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Sulfinamide Crossover Reaction and Its Applications

Sulfinamidová crossover reakce a její využití

Diploma thesis

Supervisor: doc. RNDr. Jiří Míšek, Ph.D.

Prague, 2024

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 12.12.2024

Anna Šimková

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Abstract

This thesis focuses on the application of the sulfinamide crossover reaction as an innovative approach to ligand discovery. Sulfinamides were first synthesized using a modified one-pot reductive method developed in our lab. The next step involved testing the scope of an acid-catalyzed crossover reaction previously described by our lab. The reaction demonstrated remarkable tolerance to structural variations and produced a sulfinamide library, which was subsequently oxidized using mCPBA to a sulfonamide library. The oxidation worked for most sulfinamides, except those with an indole structural motif. The sulfinamides were designed so that the library, after crossover and oxidation, included a factor Xa inhibitor known from the literature. To validate the method's suitability for library screenings, an affinity selection mass spectrometry assay was done with the sulfonamide library and factor Xa as the target. Delightfully, the factor Xa inhibitor was captured in the assay as the strongest binding member of the library. These results highlight the potential of the sulfinamide crossover reaction in ligand development.

Keywords: sulfinamides, sulfinamides crossover reaction, affinity selection mass spectrometry (AS-MS) assay, factor Xa inhibitor

Abstrakt

Tato práce se zaměřuje na aplikaci sulfinamidové crossover reakce jako inovativního přístupu k objevování ligandů. Nejprve byly syntetizovány sulfinamidy pomocí modifikované one-pot redukční metody vyvinuté v naší laboratoři. V dalším kroku byl testován rozsah kysele katalyzované crossover reakce popsané naší laboratoří. Reakce prokázala vysokou toleranci vůči různým funkčním skupinám. Crossover reakcí vznikla knihovna sulfinamidů, která byla následně oxidována na knihovnu sulfonamidů použitím mCPBA. Oxidace byla účinná pro většinu sulfinamidů, s výjimkou těch, které obsahovaly indolový strukturní motiv. Sulfinamidy použité pro crossover reakci byly navrženy tak, aby po provedení reakce a oxidace v knihovně vznikl inhibitor faktoru Xa popsaný v literatuře. Pro ověření vhodnosti metody pro screening knihoven byla využita metoda affinity selection mass spectrometry, která pracovala s knihovnou sulfonamidů a faktorem Xa jako cílovým proteinem. Dle předpokladů, inhibitor faktoru Xa vykazoval nejsilnější vazebnou afinitu k faktoru Xa. Výsledky potvrzují potenciál využití sulfinamidové crossover reakce při objevování ligandů.

Klíčová slova: sulfinamidy, sulfinamidová crossover reakce, affinity selection mass spektrometry (AS-MS) assay, inhibitor faktoru Xa

Table of Contents

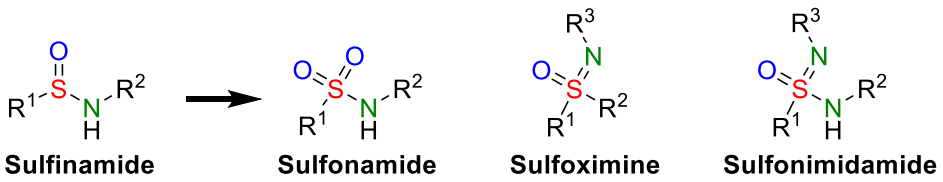
Abbreviations

Ac	acetyl
Ar	aryl
BSA	bovine serum albumin
DABSO	1,4-Diazabicyclo[2.2.2]octane bis(sulfur dioxide) adduct
DCC	*N*,*N*'-Dicyclohexylmethanediimine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
EDC	1-ethyl-3-(3'-(dimethylamino)propyl)carbodiimide
ee	enantiomeric excess
equiv.	equivalent
ESI	electrospray ionization
HTS	high throughput screening
HOBt	1*H*-1,2,3-Benzotriazol-1-ol
HPLC-MS	high performance liquid chromatograph coupled to a mass spectrometer
IR	infrared spectroscopy
mCPBA	*meta*-chloroperoxybenzoic acid
Me	methyl
NCS	*N*-chlorosuccinimide
NMR	nuclear magnetic resonance
r.t.	room temperature
TCCA	trichloroisocyanuric acid
TFA	trifluoroacetic acid
THF	tetrahydrofuran
t_R	retention time

1 Introduction

1.1 Sulfinamides

Sulfinamides are a class of organosulfur compounds characterized by a sulfinyl group attached to an amine. They can be represented by the general formula R−S(O)−NH_2 or its substituted derivatives (R−S(O)−NR'R") where R, R', and R" are organic groups or hydrogen atoms. In sulfinamides, the sulfur atom exists in the +4 oxidation state, which lies between the +2 state in thiols and the fully oxidized (+6) state in sulfonamides. The nitrogen atom in these molecules can act as a nucleophile, while the S=O group imparts electrophilic character to the molecule. Sulfinamides exhibit chirality due to the stereocenter at sulfur, which is useful in asymmetric synthesis and as chiral auxiliaries.[1–3] Sulfinamides are also very useful intermediates in preparing sulfonamides, sulfoximines, and sulfonimidamides, which are gaining popularity in medicinal chemistry (Scheme 1) .[4–8]

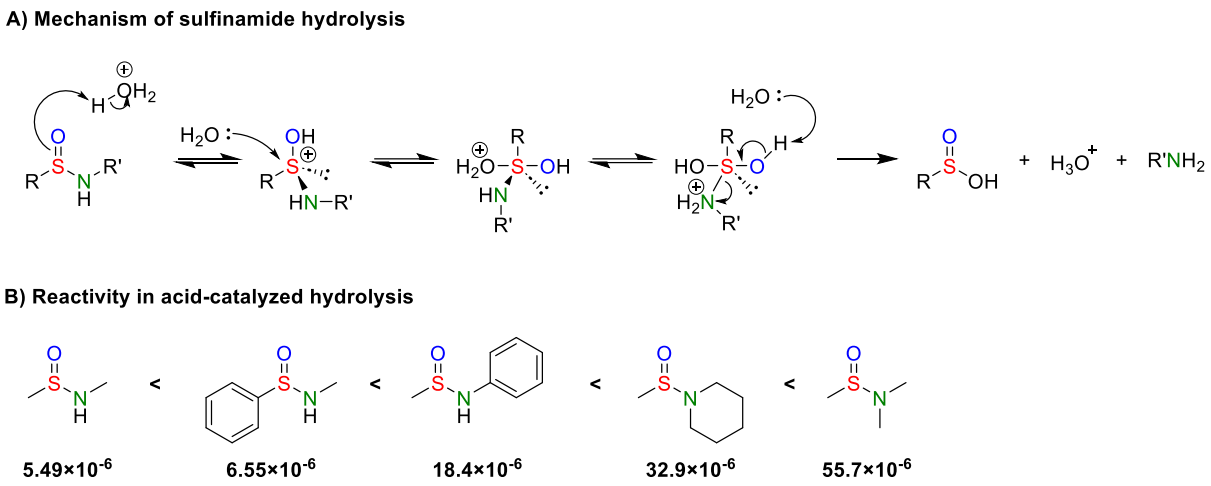


Scheme 1. Structure of sulfinamide and its derivatives.

1.1.1 Reactivity of Sulfinamides

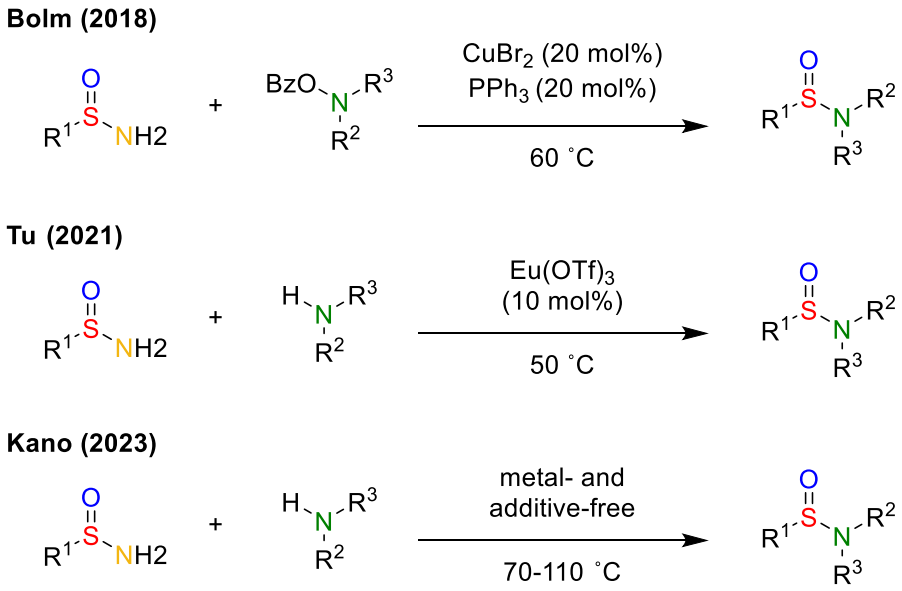
The reactivity of sulfinamides arises from their structure. The sulfur atom is double bonded to an oxygen atom, which withdraws electron density and gives sulfur an electrophilic character. This makes sulfinamides highly reactive toward nucleophiles. Sulfinamides can undergo acidic hydrolysis starting with protonation of the sulfinamide oxygen. Then, water, as a nucleophile, attacks the sulfur atom, leading to substitution and releasing the corresponding amine and sulfinic acid (Scheme 2). It has been observed that hydrolysis occurs faster with tertiary sulfinamides than with the secondary sulfinamides. Interestingly, tertiary sulfinamides derived from cyclic amines react a bit slower than acyclic amines. When an aromatic substituent is attached to sulfur instead of an aliphatic substituent, it slightly accelerates the rate of hydrolysis. It is believed that conjugation of the aromatic ring with the S=O bond enhances oxygen's protonation, resulting in a faster reaction. On the other hand, the rate of hydrolysis with aromatic N-substituent is between tertiary and secondary sulfinamides (Scheme 2).

Notably, that nucleophilic displacement reactions of sulfinamides usually proceed with inversion of configuration at a sulfur atom.[9]



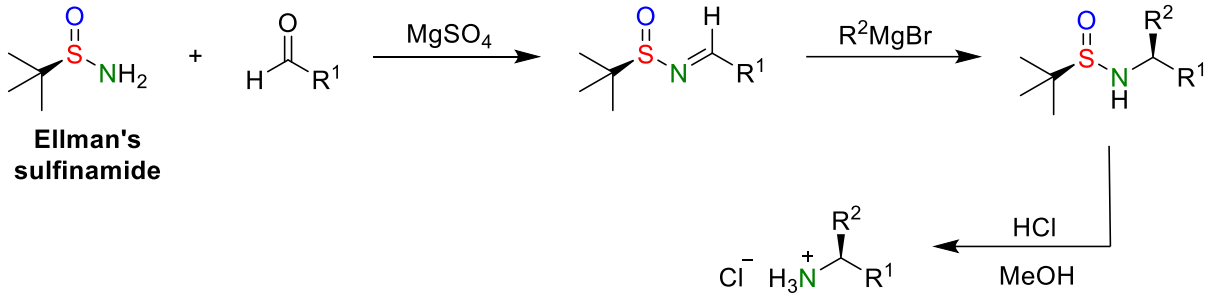
Scheme 2. A) Mechanism of sulfinamide hydrolysis. B) Sulfinamides ranked based on their rate of hydrolysis with pseudo-first-order rate constants for the specific acid-catalyzed hydrolysis.

Intringuingly, primary sulfinamides can undergo nucleophilic substitution with amines. This transsulfinamidation reaction has been described as an efficient method to enhance the diversity of sulfinamides. This reaction involves transforming primary sulfinamides with an excess of aryl-, alkyl-, or heterocyclic amines (primary or secondary). The initial methods employed copper catalysis, where amines were in the form of *O*-benzoyl hydroxylamines.[10] Later, copper was replaced with a rare-earth-metal catalyst, enabling the use of unmodified primary or secondary amines.[11] The most recent method involves thermally mediated transsulfinamidation, which takes place at temperatures of 70–110 °C, offering another variant of this approach (Scheme 3).[12]



Scheme 3. Different approaches to transsulfinamidation.

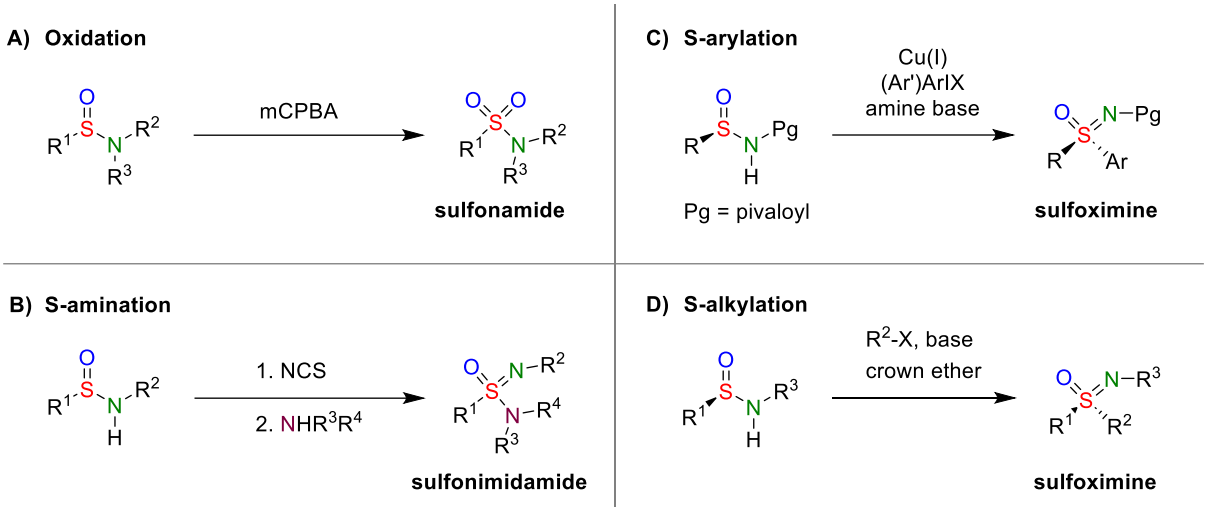
Sulfinamides also feature a nitrogen atom with a notable nucleophilicity, which allows them to create stable, imine-like structures when reacting with carbonyl groups. This property enables the use of sulfinamides in asymmetric amine synthesis, as seen with so-called Ellman's sulfinamides (Scheme 4).[1,13]



Scheme 4. Use of Ellman's sulfinamide in asymmetric synthesis of amines.

The ease of converting sulfinamides into medicinally relevant molecules is important for drug discovery. Mild oxidation of sulfinamides to sulfonamides with m-chloroperoxybenzoic acid (mCPBA) is particularly advantageous due to its tolerance of a wide range of functional groups.[10] The oxidative chlorination of sulfinamides with N-chlorosuccinimide (NCS), followed by a substitution with an amine, results in sulfonimidamides.[14] Sulfinamides can be also converted to sulfoximines through S-arylation or S-alkylation. In S-arylation, a weak base removes a proton from the secondary nitrogen,

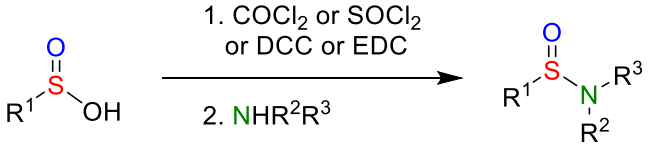
enabling the sulfur's nucleophilic lone pair to attack the metal-activated aryl halide.[15] In S-alkylation, NaH, a crown ether, and alkyl halide are used (Scheme 5).[16]



Scheme 5. Converting sulfinamides into medicinally relevant molecules.

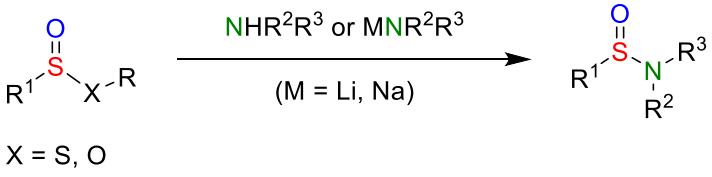
1.1.2 Preparation of Sulfinamides

Several methods for the synthesis of sulfinamides from sulfinic acid have been described. Sulfinic acid is converted to sulfinyl chloride using $(COCl)_2$, $SOCl_2$, an activated ester using DCC, or EDC. Then it reacts with an amine to produce the sulfinamide (Scheme 6).[17–20]



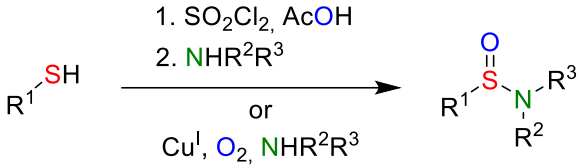
Scheme 6. Synthesis of sulfinamides from sulfinic acids.

Sulfinic acids are not readily available due to their poor stability, arising from potential disproportionation.[21] An alternative route for synthesizing sulfinamides is based on using sulfinate or thiosulfinate esters, which react with amines or metal amides through aminolysis (Scheme 7).[22]



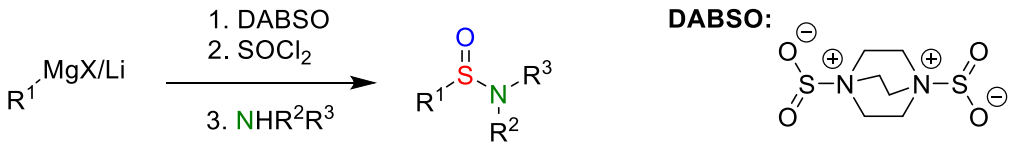
Scheme 7. Synthesis of sulfinamides from sulfinate or thiosulfinate esters.

These sulfinate or thiosulfinate esters are often synthesized from sulfinic acids or disulfides.[23,24] Oxidative routes for sulfinamide synthesis start from thiols, and use SO_2Cl_2 and acetic acid in a one-pot reaction to prepare sulfinyl chloride, which then reacts with the chosen amine.[25] Another approach involves a copper/palladium-catalyzed oxidative transformation (Scheme 8).[26,27]



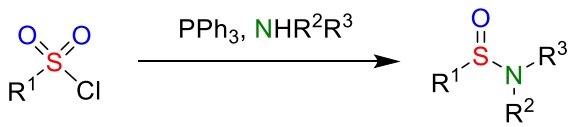
Scheme 8. Synthesis of sulfinamides from thiols.

Another option for sulfinamide diversification is a metal-catalyzed transsulfination.[11] Lo et al. recently presented a refined method for sulfinamide synthesis. They used DABSO as a sulfur dioxide surrogate. This reacted with organomagnesium or lithium compounds to form a sulfinate, which was then converted into sulfinamide (Scheme 9).[28]



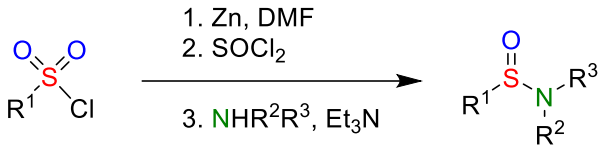
Scheme 9. Synthesis of sulfinamides from organomagnesium or lithium compounds.

Arylboron compounds have also been reported as starting materials for one-pot reactions that lead to sulfinamides.[29] Harmata et al. developed a new method using sulfonyl chlorides as a starting material. In this approach, sulfonyl chloride is reduced in situ by PPh_3, creating an intermediate that reacts directly with amine to form sulfinamide (Scheme 10).[30]



Scheme 10. Synthesis of sulfinamides from sulfonyl chlorides.

This process was further enhanced with the use of a metal-organic framework (MOF) catalyst.[31] However, it has a limited substrate range, as only certain aromatic sulfonyl chlorides yield the desired sulfinamides in a reasonable amount. In our lab, we developed a new method to address this challenge. Sulfonyl chlorides are reduced with zinc, followed by conversion to sulfinyl chloride using thionyl chloride. Finally, the sulfinyl chloride is transformed into sulfinamide through a reaction with a base and an amine (Scheme 11). This approach tolerates a wide spectrum of functional groups and structural features.[32]

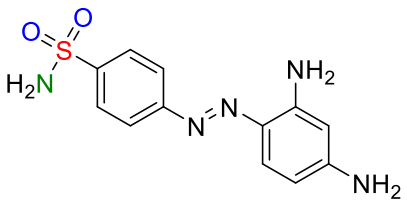


Scheme 11. Synthesis of sulfinamides from sulfonyl chlorides.

1.1.3 Medicinally Interesting Sulfinamide Derivatives

1.1.3.1 Sulfonamides

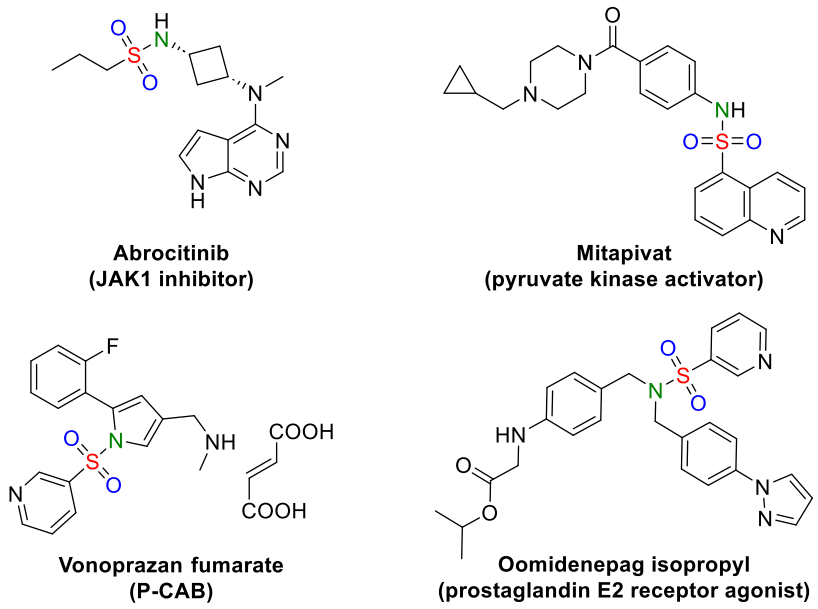
Sulfonamides have been a key component in medicinal chemistry for over ten decades. They contain hexavalent sulfur atom bonded to two oxygens, one nitrogen, and one carbon substituent. Many sulfonamide-based drugs are used in clinical practice today. These compounds are primarily known for their antibacterial activity. They are among the oldest antibacterial drugs still in use due to their high efficacy, broad spectrum of activity, simple synthesis, and low cost. An example is Prontosil (Scheme 12), its antibacterial activity was discovered in 1932 by Gerhard Domagk.[33]



Scheme 12. Structure of Prontosil.

