Summary
Of
The Thesis Entitle

SYNTHESIS, CHARACTERIZATION AND STUDY OF ANTICOAGULANT PROPERTIES OF SOME POTENTIAL COMPOUNDS

Submitted to

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

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Summary

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Anticoagulants or antithrombotic drugs are one of the most important classes of drugs which can be shortly defined as "all possible compounds that do not allow blood to clot". Anticoagulants prevent fibrin (clot) formation by targeting coagulation cascade and have established role as antithrombotic agents. Clot formation is a coordinated interplay of two fundamental processes, aggregation of platelets and formation of fibrin. Platelet aggregation involves association of platelets through physical forces following their activation, whereas fibrin formation involves the chemical synthesis of fibrin polypeptide through the action of several enzymes and cofactor. Thus, there are three classes of antithrombotic agents—anti platelets, anticoagulants and thrombolytic drugs. Antiplatelet molecules block the physical process (traditionally referred as the cellular process), whereas anticoagulants inhibit the chemical process (also referred as the humoral process).[1]

Blood flow is a complex and highly regulated physiological process, with multiple complementary and opposing mechanisms of control. Normally in the vasculature, a precise balance is achieved, permitting free flow while also allowing the trigger of nearly instantaneous clot formation at sites of vascular injury to prevent hemorrhage. Indeed, blood coagulation evolved in humans and animals as a protective mechanism against bleeding. As protective blood clots (hemostatic plugs) form and grow after injury, mechanisms exist to maintain or dissolve them as needed while allowing normal flow in the remainder of the vasculature. Imbalances in the complex regulatory network of coagulation and anticoagulation, however, can lead to a variety of pathological consequences, such as hemorrhage or obstructive clot (thrombus) formation in veins or arteries, leading to stroke, pulmonary embolism, heart attack, and other serious conditions.[2] Therefore anticoagulants are extremely useful lifesaving medicaments.

In the body clotting follows a specific mechanism through various pathways namely a) intrinsic pathway b) extrinsic pathway and c) common pathway[3] as shown below (**Figure 1**). Factor Xa is one of the enzyme responsible for blood clotting, present at the convergent of two pathways. Thus inhibition of factor Xa may results into better anticoagulant therapy as compared to other enzymes.

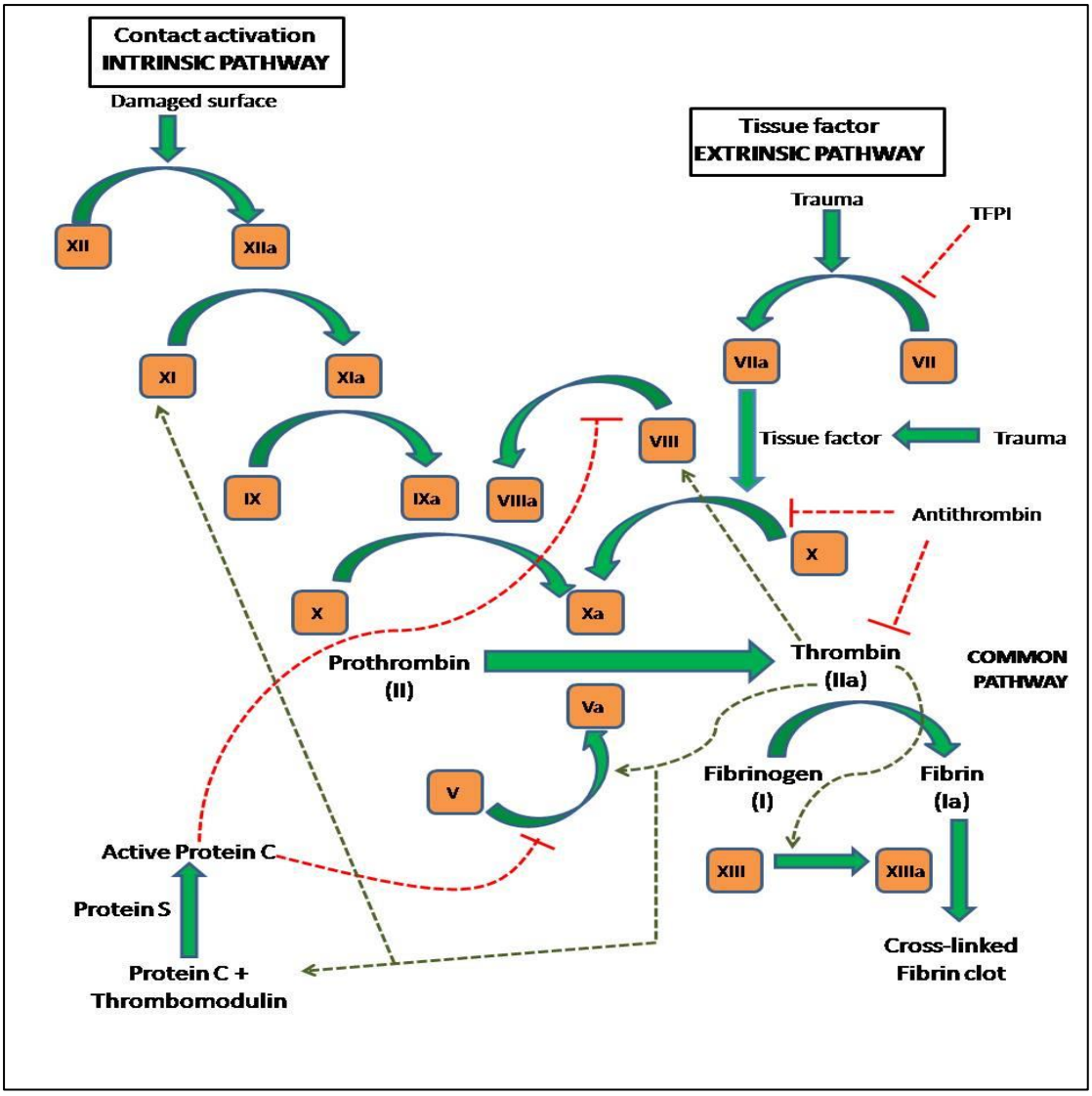


Figure 1 Blood coagulation Cascade Model

Factor Xa was first identified in 1956 by Telfer and colleagues. The role of fXa was recognized in the conversion of prothrombin to thrombin almost after seven years and it was assigned a unique position in the coagulation cascade at the point of convergence of the intrinsic and extrinsic coagulation pathways.[4] Traditional anticoagulants, including vitamin K antagonists (VKAs) (e.g warfarin), unfractionated heparin (UFH) and low-molecular-weight heparins (LMWHS, fractionated heparin with reduced activity towards thrombin compared to UFH) are the gold standard therapeutic agents for the prevention and treatment arterial and venous thromboembolic diseases. However, these anticoagulants have well known limitations. Warfarin carries the risk of serious bleeding, requires continuous monitoring for accurate dosing and its activity is also affected by food and drug

interactions. It has a narrow therapeutic window and unpredictable pharmacokinetics, with marked inter and intra- individual variability. Other anti coagulating agents like UFH, LMWHs, the direct thrombin inhibitors (DTIs) argatroban, bivalirudin and hirudin require parental administration, which makes their use outside hospital cumbersome and which can also be associated with injection site hematomas.[5] Among the novel anticoagulant drugs, orally administered direct thrombin inhibitors and factor Xa inhibitors display better efficiency and improved safety profile. Ximelagartran (exanta, Astra Zeneca) was the first approved oral direct thrombin inhibitors (DTI), was unfortunately withdrawn from the market owing to its hepatotoxicity. All of these factors have been the driving force for the development of new anticoagulants, including the direct factor Xa inhibitors. Inhibition of fXa should prevent production of new thrombin without affecting its basal level, which should ensure primary hemostasis. Hence, fXa inhibitors are predicted to have lower risk of bleeding than heparins and warfarin, and even higher therapeutic ratio than direct thrombin inhibitors (DTIs) e.g Rivaroxaban (**1**), Darexaban (**2**), Edoxaban (**3**), Betrixaban (**4**), Apixaban (**5**) etc as shown in **Figure 2**.[6]

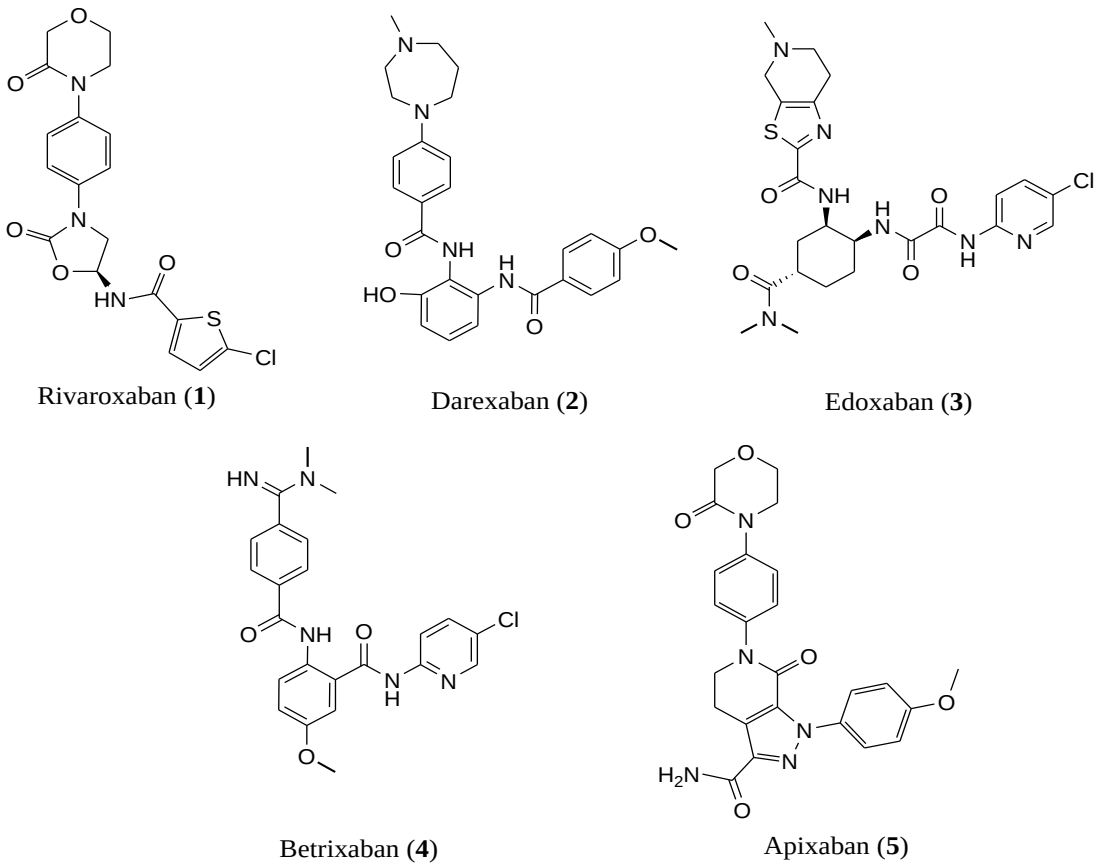


Figure 2 Factor Xa inhibitors

The proposed work has been divided into five main sections depending upon the synthetic, biological applications and development of new reagent.

First chapter of the thesis covers a detailed introduction of anticoagulants including mechanism of blood clotting, historical perspectives and current trends in anticoagulant therapy.

Second chapter describes the design, synthesis and characterization of some novel neutral 2-substituted benzamidobenzene derivatives as factor Xa inhibitors.

We decided to initiate our research in the direction of development of novel factor Xa inhibitors. Extensive literature survey was carried out before designing the molecules which could behave as factor Xa inhibitors. Factor Xa is a trypsin like serine protease which has two essential subsites named as S1 and S4 sub pockets. Molecules which bind with these active sites normally acquire V or L shape conformations, where one group of ligands resides in S1 sub pocket and the other occupies S4 sub pocket.[7] Many small molecules fXa inhibitors having basic substituents (like amidine, guanidine, amine etc) were reported to show good fXa inhibitory activity, but they were often associated with low oral bioavailability, short life and rapid clearance.[8-11] To overcome these difficulties inhibitors bearing neutral substituents were developed like rivaroxaban (**1**), darexaban (**2**) and edoxaban (**3**) (**Figure 2**). Based on the knowledge of receptor and related reports, we undertook the task of systematic development of factor Xa inhibitors. We designed some V shaped 2-substituted benzamidobenzene derivatives having neutral groups like sulfone, sulfide and sulfoxide and varing linkers connecting B and C rings as shown in **Figure 3**. The synthesis and characterization of designed molecules is discussed in **Chapter 2**.

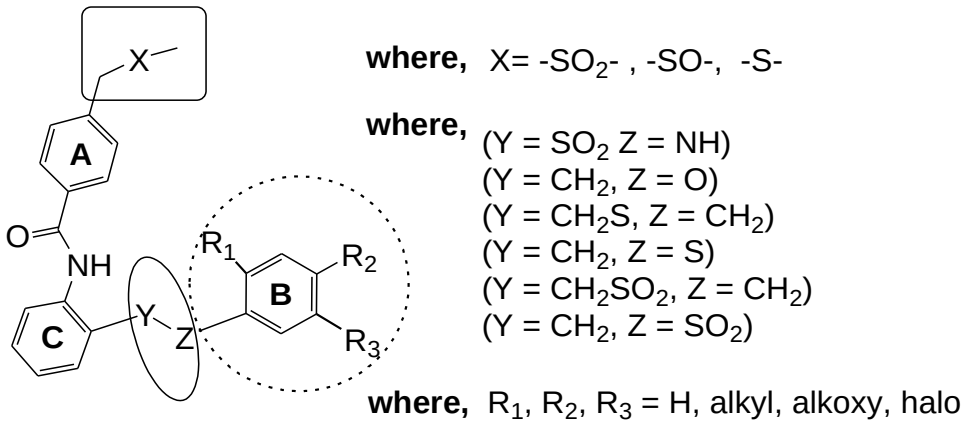
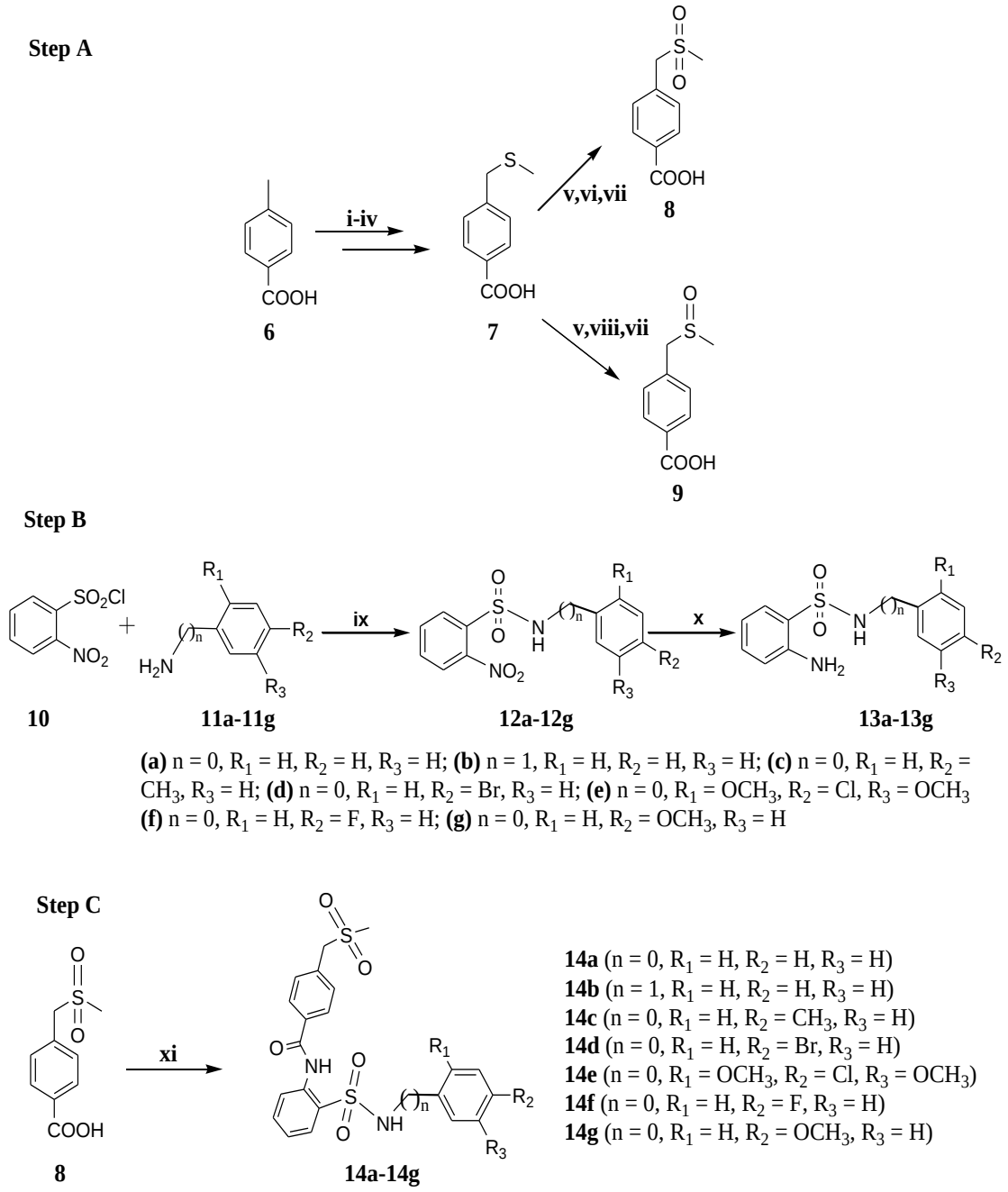


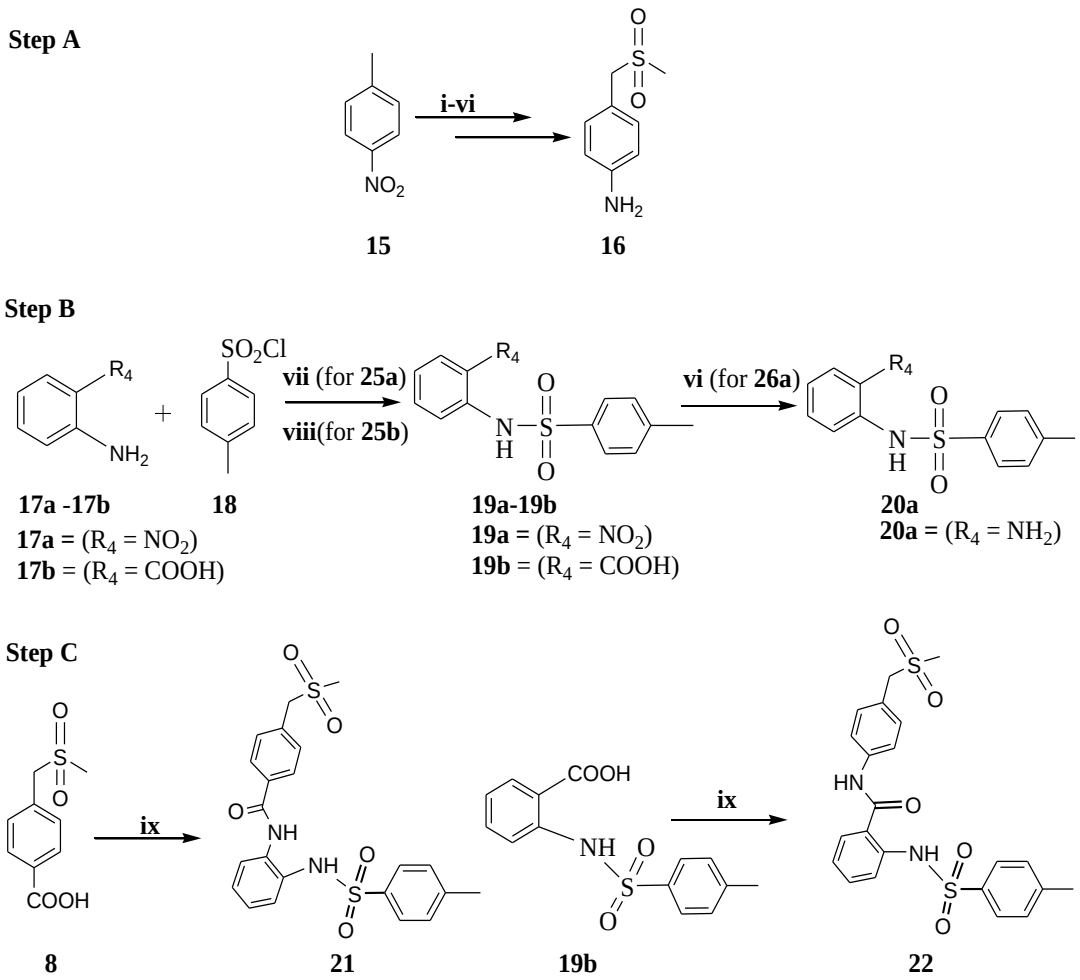
Figure 3 General structure of 2-substituted benzamidobenzene derivatives

Scheme 1, **2** and **3** describe the synthesis of newly designed molecules.



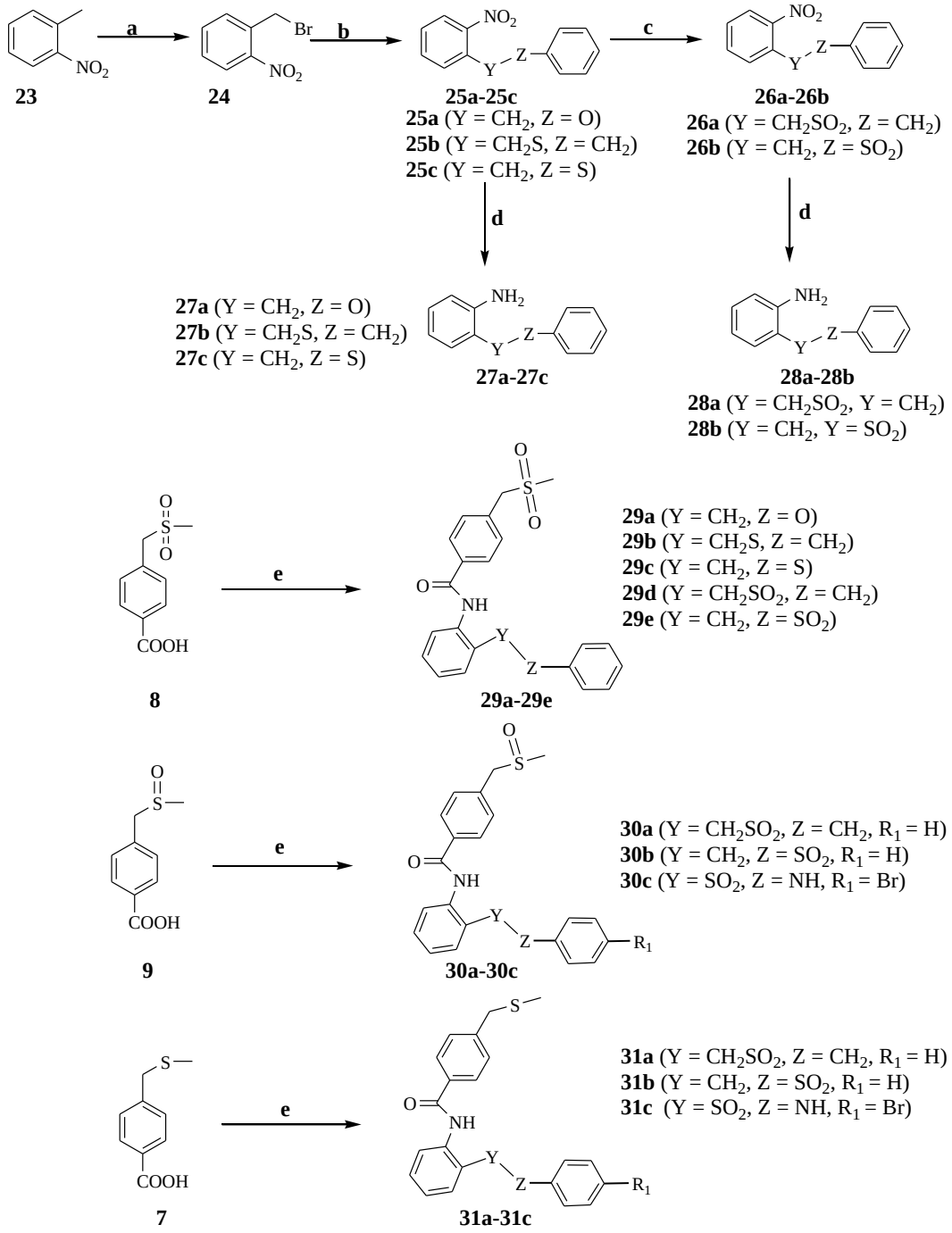
Scheme 1. Reagents and conditions: (i) NBS, cat $(PhCO_2)_2$, CCl_4, reflux, 6h; (ii) thiourea, H_2O, reflux, 3h; (iii) 10% aq. NaOH, reflux, 2h; (iv) NaH, CH_3I, THF, 0 ^oC, or CH_3ONa, CH_3I, MeOH, 0 ^oC; (v) cat H_2SO_4, CH_3OH, 65 ^oC; (vi) H_2O_2, AcOH, 100 ^oC; (vii) NaOH, MeOH, H_2O, 25 ^oC; (viii) $[C_{16}H_{33}N^+(CH_3)]IO_4^-$, 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 **13a-13g** TEA, CH_2Cl_2, 0-25^oC.

Scheme 1 Synthesis of 2-benzamidobenzenesulfonamide derivatives



Scheme 2. 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 (**20a** for **21**) and (**16** for **22**) TEA, CH_2Cl_2, 0-25^oC.

Scheme 2 Synthesis of molecules with inverted amide and sulfonamide linkers



Reagents and conditions: (a) NBS, cat $(PhCO_2)_2$, CCl_4, reflux, 6h; (b) Ph-OH(for **25a**), $PhCH_2$-SH (for **25b**), Ph-SH (for **25c**) CH_3ONa, MeOH, 0 ^oC; (c) **25b (**for **26a**)**, 25c** (for **26b**) H_2O_2, AcOH, 100 ^oC (d) Fe, HCl, EtOH, 78 ^oC; (e) oxalyl chloride, cat DMF, CH_2Cl_2, 0-25 ^oC, then **27a-c (**for **29a-c**)**, 28a-b**(for **29d-e, 30a-b** and **31a-b)**, and **13d (**for **30c** and **31c),** pyridine, CH_2Cl_2, 0-25^oC.

Scheme 3 Synthesis of molecules with alternative linkers to sulfonamide along with sulfoxide and sulfide as S4 ligands

All the compounds synthesized are characterized using ^{1}H NMR, ^{13}C NMR, HRMS and elemental analysis.

Chapter 3 is devoted to molecular docking study and biological activities of 2-substituted benzamidobenzene derivatives (synthesized as shown in chapter 2) as factor Xa inhibitors along with thrombin, activated partial thromboplastin time (APTT) and prothrombin time (PT) of most active molecules

Molecular docking is one of the most commonly used computational tool commonly applied in drug discovery projects and fundamental biological studies for molecular interactions, mainly receptor-ligand interactions. With more complex molecular mechanics program it is possible to superimpose the three dimensional structure of a potential drug like candidate on its possible target site. This process, which is often automated, is known as docking.

This method is widely used in the field of structure-based drug design, in which researchers try to find compounds, which will form a low energy stable intermolecular complex with a target protein. Initial screening of possibly millions of compounds in a laboratory is often too expensive and time-consuming process to be feasible and thus fast molecular docking methods are used to eliminate unlikely candidates.[12-14] The designed molecules in chapter 2 have been examined for their receptor interactions by means of molecular docking study, which showed good interactions between the designed molecules and the receptor active site. Compound (**31c**) docked in active site of fXa is shown in **Figure 4**. Docking studies have been performed using Glide[15] with extra precision (XP) mode. Before docking of the actual synthesized compounds, the generated grid on fXa receptor (PDB Code: 4A7I)[16] was validated by re-docking the co-crystallized ligand. A very similar interaction between ligand and receptor has been observed. The RMSD value of 0.36 Å was observed between re-docked ligand and the original coordinates of the ligand.

