HCl}$ of 463 in the absence of Al.

**Characterization of the AlKIT-5:** The powder X-ray diffraction (XRD) patterns of the AlKIT-5 catalysts with different ‘Al’ contents were collected on a Rigaku diffractometer using Cu K $\alpha$ ($\lambda = 0.154$ nm) radiation. The diffractograms were recorded in the 2$\theta$ range of 0.8–10$\degree$ with a 2$\theta$ step size of 0.01$\degree$ and a step time of 10s. Nitrogen adsorption and desorption isotherms were measured at -196°C on a Quantachrome Autosorb 1C sorption analyzer. All samples were outgassed at 250°C for 24h. The specific surface area was calculated using the Brunauer–Emmett–Teller (BET) method. The pore size distributions were obtained from the adsorption branch of the nitrogen isotherms by Barrett–Joyner–Halenda method. Elemental composition of the materials was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). The temperature-programmed desorption (NH$_3$-TPD) was carried out on a Micromeritics Autochem 2910 instrument. Approximately 0.2 g of a fresh sample was placed in a U-shaped, flow-through, quartz microreactor for each
experiment. The catalyst was activated at 500°C for 6h under ‘He’ flow (20 mL/min) and then cooled to 100°C before being exposed to ammonia. The sample was flushed again in ‘He’ for 2h to remove any physisorbed ammonia, and desorption profile was then recorded by increasing the sample temperature from 100 to 550°C at a ramp rate of 5°C/min. The diameter of the cages in AlKIT-5 materials was calculated using Equation 1 which proposed by Ravikovitch et al.\(^3\)

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D_{me} = a \times (6\varepsilon_{me}/\pi\nu)^{1/3}\]

In Equation 1, \(D_{me}\) is the diameter of the cavity of a cubic unit cell of length \(a\), \(\varepsilon_{me}\) is the volume fraction of a regular cavity and \(\nu\) is the number of cavities present in the unit cell (for Fm\(\bar{3}\)m space group, \(\nu = 4\)). The average wall thickness of the materials (\(h\)) was calculated using the Equation 2 which was derived from the mesoporosity and \(D_{me}\).

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h = (D_{me}/3)(1 - \varepsilon_{me})/\varepsilon_{me}\]

2.2. General procedure for the synthesis of 1,5-benzodiazepines using silica gel supported sulfuric acid and AlKIT-5 catalysts: The benzodiazepine derivatives were synthesized using a mixture of o-phenylenediamine \(1\) (1 mmol), ketone \(2\) (2.5 mmol), and silica gel supported sulfuric acid catalyst (10 mol%) in acetonitrile. The reaction mixture was stirred at room temperature for the appropriate time to afford the desired products.
A mixture of \( \alpha \)-phenylenediamine (OPDA) (1 mmol), ketone (2.5 mmol) and AlKIT-5 (100 mg) was stirred in acetonitrile (4 mL) at room temperature until thin layer chromatography indicated the reaction was completed. Ethyl acetate (10\%) in hexane was used as the mobile phase and both the reactant and the final product were spotted on the TLC plate. The disappearance of the reactant spot on the TLC plate indicates the completion of the reaction. After completion of the reaction, 20 ml of ethyl acetate was added to the reaction mixture and the catalyst was recovered by filtration. The resulting organic layer was concentrated and the crude product is purified by silica gel column chromatography using ethyl acetate: \( n \)-hexane (1:9) as eluent to afford the desired product. All synthesized products were characterized by IR, NMR and MALDI-MS.

2.3. General procedure for the synthesis of 3,4–dihydropyrimidin–2-ones (DHPMs) using DDQ and AlKIT-5: A solution of aromatic aldehyde (1.0 mmol), \( \beta \)-ketoester (1.2 mmol), and urea/thiourea (1.2 mmol) in acetonitrile (6 mL) was heated at reflux temperature in the presence of DDQ (5 mole\%)/ AlKIT-5(10) (150 mg) catalyst for appropriate time. TLC was monitored for completion of the reaction. For TLC, ethyl acetate (20\%) in hexane was used as the mobile phase and both the reactant and the final product were spotted on the TLC plate. The disappearance of the reactant spot on the TLC plate indicates the completion of the reaction and then reaction mixture was poured onto crushed ice and the solid product was separated by
filtration. The resulting solid product was recrystalised from methanol. In the case of AlKIT-5 catalyst, the catalyst was recovered by filtration and the reaction product was precipitated by addition of 10 mL of water and recrystalised from methanol to give pure crystals. All synthesized products were characterized by IR, $^1$H NMR & MALDI-MS.

2.4. General procedure for the synthesis of 3,4-dihydroquinoxalin-2-amine derivatives: To a solution of OPDA or substituted OPDA (1 mmol) in 3 ml of ethanol, ketone (1 mmol), and cyclohexyl isocyanide/benzyl isocyanide (1 mmol) was added PTSA (0.095g, 5 mol%)/AlKIT-5 (100 mg). The reaction mixture was stirred for appropriate time at room temperature. Completion of the reaction was indicated by TLC. For TLC, ethyl acetate (20%) in hexane was used as the mobile phase and both the reactant and the final product were spotted on the TLC plate. The disappearance of the reactant spot on the TLC plate indicates the completion of the reaction and then the resulting mixture was precipitated by addition of excess of water and then the precipitate was filtered off and washed with 5% sodium hydroxide solution and then with water. The residue was recrystallized from ethanol to give pure crystals. In the case of AlKIT-5 catalyst, the catalyst was recovered by filtration and the resulting mixture was precipitated by addition of more than 10 mL of cold water and recrystalised from ethanol to give pure crystals.
2.5. General Procedures for the antineuroinflammatory studies of the compounds:

**iNOS enzyme activity studies:** For the assay the iNOS enzyme activity in intact cells, BV-2 cells were plated in 100 mm tissue culture dishes (4×10^6 cells) and incubated with LPS (100 ng/ml) for 12 h. Then, the cells were washed twice with PBS, and cells were harvested and plated in a 96-well plate (2×10^5 cells/well), and incubated in the presence or absence of different concentrations of compounds for a further 12 h. The amount of NO in the supernatant was detected by Griess reaction.

**iNOS expression studies:** Western blot was performed to analyze iNOS expression. BV-2 cells were seeded in a 6-well plate and exposed to LPS (100 ng/ml) in the presence or absence of compounds for 6 h. The protein sample (40 µg for each) from the BV-2 cell extract was separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated with primary antibodies (rabbit anti-iNOS; Transduction Laboratories, San Diego, CA, USA) and secondary antibodies (goat anti-rabbit IgG; Amersham Pharmacia Biotech). The blots were developed using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech).

**NO scavenging activity:** Sodium nitroprusside (SNP) was dissolved in phosphate buffered saline (PBS) at 100 mM. This SNP solution (50µl) was mixed with 950 µl PBS containing various concentrations of
compounds (1, 5 and 20 µM). These mixtures were incubated at 25°C for 2.5 h, and nitrite formed through the combination of oxygen and NO released from the SNP, as measured by the Griess reaction.

References:

