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Research Article
Synthesis and Biological Evaluation of Novel Neutral 2-substituted Benzamidobenzene Derivatives as Human Factor Xa Inhibitors

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Abstract

Background and Objective: Factor Xa is an essential enzyme in the blood coagulation cascade. Inhibition of factor Xa can overcome confines of the current antithrombotic therapy. Here, the aim of this report a series of novel non-basic compounds possessing sulfide, sulfoxide and sulfone groups as S4 binding elements and sulfonamide, sulfide and sulfone bearing aryl groups as S1 binding moieties and their human factor Xa (FXa or hFXa) inhibitory activity. **Materials and Methods:** Measurement of direct Fxa was done using a chromogenic substrate hydrolysis assay using a micro plate reader (Flex Station III, Molecular Devices). Initial screening was done using two concentrations of the compounds (500 and 100 μM). The IC_{50} values were determined for those compounds that caused>50% reproducible inhibition of coagulation enzyme in the initial screening at 100 μM concentration. Clotting time was measured using a BBL Fibro system fibrometer (Becton-Dickinson, Sparles, MD) in a standard one-stage re-calcification assay. Docking studies were performed using Glide tool of Schrödinger 2009. **Results:** Among the 20 compounds so evaluated, three compounds exhibited moderate inhibition of hFXa, while two of the compounds (34 and 35c) exhibited good hFXa inhibition with IC_{50} values of 29.2 and 16.1μM with an efficacy of 70 and 75%, respectively. **Conclusion:** A series of 'V' shaped molecules were designed, synthesized and evaluated for their FXa inhibitory activity. Out of the 20 compounds screened for activity, two compounds (34 and 35c) showed activity in low micromolar range. The SAR studies indicated the presence of sulfide or sulfoxide P4 as an essential feature for FXa inhibitory activity. Sulfonamide as a linker between the P1 ligand and the main scaffold offered more potent compounds. Docking studies indicated strong interaction of the compound 35c within the active site. The compounds have advantage of possessing neutral/acidic functionalities unlike the existing FXa inhibitors having basic character which hampers their oral bioavailability.

Key words: Sulfide, sulfonamide, factor Xa, anticoagulant, 2-Substituted benzamidobenzenes, non-basic

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Thromboembolic complications such as acute coronary syndrome (ACS), atrial fibrillation (AF) and percutaneous coronary intervention (PCI) in patients are treated using anticoagulants. Coumarins and heparins having narrow therapeutic window require extensive patient monitoring and parenteral route of administration in these diseases[1-3]. Due to these limitations, research has been directed towards the development of orally available anticoagulants with better therapeutic window acting through selective inhibition of some vital enzyme involved in the coagulation process. Extensive monitoring would not be required for such orally administered selective medicaments reducing the bleeding liability as compared to the established agents[4-9].

Two specific proteases, namely FXa and thrombin (FIIa) are targeted for discovery of new oral anticoagulants. Discovery of dabigatran[10,11] a selective FIIa inhibitor and rivaroxaban[12,13] a direct FXa inhibitor was a milestone in the search for new small molecule anticoagulants. Factor Xa has a unique position in blood coagulation cascade at the junction of the intrinsic and extrinsic pathways, which catalyzes the formation of thrombin from prothrombin via prothrombinase complex. It is known that thrombin is responsible for fibrin clot formation in the cascade and it also has several thrombotic functions, including activation of platelets and feedback activation of several coagulation factors[14]. Inhibition of FXa should safely interrupt blood coagulation and prevent production of new thrombin without affecting its existing basal level. Hence FXa inhibitors are expected to cause less disturbance in hemostasis than direct thrombin inhibitors, leading to a higher safety margin[15-18]. Therefore, extensive research has been carried out during the last few decades to develop novel orally administered FXa inhibitors. In this direction many small molecule FXa inhibitors having different scaffolds have been reported[9]. Of these, betrixaban (1)[19], darexaban (2)[20] and edoxaban (3)[21] are some of the examples of the most effective FXa inhibitors (Fig. 1).

FXa, a serine protease contains two essential pockets S1 and S4. A search for ligands providing optimal interactions within these subsites has been a major focus in the design of selective FXa inhibitors. Molecules that are capable of acquiring V or L-shaped conformations are reported to inhibit FXa effectively[22]. Previously compounds bearing anthranilamide[23-25], 1,2-dibenzamidobenzene[26,27] and 2-aminobenzamide[28] scaffolds were reported to show good FXa inhibitory activity. Small molecule FXa inhibitors having basic substituents (like amidine, guanidine, amine etc.) have been often associated with low oral bioavailability, short life and rapid clearance[29-31]. In order to overcome these difficulties inhibitors bearing neutral substituents like rivaroxaban, darexaban and edoxaban have been developed[32-34].

Based on the existing knowledge of small molecule FXa inhibitors and receptor subsites, we designed some 'V' shaped 2-substituted benzamidobenzene derivatives having non-basic groups like sulfone, sulfide and sulfoxide as shown in Fig. 2. The designed molecules were studied for their receptor-ligand interactions by means of molecular modeling, which indicated good interactions between the designed molecules and the enzyme active site. The designed molecules were then synthesized and evaluated for their FXa inhibitory activity and anti-thrombotic potential.

MATERIALS AND METHODS

All solvents and reagents were used as such as obtained from commercial sources without purification except for anhydrous CH_2Cl_2, DMF and acetone. Thin layer

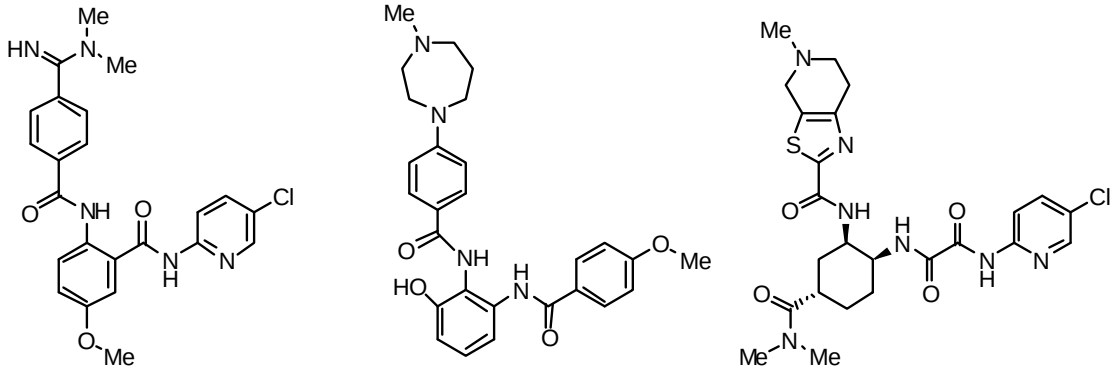


Fig. 1: Some clinically used factor Xa inhibitors

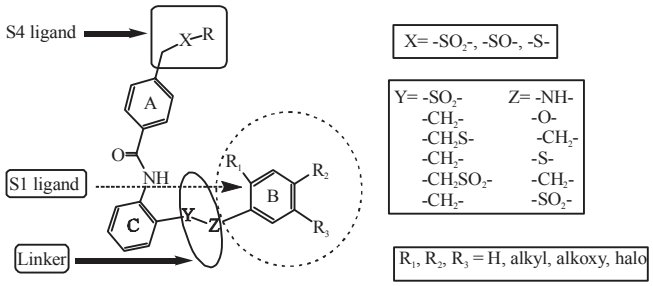


Fig. 2: General structure of the designed 2-substituted benzamidobenzene derivatives

chromatography (TLC) analysis was done on glass plates using silica gel G or on silica gel Merck 60 F254 plates. Visualization of spots was achieved by exposure to iodine vapors or ultraviolet light. Column chromatography was performed using Acme's silica gel (60-120 mesh size) and the elution was done using mixture of light petroleum (60-80) and ethyl acetate. Melting points were recorded in open capillary tubes in melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NM Rspectra were recorded on Bruker FT-NMR spectrometer (^{1}H NMR 200/400/500 MHz and ^{13}C NMR at 75/100 MHz) using $CDCl_3$ or DMSO-d_6 as solvents. Mass Spectra (HRMS) are obtained on Agilent Q-T of B.05.00 (B5042.0) higher resolution MSMS spectrometer using electrospray ionization mode. Mass spectra were recorded on a Thermo-fisher DSQ II GCMS instrument. Elemental analyses were performed on Thermo Scientific Flash 2000 organic elemental analyzer and are within 0.4% of the theoretically calculated values. The design, molecular docking and synthesis work started on 25th June, 2010 and was completed on 15th November, 2013. The design and synthesis part was completed at Applied Chemistry Department, Faculty of Technology and Engineering, The M.S. University of Baroda, Vadodara, India. Molecular docking studies were carried out at Pharmacy Department, Faculty of Technology and Engineering, The M. S. University of Baroda, Vadodara, India.

4-(Bromomethyl)benzoic acid (5): To a mixture of p-toluic acid 4 (10 g, 0.0735 mol) and N-bromosuccinimide (14.38 g, 0.0808 mol) in carbon tetrachloride (70 ml) was added a catalytic amount of benzoyl peroxide (0.74 g) under stirring and the mixture was allowed to stir under reflux for 5 h. Carbon tetrachloride was evaporated under reduced pressure, water added to the resultant solid and the slurry was allowed to warm at 50°C for 1 h. The contents of the flask were filtered with suction to obtain a crude dry solid which was stirred in light petroleum (50 mL) for 30 min and then filtered to get a white solid 5 (13.1 g, 83%)[35],mp 226°C.

4-(Mercaptomethyl) benzoic acid (6): A mixture of 4-bromomethyl benzoic acid 5 (10 g, 0.0465 mol) and thiourea (4.24 g, 0.0558 mol) was refluxed in water (70 mL) for 3 h. The reaction mixture was cooled to room temperature, a solution of aqueous NaOH (10%, 50 mL) was added and the reaction mixture was again refluxed for 2 h (monitored by TLC). Cooling the reaction mixture to 0°C and acidification using 2 M HCl furnished a light yellow solid which was filtered to obtain the crude thiol. Recrystallization from ethanol gave 6 as the pure white solid (6.64 g, 85%), mp 186°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.04 (t, 1H, exchangeable), 3.84 (d, J = 12 Hz, 2H), 7.51 (d, J = 8 Hz, 2H), 7.94 (d, J = 8 Hz, 2H), 12.96 (bs, 1H, exchangeable),MS m/z 168.06 (M^+).

4-(Methylthiomethyl) benzoic acid (7): To a stirred solution of 6 (5 g, 0.0297 mol) in dry THF (50 mL) at 0°C under nitrogen atmosphere, was added NaH (2.61 g, 0.0654 mol as 60% dispersion in paraffin oil), followed by the addition of methyl iodide (5 g, 0.0357 mol). The resulting mixture was stirred at 0°C for 1 h, diluted with water (50 mL). Aqueous layer was then acidified with dilute HCl (1:1) (20 mL) and extracted with ethyl acetate (6×25 mL).The organic layer was dried over anhydrous sodium sulfate and evaporated to afford 7 as a pale yellow solid which was purified by column chromatography (5 g, 92%)[36], mp 148°C, ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 2.00 (s, 3H) , 3.72 (s, 2H), 7.42 (d, J = 8.24 Hz, 2H), 8.07 (d, J = 8.24 Hz, 2H), 12.87 (bs, 1H, exchangeable), MS m/z 182.06 (M^+).

Methyl 4-(methylthiomethyl) benzoate (8): Compound 7 (5 g, 0.0274 mol) was taken in methanol (50 mL) and conc. H_2SO_4 (98%) was added in catalytic amounts and allowed to reflux for 10 h. Methanol was removed under reduced pressure, water (50 mL) added to the reaction mixture and the mixture was extracted with ethyl acetate (5×25 mL). Organic layer was washed with sodium bicarbonate solution (20%) (2×30 mL), dried over anhydrous sodium sulfate and

evaporated to afford 8 as light orange liquid which was purified by column chromatography (5.2 g, 96%)[36], bp 319°C, ^{1}H NMR ($CDCl_3$, 200 MHz) δ: 1.98 (s, 3H), 3.70 (s, 2H), 3.91 (s, 3H), 7.37 (d, J = 8.24 Hz, 2H), 7.99 (d, J = 8.24 Hz, 2H), MS m/z 196.12 (M^+).

4-(Methylsulfonylmethyl) benzoic acid (9): To a solution of 8 (2.5 g, 0.0127 mol) in glacial acetic acid (15 mL) at room temperature was added H_2O_2 (30%) (2.1 g, 0.0638 mol). The reaction mixture was allowed to reflux for 1 h, after which white precipitates formed on cooling were suction filtered and dried in hot air oven (52°C) to give sulfone of 8. A mixture of this sulfone (2.5 g, 0.0109 mol) and sodium hydroxide (1N,15 mL, 0.0438 mol) was stirred at room temperature for 3 h. The reaction mixture was diluted with water (15 mL) and the pH was adjusted to 2-4 using 1 N HCl. The precipitate thus obtained was filtered, washed with water and dried to give 9 (1.7 g, 76%) as white solid[36] mp 250°C.

4-((Methylsulfoxy)methyl) benzoic acid (10): To a stirred solution of 8 (2.5 g, 0.0127 mol) in aqueous methanol (8:2) (40 mL) at room temperature was added cetyltrimethyl ammonium periodate (CTAPI) (2.1 g, 0.0446 mol) in portions over a period of 15 min. The mixture was then stirred for 45 min, filtered, the filtrate extracted with ethyl acetate (6×25 mL), dried over anhydrous sodium sulfate and evaporated under reduced pressure to give sulfoxide[24] of 8. Alkaline hydrolysis of this sulfoxide was carried out by a similar procedure as reported for 9 to obtain compound 10, (1.86 g, 74%)[36].

General procedure for coupling of 11 with substituted amines (12a-12g) to yield compounds (13a-13g): To a mixture of amines 12a-12g (0.0081 mol) and triethylamine (0.98 g, 0.0097 mol) in anhydrous CH_2Cl_2 (10 mL) at 0°C was added dropwise a solution of 11 (2 g, 0.0090 mol) in anhydrous CH_2Cl_2 (10 mL). The mixture was stirred at 0°C for 30 min and then allowed to warm to room temperature for 3 h, washed successively with aqueous HCl (1:1), water (20 mL), brine (10 mL) and dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give crude products which were purified by column chromatography over silica gel using EtOAc/light petroleum.

N-Phenyl-2-nitrobenzenesulfonamide (13a): White solid, yield: 91%, mp 110°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.07-7.13 (m, 3H), 7.25-7.29 (m, 2H), 7.78-7.86 (m, 2H), 7.95-7.98 (m, 2H), 10.75 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 120.85 (C-8', C-12'), 125.10 (C-10'), 125.16 (C-3'), 129.80 (C-6'), 130.33 (C-9', C-11'), 131.76 (C-4'), 133.01 (C-1'), 135.10 (C-5'), 137.09 (C-7'), 148.40 (C-2').

N-(4-Bromophenyl)-2-nitrobenzenesulfonamide (13d): Pale yellow solid, yield: 78%, mp 106°C, ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.09 (d, J = 8.7 Hz, 2H), 7.30 (s, 1H), 7.39 (d, J = 8.7 Hz, 2H), 7.58-7.87 (m, 4H), ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 120.06 (C-10'), 124.79 (C-8', C-12'), 125.38 (C-6'), 131.77 (C-9', C-11'), 132.54 (C-4'), 132.71 (C-1'), 134.20 (C-5'), 134.55 (C-7'), 148.11 (C-2').

N-(4-Chloro-2,5-dimethoxyphenyl)-2-nitrobenzene sulfonamide (13e): Pale yellow solid, yield: 88%, mp 140°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.41 (s, 3H), 3.74 (s, 3H), 6.97 (s, 1H), 7.09 (s,1H), 7.91-8.32 (m, 4H), 10.00 (s, 1H, exchangeable), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 56.68 (OCH_3 attached to C-8' carbon), 56.90 (OCH_3 attached to C-11' carbon), 112.16 (C-12'), 114.45 (C-10'), 119.69 (C-9'), 124.06 (C-3'), 124.69 (C-7'), 130.40 (C-6'), 132.72 (C-4'), 132.75 (C-1'), 134.81 (C-5'), 147.78 (C-8'), 147.90 (C-2'), 148.66 (C-11').

General procedure for the reduction of nitro compounds to amines-preparation of compounds (14a-14g): Reduction was carried out by addition of nitro compounds (0.0066 mol) portion wise to a heated solution (65°C) of conc. HCl (36.5%, 3.2 mL) and stannous chloride dihydrate (6 g, 0.0265 mol) in ethanol (20 mL). The reaction mixture was refluxed for a sufficient period of time (monitored by TLC). After completion of the reaction, ethanol was removed under reduced pressure. Ethyl acetate (20 mL) was added to the reaction mixture for removal of organic impurities and the aqueous solution was then filtered through a celite bed and the filtrate was made alkaline with strong solution of ammonia (10 mL) and extracted with ethyl acetate (4×25 mL). The combined organic extract was washed successively with water (20 mL), brine solution (10 mL) and dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure furnished the crude amines, which were chromatographed using ethyl acetate/light petroleum (60-80).

N-(4-Chloro-2,5-dimethoxyphenyl)-2-aminobenzene sulfonamide (14e): Yellow solid, yield: 71%, mp 156°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.60 (s, 3H), 3.67 (s, 3H), 6.10 (bs, 2H, exchangeable), 6.50-6.54 (m, 1H), 6.74-6.76 (m, 1H), 6.89 (s, 1H), 7.20-7.24 (m, 1H), 7.45-7.47 (m, 1H), 9.57 (bs, 1H, exchangeable).

General procedure for the synthesis of compounds (15a-15g): To a stirred solution of 9 (1 g, 0.0046 mol) in anhydrous CH_2Cl_2 (10 mL) and DMF (0.02 mL) at 0-5°C was added oxalyl chloride (0.7 g, 0.0056 mol) under nitrogen atmosphere. The resulting mixture was stirred for 4 h at 25°C and then concentrated under vacuum to dryness. The crude yellow acid chloride obtained was dissolved in anhydrous CH_2Cl_2 (5 mL) and then added dropwise to the solution of 14a-14g (0.0051 mol) and triethylamine (0.5 g, 0.0051 mol) in anhydrous CH_2Cl_2 (5 mL) cooled to 0-5°C. After complete addition of the acid chloride, the reaction mixture was stirred at 25°C for 2 h. The mixture was then diluted with 2 N HCl (10 mL), extracted with CH_2Cl_2 (4×20 mL),the organic layer dried over anhydrous sodium sulfate and evaporated to gave crude products. Purification by column chromatography using ethyl acetate/light petroleum (60-80) afforded 15a-15g.

N-Phenyl-2-(4-(methylsulfonylmethyl) benzamido)benzene sulfonamide (15a): White solid, yield: 67%, mp 164°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.98 (s, 3H), 4.65 (s, 2H), 7.00-7.04 (m, 3H), 7.14-7.18 (m, 2H), 7.25-7.29 (m, 1H), 7.44 (d, J = 8.0 Hz, 1H), 7.61-7.67 (m, 3H), 7.90 (d, J = 8.4 Hz, 2H), 8.35 (d, J = 8.0 Hz, 1H), 10.13 (s, 1H), 10.52 (s,1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 39.27-40.49 (merged SO_2CH_3), 59.45 ($ArCH_2SO_2CH_3$), 122.25 (C-14', C-18'), 123.58 (C-16'), 124.71 (C-12'), 125.63 (C-10'), 127.95 (C-9', C-1'), 128.20 (C-3', C-5'), 129.69 (C-15', C-17'), 131.84 (C-2', C-6'), 133.75 (C-11'), 134.38 (C-4'), 134.57 (C-7'), 136.44 (C-13'), 137.03 (C-8'), 164.79 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{20}N_2O_5S_2$: 467.0711, found: 467.0710, Anal. Calcd for $C_{21}H_{20}N_2O_5S_2$: C, 56.75, H, 4.51, N, 6.08, found: C, 56.20, H, 4.58, N, 6.01.

N-Benzyl-2-(4-(methylsulfonylmethyl) benzamido)benzene sulfonamide (15b): White solid, yield: 62%, mp 180°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.95 (s, 3H), 4.04 (s, 2H), 4.63 (s, 2H), 7.24 (m, 5H), 7.34 (m, 1H), 7.59 (d, J = 8.0 Hz, 2H), 7.68 (m, 1H), 7.87 (d, J = 7.6 Hz, 1H), 7.95 (d, J = 8.0 Hz, 2H), 8.46 (d, J = 7.6 Hz, 1H), 8.68 (s, 1H), 10.31 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 39.32-40.57 (merged SO_2CH_3), 46.23 ($NHCH_2Ar$), 59.43 ($ArCH_2SO_2CH_3$), 123.26 (C-12'), 124.68 (C-16'), 127.75 (C-10'), 127.88 (C-14', C18'), 127.99 (C-1'), 128.74 (C-9'), 128.97 (C-3', C-5'), 129.55 (C-14', C-15'), 131.89 (C-3', C6'), 133.81 (C-11'), 134.15 (C-4'), 134.50 (C-7'), 136.10 (C-8'), 137.57 (C-13'), 164.78 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{22}H_{22}N_2O_5S_2$: 481.0868, found, 481.0861, Anal. Calcd for $C_{22}H_{22}N_2O_5S_2$: C, 57.64, H, 4.80, N, 6.11, found: C, 57.61, H, 4.77, N, 5.96.

(N-(4-Methylphenyl)-2-(4-(methylsulfonylmethyl) benzamido))benzene sulfonamide (15c): White solid, yield: 59%, mp 186°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.11 (s, 3H), 2.98 (s, 3H), 4.65 (s, 2H), 6.87 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.4 Hz, 2H), 7.24-7.28 (m, 1H), 7.62 (d, J = 8.4 Hz, 2H), 7.65-7.74 (m, 2H), 7.86 (d, J = 8.4 Hz, 2H), 8.35-8.37 (m, 1H), 10.07 (s, 1H), 10.32 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 20.79 ($ArCH_3$), 40.18 (SO_2CH_3), 59.46 ($ArCH_2SO_2CH_3$), 123.13 (C-14', C-18'), 124.57 (C-12'), 127.84 (C-10'), 127.94 (C-1', C-9'), 129.66 (C-16'), 130.13 (C-3', C-5'), 131.80 (C-15', C-17'), 133.76 (C-2', C-6'), 134.14 (C-11'), 134.38 (C-4', C-13'), 135.28 (C-7'), 136.48 (C-8'), 164.60 (C = O), EI-MS m/z 459.3 $[M+1]^+$, Anal. Calcd for $C_{22}H_{22}N_2O_5S_2$: C, 57.64, H, 4.80, N, 6.11, found: C, 57.33, H, 4.93, N, 6.10.

(N-(4-Bromophenyl)-2-(4-(methylsulfonylmethyl) benzamido)) benzene sulfonamide (15d): White solid, yield: 76%, mp 198°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.98 (s, 3H), 4.65 (s, 2H), 6.97 (d, J = 8.4 Hz, 2H), 7.27-7.34 (m, 3H), 7.62-7.68 (m, 3H), 7.76 (d, J = 7.6 Hz, 1H), 7.92 (d, J = 8 Hz, 2H), 8.29 (d, J = 8.4 Hz, 1H), 10.07 (s, 1H), 10.66 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 39.32-40.57 (merged SO_2CH_3), 59.49 ($ArCH_2SO_2CH_3$), 117.92 (C-16'), 124.00 (C-14', C-18'), 124.09 (C-12'), 124.91 (C-10'), 127.95 (C-1'), 128.23 (C-9'), 129.66 (C-3', C-5'), 131.81 (C-2', C-6'), 132.59 (C-11'), 133.81 (C-15', C-17'), 134.38 (C-4'), 134.72 (C-7'), 136.45 (C-13'), 136.54 (C-8'), 161.81 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{19}N_2O_5S_2Br$: 546.9780, found: 546.9784, Anal. Calcd for $C_{21}H_{19}N_2O_5S_2Br$: C, 48.19, H, 3.63, N, 5.35, found: C, 47.88, H, 3.94, N, 4.95.

(N-(4-chloro-2, 5-Dimethoxyphenyl)-2-(4-(methyl sulfonylmethyl)benzamido))benzene sulfonamide (15e): Pale yellow solid, yield: 72%, mp 224°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.03 (s, 3H), 3.32 (s, 3H), 3.64 (s, 3H), 4.68 (s, 2H), 6.93 (s, 1H), 6.96 (s, 1H), 7.30 (m, 1H), 7.64 (d, J = 8.3 Hz, 2H), 7.69-7.78 (m, 2H), 7.87 (d, J = 8.3 Hz, 2H), 8.44 (d, J = 8.2 Hz, 1H), 10.09 (s, 1H), 10.17 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 39.32-40.57 (SO_2CH_3 merged), 56.47 (OCH_3 attached to C-17'), 56.63(OCH_3 attached to C-14'), 59.46 ($ArCH_2SO_2CH_3$), 113.14 (C-18'), 114.21 (C-16'), 120.22 (C-15'), 122.73 (C-12'), 123.49 (C-10'), 124.24 (C-1'), 127.69 (C-9'), 128.74 (C-13'), 129.62 (C-3', C-5'), 131.69 (C-2', C-6'), 133.82 (C-11'), 134.00 (C-4'), 134.29 (C-7'), 136.46 (C-8'), 148.44 (C-14'), 148.49 (C-17'), 164.24 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{23}H_{23}N_2O_7S_2Cl$: 561.0553, found: 561.0553, Anal. Calcd for $C_{23}H_{23}N_2O_7S_2Cl$: C, 51.25, H, 4.27, N, 5.26, found: C, 51.04, H, 4.13, N, 5.34.

(N-(4-Fluorophenyl)-2-(4-(methyl sulfonylmethyl) benzamido))benzene sulfonamide (15f): White solid, yield: 69%, mp 200°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.98 (s, 3H), 4.64 (s, 2H), 6.99 (m, 4H), 7.28 (m, 1H), 7.68 (m, 4H), 7.88 (d, J = 8.4 Hz, 2H), 10.07 (s, 1H), 10.43 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 39.32-40.57 (merged SO_2CH_3), 59.47 ($ArCH_2SO_2CH_3$), 116.34 (C-15', C-17'), 116.56 (C-14', C-18'), 123.64 (C-12'), 124.75 (C-10'), 125.29 (C-1'), 127.87 (C-8'), 127.93 (C-3', C-5'), 129.68 (C-2', C-6'), 131.81 (C-11'), 133.08 (C-13'), 133.83 (C-4'), 134.33(C-7'), 134.65 (C-8'), 136.45 (C-16'), 164.66 C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{19}N_2O_5S_2F$: 485.0617, found: 485.0607, Anal. Calcd for $C_{21}H_{19}N_2O_5S_2F$: C, 54.55, H, 4.11, N, 6.06, found: C, 54.36, H, 4.05, N, 6.01.

(N-(4-Methoxyphenyl)-2-(4-(methylsulfonylmethyl) benzamido)) benzenesulfonamide (15g): White solid, yield: 59%, mp 176°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.98 (s, 3H), 3.58 (s, 3H), 4.64 (s, 2H), 6.67 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 7.24-7.28 (m, 1H), 7.61 (d, J = 8.4 Hz, 2H), 7.63-7.70 (m, 2H), 7.84 (d, J = 8.4 Hz, 2H), 8.39-8.41 (m, 1H), 10.08 (s, 1H), 10.12 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 39.25-40.37 (SO_2CH_3 merged), 55.55 (OCH_3 attached to C-16'), 59.48 ($ArCH_2SO_2CH_3$), 114.80 (C-15', C-17'), 122.91 (C-14', C-18'), 124.49 (C-12'), 125.95 (C-10'), 127.70 (C-1'), 127.80 (C-9'), 129.03 (C-3', C-5'), 129.69 (C-13'), 131.78 (C-2', C-6'), 133.79 (C-11'), 134.30 (C-4'), 134.48 (C-7'), 136.52 (C-8'), 157.81 (C-16'), 164.49 C = O), EI-MS m/z 475.3 $[M+H]^+$, Anal. Calcd for $C_{22}H_{22}N_2O_6S_2$: C, 55.69, H, 4.64, N, 5.90, found: C, 55.61, H, 4.70, N, 5.86.

4-(Mercaptomethyl) nitrobenzene (17): 4-(Bromomethyl) nitrobenzene was prepared following the procedure reported for preparation of 5 (5.1.1) from 16. This crude product was sufficiently pure (as indicated by TLC) to be used directly for the preparation of 17. To a stirred solution of 4-(bromomethyl) nitrobenzene (5 g, 0.0231 mol) in acetone (50 mL) was added potassium thioacetate (3.9 g, 0.0347 mol). The resulting mixture was allowed to stir at room temperature for 2 h. Acetone was removed under vacuum, water (50 mL) was added to the reaction mixture and extracted with ethyl acetate (5×30 mL). The organic layer was washed successively with sodium bicarbonate solution (20%, 3×20 mL), water, brine and dried over anhydrous sodium sulfate. Solvent was removed *in vacuo* and the residue was taken up in methanol (50 mL). Aqueous sulfuric acid (50%, 8.3 mL) was added and the solution was allowed to reflux for 3 h. Methanol was rotary evaporated and water (40 mL) was added to it. Extraction was carried out using ethyl acetate (5×20 mL) and dried over anhydrous sodium sulfate. Removal of solvent afforded 17 as light yellow solid which was chromatographed to yield the pure product (2.5 g, 73%), mp 50°C[37,38].

4-((Methylthio) methyl) nitrobenzene (18): This compound was prepared from 17 by following the procedure similar to that reported for 7. Column chromatography afforded pure 18 as colorless liquid, yield 79%, mp 68°C[39].

4-((Methylsulfonyl)methyl) nitrobenzene (19): This compound was prepared from 18 by following the procedure similar to that reported for 9. The solid thus obtained was directly used for the next step without further purification. Yield: 85%, mp 168°C. Identity of the compound was confirmed by comparison with the reported compound[39].

4-((Methylsulfonyl) methyl) aniline (20): Reduction of 19 by a procedure similar to that reported for 14a-14g afforded a crude product, which was chromatographed to give pure 20 as white solid, yield[40] 56%, mp 170°C.

N-(2-Nitrophenyl)-4-methylbenzene sulfonamide (23a): To a stirred solution of 21a (2.0 g, 0.0144 mol) in dry pyridine (8 mL) was added 22 (2.7 g, 0.0144 mol) in portions over a period of 5 min and the reaction mixture was heated to 120°C. The reaction mixture was poured into cold water (50 mL) and extracted using ethyl acetate (4×25 mL). The organic layer was washed with dil. HCl (1:1) (10 mL), water (20 mL) and dried over anhydrous sodium sulfate. Removal of solvent under *vacuo* afforded 23a as yellow solid (2.3 g, 55%). Recrystallization from EtOAc gave (1.6 g) yellow crystals[41], mp 114°C.

N-(2-Carboxyphenyl)-4-methylbenzene sulfonamide (23b): p-Toluenesulfonyl chloride 22 (1.7 g, 0.0087 mol) was added to a stirred solution of anthranilic acid 21b (1.0 g, 0.0073 mol) and sodium carbonate (1.9 g, 0.0175 mol) in water (10 mL) at 40°C over a period of 5 min and the reaction mixture was stirred for 6 h at 80°C. After completion of the reaction, it was cooled to room temperature and acidified with 6 N HCl (20 mL). The solid thus obtained was filtered under vacuum and washed with water (20 mL) followed by drying in hot air oven at 60°C to afford 23b as brownish solid (1.8 g). Recrystallization form ethanol gave (1.2 g, 57%) pure white crystals, mp 210°C[42].

N-(2-Aminophenyl)-4-methylbenzene sulfonamide (24a): It was synthesized according to the general procedure for

reduction of nitro compound14a-14g. The crude product so obtained was purified by column chromatography to obtain 24a as white solid, yield[43]: 83%, mp 136°C.

4-((Methylsulfonyl) methyl)-N-2-(tosylamino) phenyl) benzamide (25): Synthesized according to the general procedure for the synthesis of compounds 15a-15g. The crude product was purified by column chromatography to obtain 25 as white solid, yield: 62%, mp 190°C, ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.33 (s, 3H), 3.03 (s, 3H), 4.68 (s, 2H), 7.16-7.32 (m, 5H), 7.52 (d, J = 8.1 Hz, 2H), 7.64 (d, J = 8.1 Hz, 2H), 7.78 (d, J = 7.9 Hz, 1H), 7.88 (d, J = 8.2 Hz, 2H), 9.59 (s, 1H), 9.64 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 21.42 (CH_3 attached to C-16), 39.51-40.34 (merged SO_2CH_3), 59.52 ($ArCH_2SO_2CH_3$), 125.23 (C-9'), 125.92 (C-11'), 126.92 (C-12'), 127.15 (C-7'), 127.22 (C-10'), 128.06 (C-14', C-18'), 128.94 (C-1'), 130.12 (C-15', C-17'), 131.46 (C-3', C-5'), 133.15 (C-2', C-6'), 133.43 (C-8'), 134.40 (C-4'), 137.11 (C-13'), 143.76 (C-16'), 165.11 (C = O), HRMS (ESI) m/z [M+Na]$^+$ calculated for $C_{22}H_{22}N_2O_5S_2$: 481.0868, found: 481.0861 , Anal. Calcd for $C_{22}H_{22}N_2O_5S_2$: C, 57.64, H, 4.80, N, 6.11, found: C, 57.43, H, 4.73, N, 6.14.

N-(4-((Methylsulfonyl)methyl)phenyl)-2-(tosylamino) benzamide (26): This compound was prepared from 23b using the procedure similar to that reported for compounds 15a-15g. The crude product was purified by column chromatography to obtain 26 as white solid, yield: 66%, mp 228°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.26 (s, 3H), 2.91 (s, 3H), 4.47 (s, 2H), 7.22-7.26 (m, 3H), 7.40-7.52 (m, 4H), 7.61 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.4 Hz, 2H), 7.77-7.74 (m, 1H), 10.42 (s, 1H), 10.56 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 21.42 (CH_3 attached to C-16'), 39.47-40.14 (merged SO_2CH_3), 59.44 ($ArCH_2SO_2CH_3$), 121.29 (C-9'), 121.83 (C-8'), 124.61 (C-11'), 125.27 (C-1'), 127.30 (C-3', C-5'), 129.58 (C-14', C18'), 130.26 (C-12'), 131.67 (C-15', C-17'), 132.88 (C-2', C-6'), 136.30 (C-10'), 137.61 (C-4', C-13'), 138.93 (C-16'), 144.20 (C-7'), 167.14 (C = O), HRMS (ESI) m/z [M+H]$^+$ calculated for $C_{22}H_{22}N_2O_5S_2$: 459.0970, found: 459.0904, Anal. Calcd for $C_{22}H_{22}N_2O_5S_2$: C, 57.64, H, 4.80, N, 6.11, found: C, 57.55, H, 4.66, N, 5.98.

2-Nitrobenzylbromide (28): The title compound was synthesized from 27 according to the general procedure reported for the preparation of 5. The product was obtained as lachrymatory yellow needles, yield[37]: 48%, mp 46°C.

General procedure for the synthesis of compounds (29a-29c): To a stirred solution of sodium metal (0.25 g, 0.0111 mol) in methanol (20 mL) at 0°C under nitrogen was added phenol/thiophenol/benzyl mercaptan (0.0093 mol). The reaction mixture was stirred for 30 min at 0°C after addition of 28 (2 g, 0.0093 mol). The mixture was then warmed to room temperature and allowed to stir for 2 h. The solvent was removed *in vacuo* and the residue was extracted with ethyl acetate (4×25 mL), washed with water (30 mL) and brine (25 mL) and dried over anhydrous sodium sulfate. Removal of the solvent *in vacuo* gave the crude product which on purification by column chromatography afforded compound 29a-29c.

Benzyl 2-nitrobenzyl sulfide (29b): Yellow liquid, yield: About 78%, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 3.74 (s, 2H), 4.05 (s, 2H), 7.35-8.06 (m, 9H), MS m/z 259.1 (M^+).

Procedure for the synthesis of30a, 30b: To a solution of sulfides 29a,29b (0.0077 mol) in glacial acetic acid (10 mL) at room temperature was added H_2O_2(30%) (1.3 g, 0.0385 mol). The reaction mixture was heated at 100°C for 1 h. White precipitates thus formed on cooling were suction filtered and dried in hot air oven (52°C) to give sufficiently pure (as indicated by TLC) compounds 30a, 30b which were used as such in the next step.

General procedure for the preparation of 31a-31c and 32a, 32b: To a solution of nitro compound (29a, 29b and 30a, 30b) (0.00612 mol) in ethanol was added iron powder (2 g, 0.0369 mol) followed by conc. HCl (3.0 mL) at room temperature. The resulting mixture was then allowed to reflux for 3 h. Ethyl acetate was added to the reaction mixture and was made alkaline with strong solution of ammonia (10 mL). The reaction mixture was filtered through celite bed and the filtrate was extracted with ethyl acetate (4×25 mL). The combined organic extract was washed with water (30 mL), brine (25 mL) and dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude residue was then subjected to column chromatography to obtain pure products 31a-31c and 32a, 32b.

2-((Benzylsulfonyl)methyl)aniline(32a): Light brown solid, yield: 65%, mp 124°C ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.30 (s, 2H), 4.36 (s, 2H), 6.86-7.30 (m, 4H), 7.35 (s, 2H), 7.50-7.54 (m, 5H), MS m/z 261.1 (M^+).

General procedure for the synthesis of33a-33e: These compounds were synthesized according to the general procedure reported for synthesis of 15a-15g (*vide supra*). Here pyridine was used instead of triethylamine.

4-((Methylsulfonyl)methyl)-N-(2-(phenoxymethyl)phenyl) benzamide (33a): White solid, yield: 76%, mp 152°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.83 (s, 3H), 4.33 (s, 2H), 5.24 (s, 2H), 7.02-7.06 (m, 3H), 7.16-7.20 (m, 1H), 7.31-7.38 (m, 3H), 7.43-7.48 (m, 3H), 7.88-7.90 (m, 2H), 8.32 (d, J = 8.4 Hz, 1H), 9.21 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 39.37 (SO_2CH_3), 60.86 ($ArCH_2SO_2CH_3$), 69.89 (CH_2O), 114.93 (C-14', C-18'), 122.21 (C-16'), 122.59 (C-12'), 124.67 (C-10'), 126.00 (C-9'), 127.79 (C-1'), 129.61 (C-11'), 129.81 (C-3', C-5'), 129.92 (C-15', C-17'), 131.01 (C-2', C-6'), 131.97 (C-7'), 135.36 (C-4'), 137.62 (C-8'), 157.55 (C-13'), 164.26 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{22}H_{21}NO_4S$: 418.1089, found: 418.1088, Anal. Calcd for $C_{22}H_{21}NO_4S$: C, 66.82, H, 5.35, N, 3.54, found: C, 66.88, H, 5.09, N, 3.65.

N-(2-((Benzylthio)methyl)phenyl)-4-((methylsulfonyl) methyl) benzamide (33b): White solid, yield: 74%, mp 196°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.01 (s, 3H), 3.69 (s, 2H), 3.80 (s, 2H), 4.67 (s, 2H), 7.21-7.31 (m, 5H), 7.35-7.39 (m, 1H), 7.44-7.46 (m, 1H), 7.50-7.52 (m, 1H), 7.62 (d, J = 8.3 Hz, 2H), 7.98 (d, J = 8.3 Hz, 2H), 10.52 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 33.57 (CH_2S attached to C-8'), 35.52 (SCH_2 attached to C-13'), 39.45 (merged SO_2CH_3), 60.94 ($ArCH_2SO_2CH_3$), 123.87 (C-12'), 124.85 (C-10'), 126.83 (C-8'), 127.41 (C-16'), 128.03 (C-1', C-11'), 128.70 (C-3', C-5'), 128.88 (C-15', C-17'), 130.99 (C-14', C-18'), 131.04 (C-9'), 131.90 (C-2', C-6'), 135.41 (C-7'), 136.64 (C-4'), 137.17 (C-13'), 164.40 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{23}H_{23}NO_3S_2$: 448.1017 found: 448.1013, Anal. Calcd for $C_{23}H_{23}NO_3S_2$: C, 64.91, H, 5.45, N, 3.29, found: C, 64.36, H, 5.05, N, 3.01.

4-((Methylsulfonyl)methyl)-N-(2-((phenylthio) methyl)phenyl) benzamide (33c): White solid, yield: 69%, mp 170°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.83 (s, 3H), 4.18 (s, 2H), 4.35 (s, 2H), 7.11-7.24 (m, 2H), 7.26-7.30 (m, 3H), 7.31-7.38 (m, 3H), 7.55 (d, J = 8.4 Hz, 2H), 8.00 (d, J = 8.4 Hz, 2H), 8.05 (d, J = 8.0 Hz, 1H), 8.96 (s, 1H),^{13}C NMR ($CDCl_3$, 100 MHz) δ: 37.58 (CH_2S attached to C-8'), 39.47 (SO_2CH_3), 60.86 ($ArCH_2SO_2CH_3$), 124.09 (C-12'), 125.23 (C-10'), 127.37 (C-16'), 127.70 (C-14', C-18'), 127.99 (C-11'), 128.81 (C-1'), 129.18 (C-9'), 130.75 (C-3', C-5'), 131.09 (C-8'), 131.37 (C-15', C-17'), 132.00 (C-2', C-6'), 133.74 (C-7'), 135.27 (C-4'), 136.25 (C-13'), 164.52 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{22}H_{21}NO_3S_2$: 434.0860 found: 434.0859, Anal. Calcd for $C_{22}H_{21}NO_3S_2$: C, 64.21, H, 5.14, N, 3.40, found: C, 64.20, H, 4.88, N, 3.11.

N-(2-((Benzylsulfonyl)methyl)phenyl)-4-((methylsulfonyl) methyl)benzamide (33d): White solid, yield: 62%, mp 230°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.02 (s, 3H), 4.63 (s, 2H), 4.68 (s, 4H), 7.33-7.41 (m, 4H), 7.43-7.53 (m, 4H), 7.60 (d, J = 8.4 Hz, 2H), 7.70 (d, J = 8.0 Hz, 1H), 7.88 (d, J = 8.4 Hz, 2H), 9.91 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 39.45 (SO_2CH_3), 55.22 (CH_2SO_2 attached to C-8'), 58.83 (SO_2CH_2 attached to C-13'), 60.94 ($ArCH_2SO_2CH_3$), 126.27 (C-8'), 126.84 (C-12'), 128.17 (C-13'), 129.28 (C-10'), 129.53 (C-1'), 130.40 (C-3', C-5'), 130.76 (C-15', C-16', C-17'), 130.99 (C-11'), 131.99 (C-14', C-18'), 132.82 (C-2', C-6'), 134.58 (C-9'), 136.84 (C-4'), 137.57 (C-7'), 164.90 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{23}H_{23}NO_5S_2$: 480.0915 found: 480.0916, Anal. Calcd for $C_{23}H_{23}NO_5S_2$: C, 60.37, H, 5.07, N, 3.06, found: C, 59.99, H, 4.95, N, 3.15.

4-((Methylsulfonyl)methyl)-N-(2-((phenylsulfonyl) methyl)phenyl) benzamide (33e): White solid, yield: 66%, mp 194°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.58 (s, 3H), 4.36 (s, 2H), 4.44 (s, 2H), 6.65-6.67 (m, 1H), 6.98-7.02 (m, 1H), 7.39-7.43 (m, 1H), 7.50-7.54 (m, 2H), 7.61 (d, J = 8.3 Hz, 2H), 7.66-7.72 (m, 3H), 7.94 (d, J = 7.7 Hz, 1H), 8.15 (d, J = 8.3 Hz, 2H), 9.72 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 39.40 (SO_2CH_3), 60.28 (CH_2SO_2), 60.99 ($ArCH_2SO_2CH_3$), 120.90 (C-12'), 125.82 (C-10'), 126.46 (C-11'), 128.27 (C-1'), 128.51 (C-3', C-5'), 129.29 (C-14', C-18'), 130.10 (C-9'), 131.10 (C-8'), 132.27 (C-15', C-17'), 132.52 (C-2', C-6'), 134.47 (C-16'), 134.68 (C-7'), 136.61(C-4'), 137.36 (C-13'), 164.84 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{22}H_{21}NO_5S_2$: 466.0758 found: 466.0757, Anal. Calcd for $C_{22}H_{21}NO_5S_2$: C, 59.57, H, 4.77, N, 3.16, found: C, 59.61, H, 4.70, N, 3.56.

General procedure for the synthesis of 34a-34c: These compounds were synthesized according to the general procedure for synthesis of compounds 15a-15g (*vide supra*), from acid 10 (1 g, 0.0050 mol) and amines 32a, 32b, 14e (0.0050 mol) using pyridine.

N-(2-((Benzylsulfonyl)methyl)phenyl)-4-((methylsulfinyl) methyl) benzamide (34a): White solid, yield: 56%, mp 224°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.52 (s, 3H), 4.04 (d, J = 12.8 Hz, 1H), 4.26 (d, J = 12.8 Hz, 1H), 4.58 (s, 2H), 4.63 (s, 2H), 7.26-7.30 (m, 1H), 7.33-7.35 (m, 3H), 7.37-7.42 (m, 2H), 7.44-7.46 (m, 4H), 7.64 (d, J = 7.6 Hz, 1H), 7.82 (d, J = 8.4 Hz, 2H), 9.86 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 37.76 ($SOCH_3$), 54.30 (CH_2SO_2 attached to C-8'), 58.38 (SO_2CH_2 attached to C-13'), 58.42 ($ArCH_2SOCH_3$), 122.78 (C-8'), 126.23 (C-12'), 126.73 (C-13'), 128.02 (C-10'), 128.58 (C-3', C-5'), 128.95 (C-1'), 128.99 (C-15', C-17'), 129.64 (C-16'), 130.90 (C-11'), 131.53 (C-2', C-6'), 133.42 (C-14', C-18'), 133.98 (C-9'), 135.63 (C-4'), 138.07 (C-7'), 165.38 (C=O), MS m/z 441.43 (M^+)., Anal. Calcd for $C_{23}H_{23}NO_4S_2$: C, 62.58, H, 5.21, N, 3.17, found: C, 62.11, H, 5.25, N, 3.03.

4-((Methylsulfinyl)methyl)-N-(2-((phenylsulfonyl)methyl) phenyl)benzamide (34b): White solid, yield: 60%, mp 166°C, ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 2.52 (s, 3H), 4.08 (d, J = 12.8 Hz, 1H), 4.26 (d, J = 12.8 Hz, 1H), 4.89 (s, 2H), 7.09-7.11 (m, 2H), 7.35-7.39 (m, 1H), 7.49 (d, J = 8.4 Hz, 2H), 7.53-7.57 (m, 2H), 7.66 (d, J = 8.0 Hz, 1H), 7.68-7.71 (m, 3H), 7.92 (d, J = 8.4 Hz, 2H), 9.88 (s, 1H), ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 37.79 ($SOCH_3$), 58.28 (CH_2SO_2), 58.43 ($ArCH_2SOCH_3$), 122.86 (C-12'), 125.70 (C-10'), 126.25 (C-11'), 128.15 (C-3', C-5'), 128.32 (C-14', C-18'), 129.56 (C-1'), 129.70 (C-9'), 130.86 (C-8'), 133.39 (C-15', C-16'), 134.27 (C-2', C-6'), 134.45 (C-16'), 135.60 (C-4'), 138.02 (C-7'), 138.73 (C-13'), 165.41 (C = O), MS m/z 427.83 (M^+)., Anal. Calcd for $C_{22}H_{21}NO_4S_2$: C, 61.74, H, 4.91, N, 3.27, found: C, 61.47, H, 4.87, N, 3.30.

(N-(4-Bromophenyl)-2-(4-(methylsulfinylmethyl) benzamido)) benzenesulfonamide (34c): White solid, yield: 57%, mp 144°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.49 (s, 3H), 3.97 (d, J = 12.8 Hz, 1H), 4.02 (d, J = 12.8 Hz, 1H), 6.88-6.91 (m, 2H), 7.13-7.21 (m, 3H), 7.35 (d, J = 8.0 Hz, 2H), 7.56-7.61 (m, 1H), 7.71-7.73 (m, 1H), 7.88 (d, J = 8.0 Hz, 2H), 8.43 (s, 1H), 8.53 (d, J = 8.4 Hz, 2H), 10.11 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 37.50 ($SOCH_3$), 59.31 ($ArCH_2SOCH_3$), 119.88 (C-16'), 122.71 (C-14', C-18'), 123.98 (C-12'), 125.41 (C-10'), 126.57 (C-9'), 127.99 (C-3', C-5'), 129.49 (C-1'), 130.62 (C-2', C-6'), 132.36 (C-11'), 133.82 (C-15', C-17'), 134.03 (C-4'), 134.49 (C-7'), 134.91 (C-13'), 136.14 (C-9'), 164.49 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{19}N_2O_4S_2Br$: 530.9677 found: 530.9676, Anal. Calcd for $C_{21}H_{19}N_2O_4S_2Br$: C, 49.71, H, 3.75, N, 5.52, found: C, 49.64, H, 3.53, N, 5.29.

General procedure for the synthesis of 35a-35c: These compounds were synthesized according to the general procedure for the synthesis of compounds 15a-15g (*vide supra*), from the acid 7 (1 g, 0.0055 mol) and amines 32a, 32b, 14e (0.0055 mol) using pyridine.

N-(2-((Benzylsulfonyl)methyl)phenyl)-4-((methylthio) methyl) benzamide (35a): White solid, yield: 62%, mp 182°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.04 (s, 3H), 3.75 (s, 2H), 4.25 (s, 2H), 4.34 (s, 2H), 7.26-7.28 (m, 2H), 7.37-7.39 (m, 3H), 7.42-7.43 (m, 4H), 7.44-7.50 (m, 1H), 7.86-7.92 (m, 3H), 10.14 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 15.00 (SCH_3), 38.04 ($ArCH_2SCH_3$), 55.22 (CH_2SO_2 attached to C-8'), 58.77 (SO_2CH_2 attached to C-13'), 119.92 (C-8'), 126.08 (C-12'), 126.88 (C-13'), 126.93 (C-10'), 127.65 (C-3', C-5'), 129.24 (C-2', C-6'), 129.45 (C-15', C-16', C-17'), 130.33 (C-11'), 130.77 (C-14', C-18'), 132.46 (C-9'), 132.75 (C-1'), 137.74 (C-7'), 142.74 (C-4'), 165.44 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{23}H_{23}NO_3S_2$: 448.1017 found: 448.1020, Anal. Calcd for $C_{23}H_{23}NO_3S_2$: C, 64.92, H, 5.41, N, 3.29, found: C, 64.91, H, 5.38, N, 3.10.

4-((Methylthio)methyl)-N-(2-((phenylsulfonyl) methyl)phenyl) benzamide (35b): White solid, yield: 68%, mp 176°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.04 (s, 3H), 3.76 (s, 2H), 4.45 (s, 2H), 6.67-6.69 (m, 1H), 6.98-7.02 (m, 1H), 7.39-7.43 (m, 1H), 7.49 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.0 Hz, 2H), 7.66-7.74 (m, 3H), 7.94 (m, 1H), 8.06 (d, J = 8.4 Hz, 2H), 9.60 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 14.97 (SCH_3), 38.01 ($ArCH_2SCH_3$), 60.22 (CH_2SO_2), 120.88 (C-12'), 125.62 (C-10'), 126.55 (C-11'), 127.70 (C-3', C-5'), 128.53 (C-14', C-18'), 129.26 (C-2', C-6'), 129.37 (C-9'), 130.04 (C-8'), 132.46 (C-15', C-17'), 132.53 (C-1'), 134.40 (C-16'), 136.73 (C-7'), 137.59 (C-13'), 142.90 (C-4'), 165.43 (C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{22}H_{21}NO_3S_2$: 434.0861 found: 434.0862, Anal. Calcd for $C_{22}H_{21}NO_3S_2$: C, 64.15, H, 5.10, N, 3.40, found: C, 63.88, H, 4.94, N, 3.42.

(N-(4-Bromophenyl)-2-(4-(methylthiomethyl) benzamido)) benzenesulfonamide (35c): Light yellow solid, yield: 66%, mp 190°C, ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.04 (s, 3H), 3.74 (s, 2H), 6.86-6.88 (m, 2H), 7.14-7.18 (m, 3H), 7.19 (s, 1H) 7.42 (d, J = 8.4 Hz, 2H), 7.56-7.60 (m, 1H), 7.75-7.77 (m, 1H), 7.80 (d, J = 8.4 Hz, 2H), 8.44-8.47 (m, 1H), 9.93 (s, 1H), ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 15.08 (SCH_3), 38.03 ($ArCH_2SCH_3$), 120.64 (C-16'), 123.13 (C-14', C-18'), 124.11 (C-12'), 126.01 (C-10'), 126.55 (C-3', C-5'), 127.56 (C-9'), 129.33 (C-2', C-6'), 132.04 (C-11'), 132.49 (C-1', C-15', C-17'), 134.25 (C-7'), 134.65 (C-13'), 136.36 (C-8'), 143.33 (C-4'), 164.77 C = O), HRMS (ESI) m/z $[M+Na]^+$ calculated for $C_{21}H_{19}N_2O_3S_2Br$: 514.9728 found: 514.9732, Anal. Calcd for $C_{21}H_{19}N_2O_3S_2Br$: C, 51.28, H, 3.86, N, 2.84, found: C, 50.99, H, 3.90, N, 2.62.

Biology

Proteins: Human antithrombin (AT) and human coagulation factors Xa and IIa were purchased from Haematologic Technologies (Essex Junction, VT). Stock solutions of proteins were prepared in Tris-HCl buffer (20 mM), pH 7.4, containing NaCl (100 mM) and $CaCl_2$ (2.5 mM), polyethylene glycol (PEG) 8000 (0.1%) and Tween80 (0.02%). Chromogenic substrate Spectrozyme TH was purchased from American Diagnostica (Greenwich, CT), while substrate S-2222 was purchased from DiaPharma Group, Inc (West Chester, OH). Biological activity started on 16th December, 2013 and accomplished on 3rd January, 2014 at Department of Medicinal Chemistry and

Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, Virginia 23219, USA.

FXa and thrombin inhibition studies: Measurement of direct FXa and thrombin inhibition was done using a chromogenic substrate hydrolysis assay as reported earlier[44] using a microplate reader (Flex Station III, Molecular Devices). Initial screening was done using two concentrations of the compounds (500 and 100 μM) after which IC_{50} values were determined for compounds that led to >50% reproducible inhibition of coagulation enzyme in the initial screening. Relative residual enzyme activity at each concentration of the inhibitor was calculated from the ratio of the enzyme activity in the presence and absence of the inhibitor. For IC_{50} determination, stocks of the potential inhibitors were serially diluted to give 18 different aliquots in the wells with final concentrations ranging from 500-0.000833 μM.

For FXa inhibition studies incubation was done at 37°C and in pH 7.4 buffer containing Tris-HCl (20 mM), $CaCl_2$ (2.5 mM), NaCl (100 mM), polyethylene glycol (PEG) 8000 (0.1%) and Tween80 (0.02%) was used. Buffer solution (180 μL) of pH 7.4 and the test solution (5 μL) (or solvent reference) was added to the wells and FXa (10 μL, 10 nM final concentration) were consecutively added. Incubation was done for 10 min after which, FXa substrate (5 μL, 125 μM) was added rapidly and the Fxa residual activity was measured from the initial rate of increase in absorbance at 405 nm. The ratio of FXa activity in the presence and absence of inhibitor was used to calculate the relative residual FXa activity at each concentration of the inhibitor. To obtain the potency (IC_{50}) and efficacy (ΔY) of inhibition, logistic Eq. 1 was used to fit the dose-dependence of residual protease activity:

$$Y = Y_0 + \frac{Y_M - Y_0}{1 + 10^{(\log[I]_0 - \log IC_{50})(HS)}} \quad (1)$$

where, Y is fractional residual factor Xa activity in the presence of inhibitor to that in its absence, Y_M is the maximum possible value of the fractional residual factor Xa activity and Y_0 is the minimum possible value of the fractional residual factor Xa activity respectively, IC_{50} is the inhibitor's concentration that results in 50% inhibition of the enzyme activity and HS is the Hill slope. Nonlinear curve fitting resulted in Y_M, Y_0, IC_{50} and HS values.

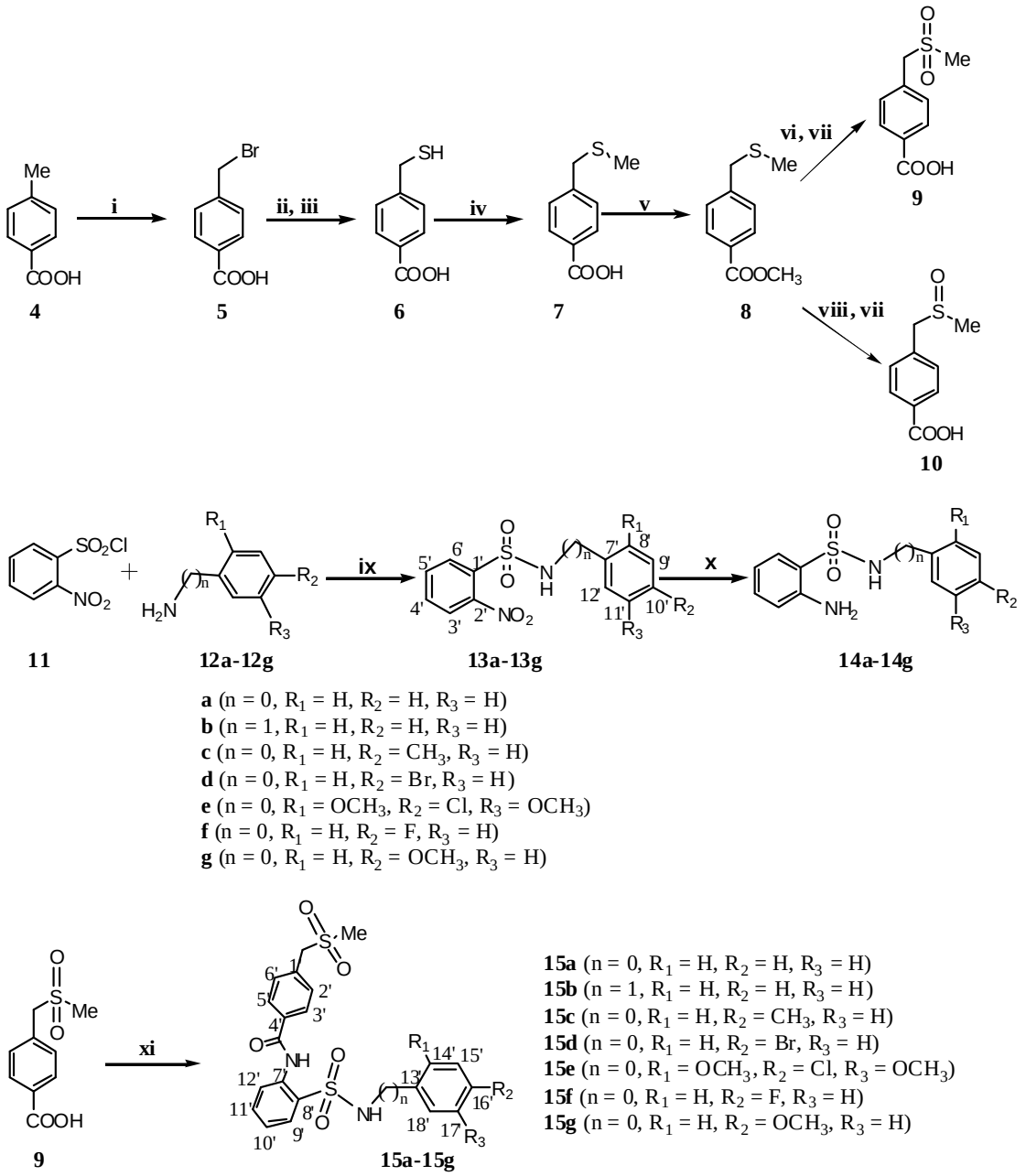
Thrombin inhibition studies were conducted at 25°C and the buffer used was Tris-HCl buffer (20 mM), pH 7.4, containing NaCl (100 mM), $CaCl_2$ (2.5 mM) and polyethylene glycol (PEG) 8000 (0.1%). Generally, buffer solution of pH 7.4 (192 μL) was added to the wells and potential thrombin inhibitor (1 μL) (or DMSO) and thrombin (5 μL, 6 nM final concentration) were sequentially added. After a 10 min incubation, thrombin substrate (5 μL, 125 μM) was added rapidly and the residual thrombin activity was measured from the initial rate of increase in absorbance at 405 nm. Relative residual thrombin activity was calculated as with FXa above.

Prothrombin time (PT) and activated partial thromboplastin time (APTT) in human plasma: Clotting time was measured using a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparles, MD) in a standard one-stage re-calcification assay as described previously[45]. For PT assay, thromboplastin was reconstituted according to the manufacturer's directions and warmed to 37°C. Sample of the compound(10 μL) was added to citrated human plasma (90 μL) to give the desired concentration which was then incubated for 30 sec at 37°C. Pre-warmed thromboplastin (200 μL) was added to it and the time to clot was recorded. In the absence of an inhibitor clotting time was determined using DMSO (10 μL). For the APTT assay, sample (10 μL) was mixed with citrated human plasma (90 μL) and pre-warmed APTT reagent (100 μL) (0.2% ellagic acid). After 4 min of incubation, clotting was initiated by addition of $CaCl_2$ (25 mM,100 μL) (37°C) and the time to clot was noted. Each clotting assay was performed in triplicate.

Docking studies: Docking studies were performed using Glide tool of Schrodinger[46]. It executes grid-based ligand docking and looks for favorable interactions between the ligand and the receptor. The 3D structures of the test compounds were built within Maestro using the Build module and a single low energy conformation search was carried out for all the molecules using OPLS_2005 force field at physiological pH condition using LigPrep module of Schrodinger[46]. The 3D crystallographic structure for factor Xa (FXa) was obtained from RCSB Protein Data Bank (PDB Code: 4A7I). Docking calculations for energy optimized 3D ligands were performed in extra precision (XP) mode within the active site of the enzyme structure.

RESULTS AND DISCUSSION

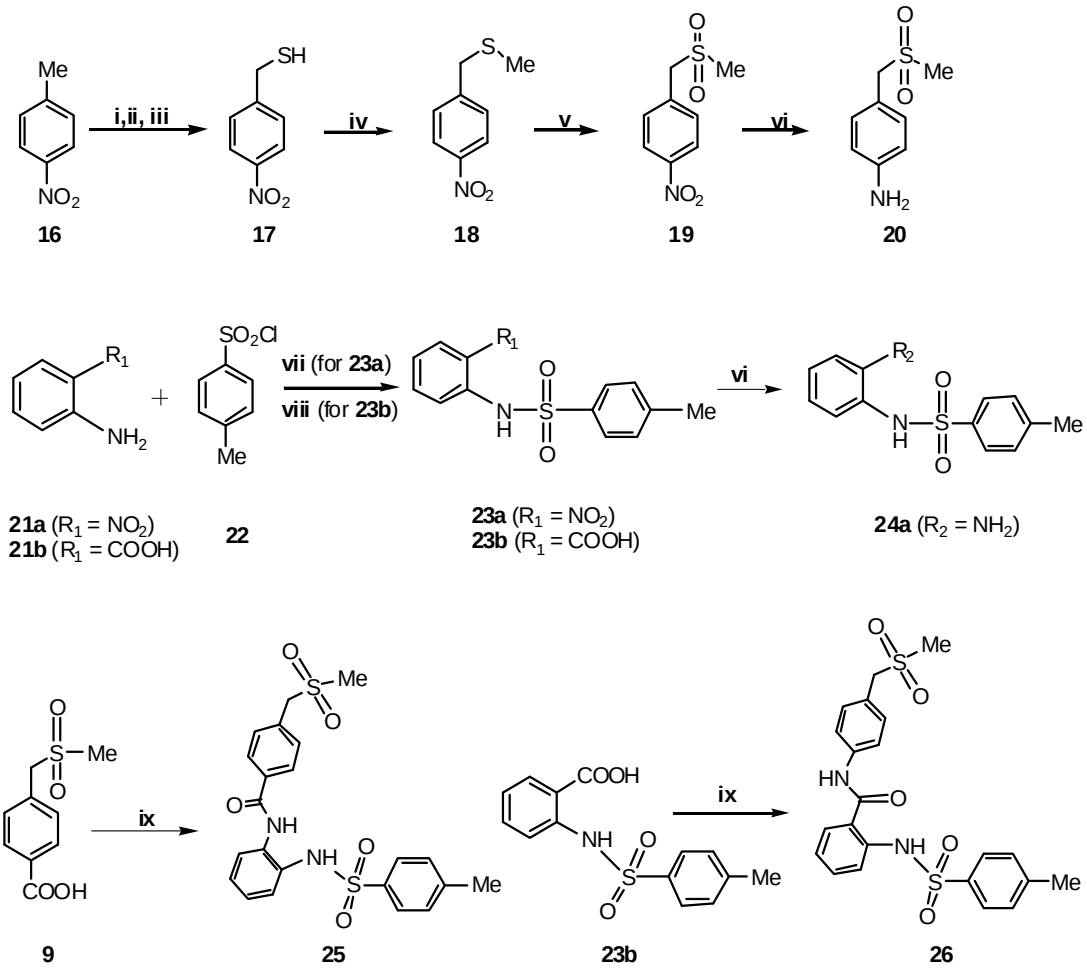
Chemistry: The synthesis of benzamido benzenesulfonamide derivatives was described in Scheme 1. The starting material used for the preparation of sulfone, sulfoxide and sulfide fragments was p-toluic acid 4. Benzylic bromination of 4 with catalytic amounts of benzoyl peroxide and NBS gave 5 which on treatment with thiourea followed by hydrolysis with



Reagents and conditions: (i) NBS, cat $(PhCO_2)_2$, CCl_4, reflux, 6h; (ii) thiourea, H_2O, reflux, 3h; (iii) 10% aq. NaOH, reflux, 2h; (iv) NaH, CH_3I, THF, 0 ^oC, or CH_3ONa, CH_3I, MeOH, 0 ^oC; (v) cat H_2SO_4, CH_3OH, 65 ^oC; (vi) H_2O_2, AcOH, 100 ^oC; (vii) NaOH, MeOH, H_2O, 25^oC; (viii) $[C_{16}H_{33}N^+(CH_3)_3]IO_4^-$, H_2O-MeOH, 25 ^oC; (ix) TEA, CH_2Cl_2, 0-25 ^oC; (x) $SnCl_2.2H_2O$, HCl, EtOH, 78 ^oC; (xi) oxalyl chloride, cat DMF, CH_2Cl_2, 0-25 ^oC, then **14a-14g** TEA, CH_2Cl_2, 0-25^oC.

Scheme 1: Synthesis of benzamido benzenesulfonamide derivatives

sodium hydroxide gave the corresponding thiol derivative[47] 6. The thiol group was then methylated using methyl iodide and a strong base like sodium hydride or sodium methoxide. Acid 7 thus obtained was converted into its methyl ester 8 which upon oxidation using hydrogen peroxide[36] and cetyltrimethyl ammonium periodate[48] gave corresponding sulfone9 and sulfoxide10 respectively subsequent to alkaline hydrolysis. Condensation of 2-nitrobenzenesulfonyl chloride 11 with amine derivatives 12a-12g afforded the intermediates 13a-13g[49-51], which on reduction using stannous chloride[52,53]



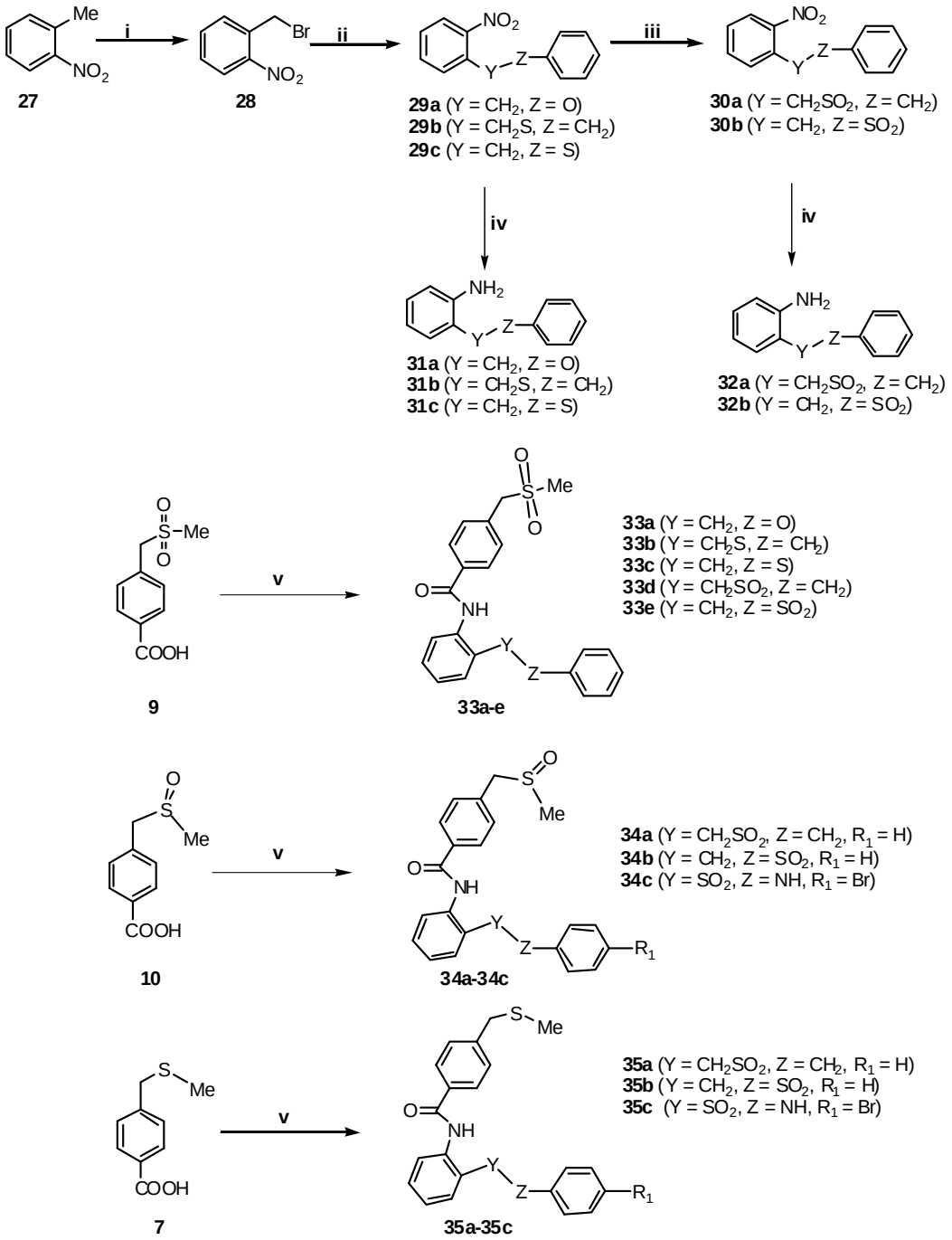
Reagents and conditions: (i) NBS, cat $(PhCO_2)_2$, CCl_4, reflux, 6h; (ii) CH_3COSK, acetone, rt; (iii) aq. H_2SO_4, CH_3OH, reflux; (iv) CH_3ONa, CH_3I, MeOH, 0 ^oC; (v) H_2O_2, AcOH, 100 ^oC ; (vi) $SnCl_2.2H_2O$, HCl, EtOH, 78 ^oC; (vii) pyridine, 120 ^oC; (viii) H_2O, 80 ^oC; (ix) oxalyl chloride, cat DMF, CH_2Cl_2, 0-25 ^oC, then (**24a** for **25**) and (**20** for **26**) TEA, CH_2Cl_2, 0-25 ^oC.

Scheme 2: Synthesis of benzamido benzenesulfonamide with reversal of linking of amide and sulfonamide groups

resulted into aniline derivatives 14a-14g. Acylation of 14a-14g using acid chloride obtained from 9 using oxalyl chloride yielded compounds 15a-15g. All these compounds were characterized using their spectral data. In ^{13}C NMR spectra of 15a-15g,the peak for methyl carbon attached to the sulfone group gets merged in the solvent residual peak of DMSO-d_6 between δ 39.0-41.0, which was confirmed by HSQC spectrum of 15a and DEPT-135 of 15c.

Scheme 2 illustrated the reversal of orientation of sulfonamide linker in 25 and reversal of both amide and sulfonamide linkers in 26. Preparation of 25 was achieved by acylation of 24a with acid chloride obtained from 9 as discussed above. Compound 24a was prepared by condensation of o-nitroaniline 21a with p-toluenesulfonyl chloride 22 followed by reduction using stannous chloride. Acylation of 20 with acid chloride of 23b obtained using oxalyl chloride resulted into compound 26. Compound 20 was prepared from p-nitrotoluene16 by treating it with NBS to obtain brominated derivative which was further converted into the thiol 17 using potassium thioacetate[37,38] followed by acidic hydrolysis. Methylation, oxidation and reduction of 17 were accomplished as discussed in Scheme 1. Treatment of anthranilic acid 21b with 22 resulted into sulfonamide 23b.

The methods used for the preparation of alternative linkers to sulfonamide for connecting the central 'C' ring to S1 binding elements 'B' are illustrated in Scheme 3. The starting material used was o-nitrotoluene 27 which was converted into bromo derivative28 by a similar procedure as



Reagents and conditions: (i) NBS, cat $(PhCO_2)_2$, CCl_4, reflux, 6h; (ii) Ph-OH (for **29a**), $PhCH_2$-SH (for **29b**), Ph-SH (for **29c**) CH_3ONa, MeOH, 0 ^oC; (iii) **29b** (for **30a**), **29c** (for **30b**) H_2O_2, AcOH, 100 ^oC (iv) Fe, HCl, EtOH, 78 ^oC; (v) oxalyl chloride, cat DMF, CH_2Cl_2, 0-25 ^oC, then **31a**-c (for **33a**-c), **32a,b** (for **33d,e**, **34a,b** and **35a,b**), and **14e** (for **34c** and **35c**), pyridine, CH_2Cl_2, 0-25 ^oC.

Scheme 3: Synthesis of alternative linkers to sulfonamide for connecting the central 'C' ring to S1 binding elements 'B'

discussed above. Preparation of compounds containing ether 29a or thioether 29b, 29 clinkers, was achieved using Williamson's synthesis[54,55] by reacting 28 with appropriate phenol, thiophenol or mercaptan. Reduction of the nitro group using Fe/HCl resulted in intermediates 31a-31c, while compounds having thioether linkages were oxidized using

H_2O_2 followed by reduction of the nitro group to furnish other intermediates 32aand 32b. Condensation of the acid chloride of 9 with appropriate intermediate 31a-31c and 32a, 32b afforded 33a-33e. Compounds 34a-34c and 35a-35c having sulfide and sulfoxide groups attached toring A have been synthesized by converting the acids 7 and 10 to corresponding acid chlorides and reacting them with appropriate aniline intermediates 15e and 32a-32b. The overall yield of the most active compound 35c was found to be 42.8%.

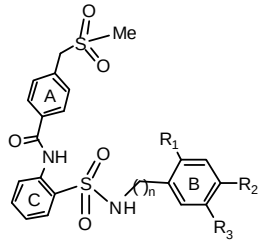
Biological activity and SAR: The synthesized compounds were initially screened using two concentrations (500 and 100 μM). IC_{50} values were determined for only those compounds that led to approximately 50% inhibition of the enzyme at 100 μM.

The human factor Xa inhibition activity of 15a-15g having sulfone group as the P4 group and sulfonamide linker connecting P1 group were studied (Table 1). It was observed that 15a having no substituents in ring B displays 37% inhibition of FXa activity at 500 μM while insertion of methylene group between the phenyl ring and the sulfonamide group in 15b, led to decrease in FXa inhibition activity (9% inhibition). Methyl and bromo substituents at 4th position of ring B in 15c and 15d showed 23 and 38% inhibition of FXa activity. Introduction of a methoxy group at 2 and 5 positions and achloro group at 4th position in 15e showed no inhibition. Replacement of ring B with 4-fluorophenyl and 4-methoxyphenyl afforded inactive compounds (15f and 15g).

Effect of interchanging of linking of atoms of ring A and ring B on FXa inhibitor activity was also studied in two compounds (25, 26) as shown in Table 2. Inversion of ring B linker as in 25 resulted in further diminished potency (18% inhibition) while the activity was totally lost when linker atoms of both the groups (i.e., -SO_2NH- and -NHCO-) were interchanged in compound 26.

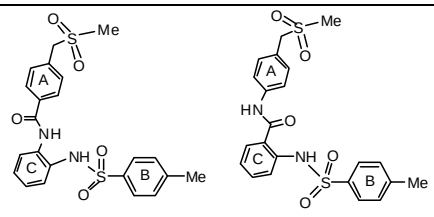
In order to determine the structural requirement for FXa inhibitory activity, alternative linkers to sulfonamide were also explored. As the data in Table 3 indicated, compound 33 a

Table 1: Effect of anilino ring substituents on FXa inhibition



Compounds	R_1	R_2	R_3	n	Inhibition of Fxa (%) (500 μM)
15a	H	H	H	0	37
15b	H	H	H	1	9
15c	H	CH_3	H	0	23
15d	H	Br	H	0	38
15e	OCH_3	Cl	OCH_3	0	No inhibition
15f	H	F	H	0	No inhibition
15g	H	OCH_3	H	0	No inhibition

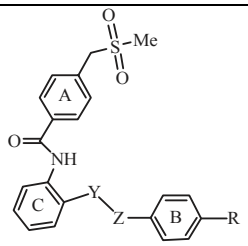
Table 2: Effect of inversion of linker atoms of sulfonamide and carboxamide groups on FXa inhibitory activity



25 **26**

Compounds	Inhibition of Fxa (%) (500 μM)
25	18
26	No inhibition

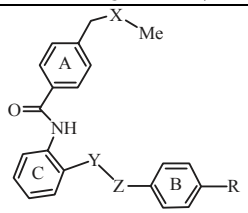
Table 3: Alternative sulfonamide linkers and their effect on activity



Compounds	Y	Z	R	Inhibition of Fxa (%) (500 µM)
33a	CH_2	O	H	84[a]
33b	CH_2S	CH_2	H	No inhibition
33c	CH_2	S	H	No inhibition
33d	CH_2SO_2	CH_2	H	No inhibition
33e	CH_2	SO_2	H	41

[a]No inhibition at 100 µM

Table 4: Sulfides and sulfoxides as P4 binding elements and their effect on biological activity



					Inhibition of FXa inhibitory activity (%)		
Compounds	X	Y	Z	R	500 µM	100 µM	Thrombin inhibition (%) (100 µM)
34a	SO	CH_2SO_2	CH_2	H	28	nd[a]	nd[a]
34b	SO	CH_2	SO_2	H	47	nd[a]	nd[a]
34c	SO	SO_2	NH	Br	72	66	21
35a	S	CH_2SO_2	CH_2	H	100	26	3
35b	S	CH_2	SO_2	H	50	25	nd[a]
35c	S	SO_2	NH	Br	100	45	No inhibition

[a]Not determined

containing an ether linker displayed 84% inhibition of FXa at 500 µM but showed no inhibition at 100 µM concentration, while thioether linkers resulted into inactive compounds (33b and 33c). Compound 33d having methyl sulfonylmethyl linker showed no inhibition while reducing the chain length to methylsulfonyl in compound 33e led to 41% inhibition of the enzyme activity at 500 µM.

In the next step it was decided to replace sulfone group at P4 ligand (4th position of ring A) by a sulfoxide or sulfide group in the designed molecules to observe their effect on FXa inhibiting activity (Table 4). Replacement of sulfone group in 15d by sulfoxide and sulfide resulted in 34 and 35c, respectively with improved FXa inhibition activity of 72 and 100% inhibition at 500 µM concentration while offering 66 and 45% inhibition at 100 µM. Similarly, 34a having methylsulfonylmethyl as the linker for P1 ligand (ring B) with the sulfoxide group at P4 ligand showed somewhat higher inhibition as compared to 33d having sulfone group while 35a with a sulfide group at this position exhibited a sudden augmentation in activity to 100% inhibition at 500 µM and 26% at 100 µM. Compounds 34b and 35b having methylsulfonyl linker with sulfoxide and sulfide groups at P4 ligand (4th position of ring A)showed comparable inhibition of FXa to that of 33e indicating that the presence of sulfonamide groups as a linker forP1 ligand (ring B) and sulfide or sulfoxide groups atP4 ligand (4th position in ring A)are more favorable for this activity as compared to the sulfone at this position.

Table 5: FXa inhibition features for compounds (34c and 35c)

Compounds	IC_{50} (µM)	HS	Y
34c	29.2±2.3	1.3±0.2	70.9±4.6
35c	16.1±1.4	1.1±0.2	75.3±4.9

IC_{50} values for two most potent compounds (34 and 35c) were determined (Table 5) as discussed in experimental section 6.2.2. Chromogenic substrate hydrolysis assay was used to measure direct inhibition of FXa as reported

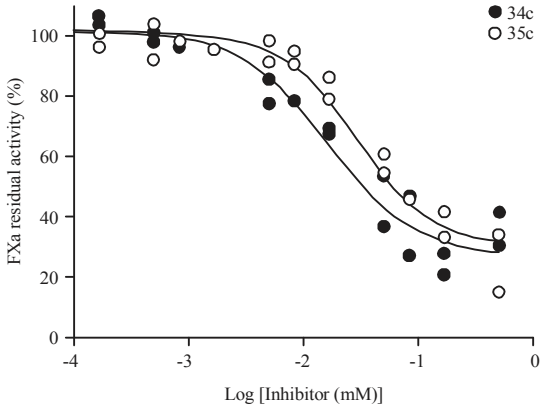


Fig. 3: Direct inhibition of FXa by compounds (34c and 35c). Solid lines represent sigmoidal dose-response fits to the data to obtain the values of IC_{50}, Y_M, Y_0 and HS as described in the experimental section

Table 6: Human Plasma clotting assays

Compounds[a]	Clotting time in APTT (sec)	Clotting time in PT (sec)
DMSO	35.9±1.3	20.4±0.9
34c	48.7±0.8	37.9±1.9
35c	41.9±10.7	24.4 ±1.2

[a]2000 μM concentration

previously[56]. This assay results in a linear augmentation in absorbance at 405 nm due to substrate hydrolysis caused by FXa. The slope is attribute of the residual enzyme activity. The change in Fxa residual activity as a function of the concentration of the potential inhibitors is plotted on a logarithmic scale (Fig. 3) and fitted by the logistic dose-response relationship (Eq. 1) to determine the potency (IC_{50}), efficacy ($\Delta Y = Y_M - Y_0$) and Hill Slope (HS) of inhibition[57].

Selectivity is an important issue for potential FXa inhibitors. In order to access selectivity against thrombin, some potent FXa inhibitors (34c, 35a and 35c) were evaluated for their inhibitory activity against thrombin. All these compounds showed either very poor or no inhibition at 100 μM concentration (Table 4). Compounds (34c and 35c) selected on the basis of FXa inhibition data were also tested for anticoagulant activity using prothrombin and activated partial thromboplastin time (PT and APTT) assay. These compounds failed to double the PT and APTT time, even at a concentration as high as 2000 μM (Table 6). This may be attributed to their poor solubility and high lipophilicity resulting in high plasma protein binding. Similar results have been reported previously by many research groups where the PT and APTT assays did not correlate with the activity against FXa or thrombin[25,57,58].

On the basis of the results obtained from biological evaluation of the synthesized compounds it can be inferred that sulfonyl group as the P4 binding ligand is not favourable for FXa inhibition while the thioether group offered the most active compounds followed by the sulfoxide moiety. Groups linking the P1 binding ligands to the C-ring of the carboxamide scaffold at the ortho position do not make an appreciable impact on FXa inhibitory activity of the resulting compounds however, the sulfonamide group has offered the most potent compounds (34c and 35c).

Docking studies: Factor Xa (FXa) has a well-recognized active site comprising of mainly four regions. These are S1, S2, S4 and an ester binding pocket (EBP) in the active binding site. The S1 and S4 sites are more important for ligand binding while the S2 is a small sack separated from S4 by Tyr[59] 99. Docking studies of the most potent compound 35c was performed using Glide[46] with extra precision (XP) mode. Before docking of 35c, the generated grid on FXa receptor (PDB Code: 4A7I)[59] was validated by re-docking the co-crystallized ligand. Very similar interactions between ligand and the receptor were observed after re-docking the co-crystallized ligand. The RMSD value of 0.36 Å was observed between the re-docked and the original coordinates of the ligand.

Docking interactions between the compound 35c and the active site of Fxa are reproduced in Fig. 4. The bromophenyl group shows good lipophilic interactions within the S1 sub-pocket. The non-covalent interactions between Br located at 3.7 Å from the centroid of Tyr228 aromatic ring and π electron system of Tyr228 of S1 site appear to further stabilize the ligand-receptor complex. NH of SO_2NH group imparts stability to the ligand-receptor complex by forming hydrogen bond with C = O of Gly219 (1.74 Å). The SO_2 group of the ligand also interacts with the enzyme by means of two hydrogen bonds, one with NH of Gln192 (1.83 Å) and the other with NH of Gly216 (1.99 Å). The amide NH of the ligand on the other hand provides stability to the complex by forming hydrogen bond with C=O of Gly216 (1.98 Å) of the enzyme. The benzyl methyl sulfide group in ring A of the ligand fits into the S4 sub-pocket of the active site with centroid to centroid distance of ~4.7 Å, ~5 Å and ~5.9 Å between the aromatic part of the ligand and Tyr 99, Phe 174 and Trp 215, respectively yielding into strong van der Waals interaction among these moieties.

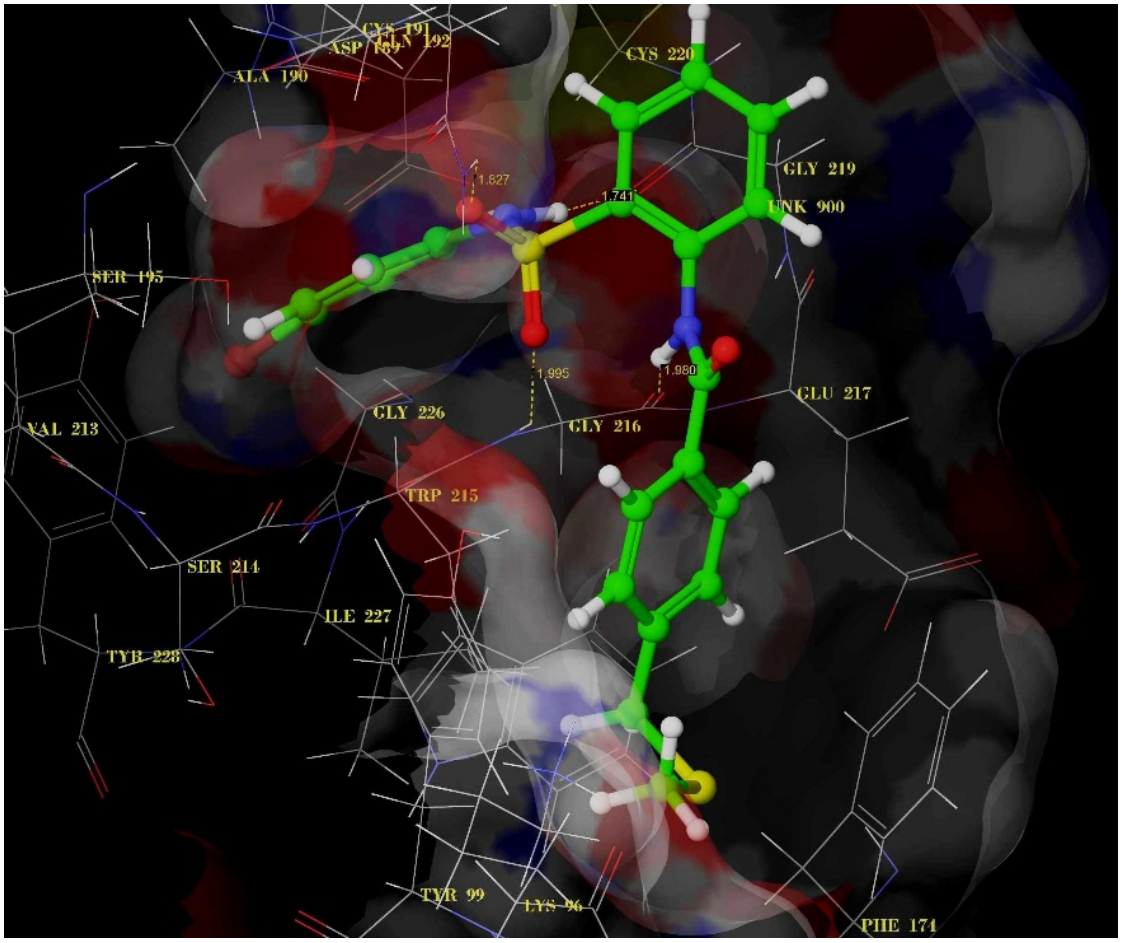


Fig. 4: Compound (35c) docked into the active site of FXa

CONCLUSION

The most potent compound (35c) showed an IC_{50} value of 16.1 μM with 75.3% efficacy. This compound did not cause any inhibition of thrombin at a concentration of 100 μM proving its high selectivity for FXa over thrombin. Unlike the existing FXa inhibiting drugs having basic P1 binding ligands, the most active compounds have neutral (i.e., sulfide, sulfone or sulfoxide) groups at this position. The neutral or acidic character should prove to be an advantage as such compounds are liable to be orally bioavailable unlike the basic amidine derivatives. The two most potent compounds (34c and 35c) reported herein are important lead molecules for further structural optimization as potential antithrombotic drugs acting through the inhibition of FXa, a vital enzyme in the clotting cascade.

SIGNIFICANCE STATEMENT

This study discovers the potential of introducing neutral groups like sulfone, sulfoxide and sulfide as the P4 ligands in the newly designed molecules unlike basic groups at this position which hamper the oral bioavailability of a large number of existing drugs/test compounds. Neutral groups as P4 and neutral/slightly acidic groups as P1 ligands should be beneficial in increasing the oral bioavailability of the synthesized compounds, which is a major challenge in designing potent FXa inhibitors as anti-thrombotic drugs. This study will help researchers working in this area to uncover the potential of neutral groups like sulfones, sulfides or sulfoxides as P4/P1 ligands in the 'V' shaped benzamidobenzene derivatives as FXa inhibitors. This study has paved the path for designing novel FXa inhibitors containing sulfur functionalities in the molecule which could further be explored by

researchers working in this area. This work may lead to redefining the structural requirements for potential orally bioavailable FXa inhibitors.

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