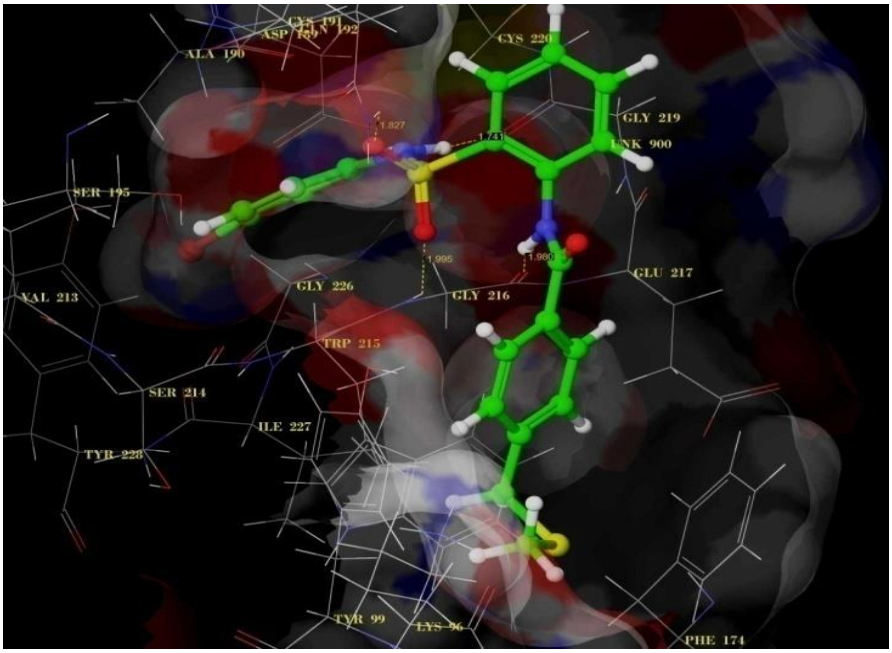


Figure 4 Compound (**31c**) docked into the active site of fXa

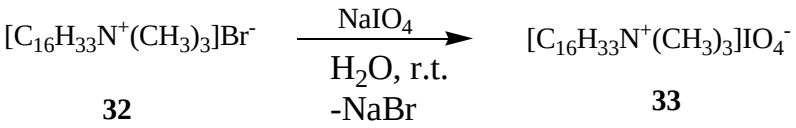
The synthesized molecules have been screened for their biological activity using two concentrations of the compounds (500 µM and 100 µM) after which IC_{50} values have been determined for only those compounds that led to approximately 50 % reproducible inhibition of the coagulation enzyme at 100 µM. Among the twenty compounds tested, three (**29a**, **31a**, **31b**) exhibited reasonably good inhibition of hfXa, while two (**30c** and **31c**) exhibited good hfXa inhibition with IC_{50} values of 29.2 µM and 16.1µM with an efficacy of 70 % and 75 % respectively. Selectivity against thrombin has also been examined for a few representative candidates **30c**, **31a,** and **31c** which showed good fXa inhibitory activity. All the members evaluated for this activity showed either very poor or no inhibition for thrombin at 100 µM concentration. Prothrombin and activated partial thromboplastin time (PT and APTT) assay has also been carried out for the compounds (**30c** and **31c).** Measurements of direct fXa and thrombin inhibition have been done using a chromogenic substrate hydrolysis assay. Prothrombin and activated partial thromboplastin time have been measured in a standard one-stage re-calcification assay with a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparles, MD).

Development of a novel reagent, **Cetyltrimethyl ammonium Periodate (CTAPI), for selective oxidation of sulfides to sulfoxides is subject matter of chapter 4**

In continuation of our research in the development of novel anticoagulants we sought an oxidant which could selectively oxidize sulfide (**7**) to sulfoxide (**9**). A number of oxidizing agents such as hydrogen peroxide (H_2O_2), sodium periodate ($NaIO_4$), *m*-CPBA, ozone, oxygen and *t*-BuOOH have been employed previously however they oxidize sulfides either slowly or over-oxidize them to sulfone.[17-18] Many of the reagents and catalysts employed for the transformation suffer from drawbacks such as over-oxidation, long reaction times, low selectivity and low yields.

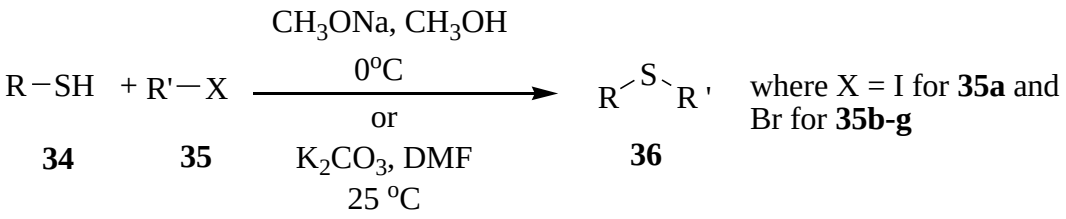
Therefore, in a search of a novel reagent which can overcome the confines of the existing reagents, we found oxidizing agents based on quaternary ammonium salts exploited in many organic reactions.[19] These phase transfer reagents have several characteristics such as solubility in several solvents, operational simplicity, selectivity, high reaction rates, low reaction temperatures and absence of side-reactions. However, some of them require catalysts for activation. For example tetrabutylammonium periodate (TBAPI) oxidizes sulfide to sulfoxide in the presence of lewis acid as catalyst at reflux temperature.[20] Therefore it was thought worthwhile to develop novel reagent cetyltrimethylammonium periodate (CTAPI) (**32**) and explore its application for the oxidation of sulfides which has been hitherto unknown to the best of our knowledge.

CTAPI (**33**) was obtained as a white crystalline solid in excellent yield by mixing aqueous solutions of sodium metaperiodate and cetyltrimethylammonium bromide (CTAB) (**32**) at room temperature (**Scheme 4**).[21]



Scheme 4 Preparation of cetyltrimethylammonium periodate (CTAPI)

In order to explore the application of CTAPI (**33**), various structurally different sulfides have been prepared by reported methods in literature (**Scheme 5**) and some were purchased from Sigma Aldrich.

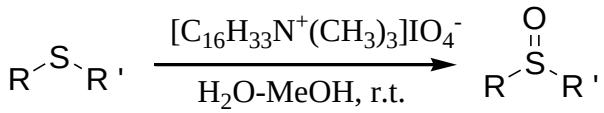


34 **35** **36**

(a) R = p-$CH_3COO(C_6H_4)CH_2$, R’ = Me; (b) R = Ph, R’ = $PhCH_2$; (c) R = $PhCH_2$, R’ = C_2H_5; (d) R = R’ = $PhCH_2$; (e) R = Ph, R’ = n-C_4H_9; (f) R = Ph, R’ = C_2H_5; (g) R = $CH_2COOC_2H_5$, R’ = C_2H_5.

Scheme 5 Preparation of structurally different sulfides

Initially sulfide (**36a**) was subjected to oxidation using varying mole proportions of CTAPI (**33**) under different solvents in order to optimize the reaction condition. The best molar ratio of sulfide and CTAPI was found to be 1.0:3.5 in aqueous methanol as solvent. Then the structurally different sulfides were subjected to oxidation using CTAPI under the optimized reaction condition. (**Scheme 6**) The oxidant worked smoothly in all cases with a variety of structurally different sulfides under the optimized reaction conditions and exhibited very high selectivity for sulfoxides in relatively short reaction time.



36 **37**

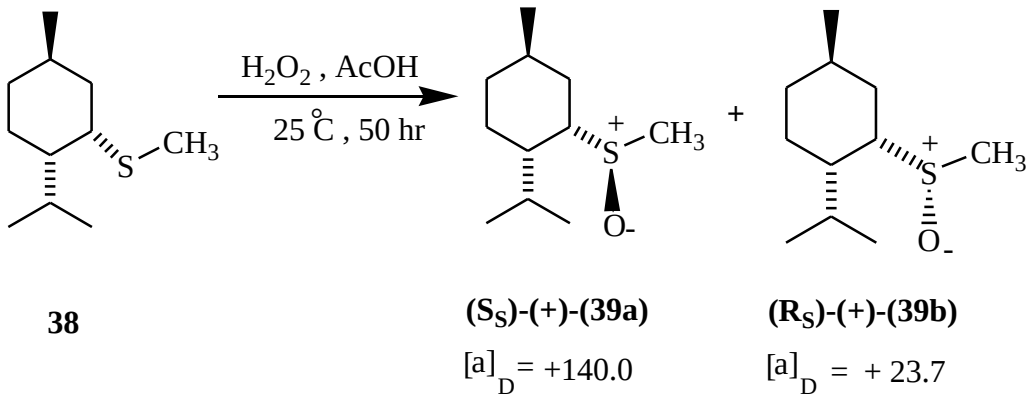
(a) R = p-$CH_3COO(C_6H_4)CH_2$, R’ = Me; (b) R = Ph, R’ = $PhCH_2$; (c) R = $PhCH_2$, R’ = C_2H_5; (d) R = R’ = $PhCH_2$; (e) R = R’ = Ph; (f) R = Ph, R’ = n-C_4H_9; (g) R = Ph, R’ = C_2H_5; (h) R = $CH_2COOC_2H_5$, R’ = C_2H_5; (i) R = R’ = n-Bu.

Scheme 6 Selective oxidation of sulfides to sulfoxides using CTAPI

Chapter 5 explores the application of CTAPI in diastereoselective oxidation of (-) and (+) S-Phenyl-S-Neomenthyl sulfides to corresponding sulfoxides

In the path of literature search for oxidation of sulfides, we came across chiral (+)-S-methyl-S-neomenthyl sulfoxides (**39a** and **39b**) which were used in asymmetric methylene transfer reactions to carry out asymmetric synthesis of oxiranes. These

sulfoxides (**39a** and **39b**) were synthesized from corresponding (+)-S-methyl-S-neomenthyl sulfide (**38)** using hydrogen peroxide in a ratio of 35:65.[22] (**Scheme 7**)



38

(S_S)-(+)-(39a) $[a]_D$ = +140.0

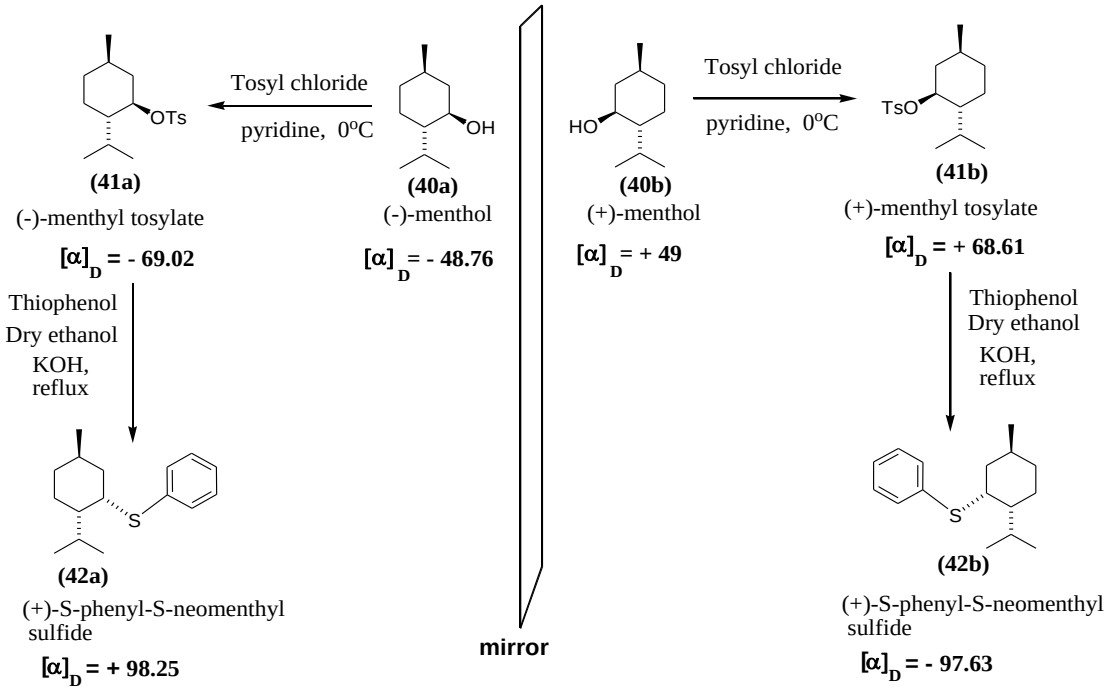
(R_S)-(+)-(39b) $[a]_D$ = + 23.7

Scheme 7 Oxidation of **38** using hydrogen peroxide-acetic acid

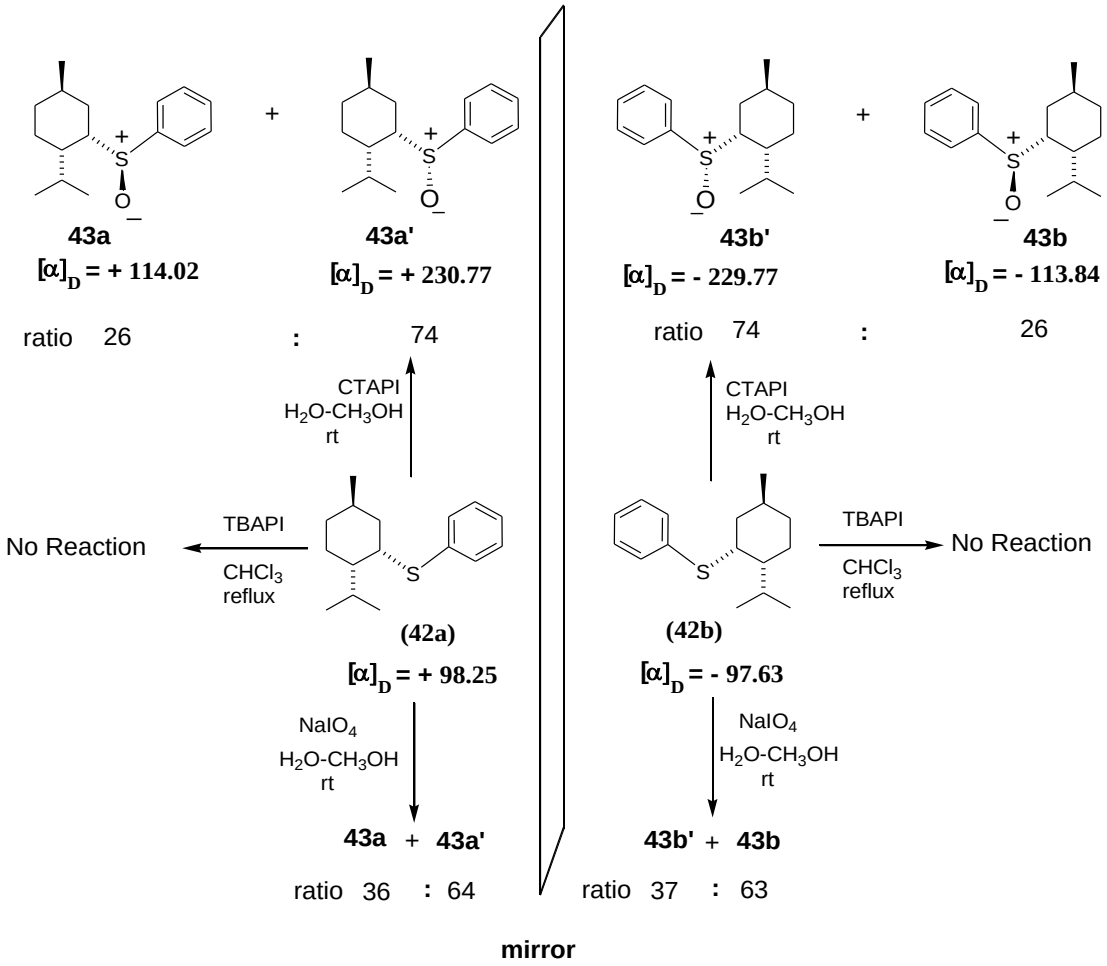
In depth literature search showed that many chiral sulfoxides containing heterocyclic fragment exhibit interesting biological properties and are promising therapeutic agents.[23] Recently Demakova *el al* have also reported the oxidation of hetaryl neomenthyl sulfides using m-CPBA to give two diastereomeric hetaryl neomenthyl sulfoxides in ratio 34:66.[24] Thus, the major stereoisomer, in the cases shown above, is that in which the sulfoxide oxygen atom is oriented away from the isopropyl group.

It was envisioned that the oxidation of neomenthyl sulfides using bulky oxidant like CTAPI (**33**) might further improve the diastereomeric ratio due to the steric effect of equatorial isopropyl group in neomenthyl sulfide with the bulky headed oxidants. The bulky oxidant due to its larger head volume would not be able to approach sulfur atom from the side of the isopropyl group due to steric hindrance making it less favored.

To test this hypothesis we synthesized (-) and (+)-S-phenyl-S-neomenthyl sulfide (**42a** and **42b**) from (-) and (+)–menthols (**40a** and **40b**) as shown in **Scheme 8**. These two enantiomerically pure sulfides were then subjected to oxidation using $NaIO_4$, CTAPI (**33**) (**Scheme 9**). Slight improvement in the diastereomeric ratio was observed in the oxidation using CTAPI (**33**) as compared to that with $NaIO_4$ as was envisioned earlier. It was then thought to employ tetrabutylammonium periodate (TBAPI) as oxidant having bulkier head group, which might give further increase in the diastereomeric ratio as compared to **33**. However, using TBAPI the reaction did not precede at all (**Scheme 9**), perhaps due to its too large head volume completely blocking the sulfur centre for oxidation.



Scheme 8 Preparation of (-) and (+)-S-Phenyl-S-neomenthyl sulfides



Scheme 9 Oxidation of **42a** and **42b** using $NaIO_4$, cetyltrimethylammonium periodate (CTAPI) and tetrabutylammonium periodate (TBAPI)

The ratio of diastereomers has been determined using ^{1}H NMR while all the compounds have been characterized using ^{1}HNMR, ^{13}CNMR, and HRMS analysis. Absolute configuration at sulfur of **43a** has been determined by single crystal X-ray structure analysis and is found to be *Ss*. Therefore the epimeric sulfoxide **43a'** should have *Rs* configuration at sulfur. Similarly **43b** and **43b'** are enantiomers of **43a** and **43a**' thus their relative configuration at sulfur should be *Rs* and *Ss* respectively.[25]

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