Over time, the range of drugs containing the sulfonamide functional group has expanded including antiviral, antiinflammatory, anticancer, antifungal, antidiuretic, and antidiabetic.[34] For instance, in 2022, four sulfonamide-containing drugs were approved (Scheme 13). Abrocitinib is a selective Janus kinase 1 (JAK1) inhibitor used to treat atopic dermatitis.[35] Mitapivat allosterically activates the pyruvate kinase enzyme and it is approved for treating

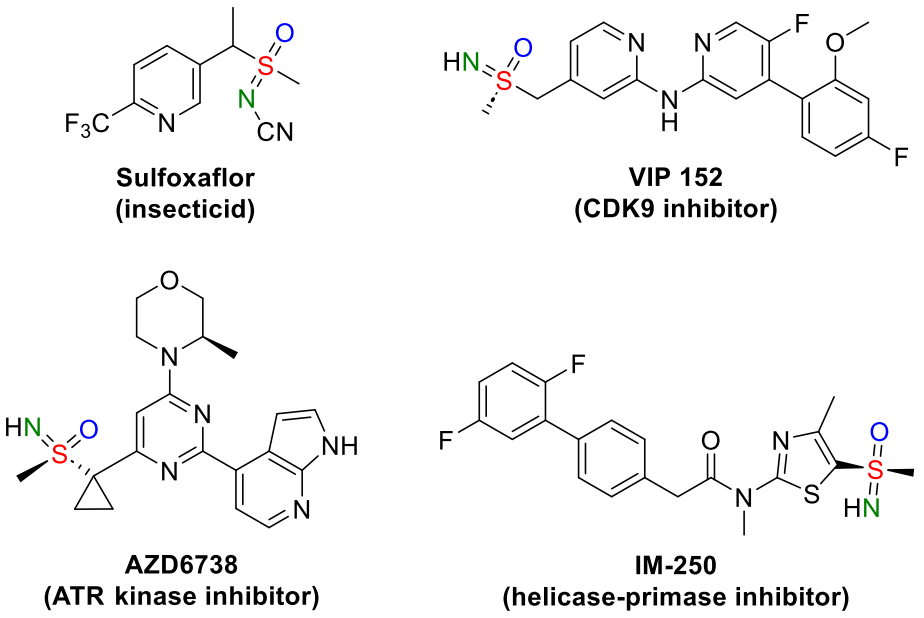
hereditary hemolytic anemias.[36] Vonoprazan fumarate, potassium-competitive acid blocker (P-CAB), is used for the treatment of *Helicobacter pylori* infection in combination with other molecules.[37] Oomidenepag isopropyl is a selective prostaglandin E2 receptor agonist and reduces elevated intraocular pressure.[38] Sulfonamide structure allows for various modifications, making them promising candidates for developing new therapies for many diseases.



Scheme 13. Structure of sulfonamide containing drugs approved in 2022.

1.1.3.2 Sulfoximines

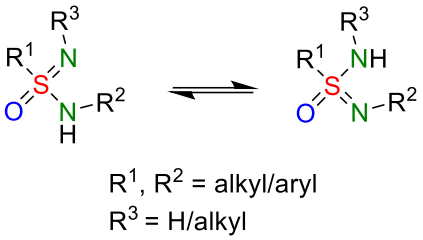
Sulfoximines feature a hexavalent sulfur atom bonded to one oxygen, one nitrogen, and two carbon substituents. When the carbon substituents differ, the sulfur becomes a stereogenic center. Due to their unique characteristics, such as a basic nitrogen atom and high solubility in polar solvents, chiral sulfoximines are now viewed as promising bioisosteres in medicinal chemistry.[39] The stereochemistry at the sulfur atom significantly influences their biological activity. Recently, asymmetric S-alkylations and S-arylations of chiral sulfinamides have been reported, resulting in the synthesis of chiral sulfoximines.[15,16] So far, no pharmaceutical products containing sulfoximines are currently available on the market. The only agrochemical product that has made it to market is the insecticide Sulfoxaflor, which received approval in 2013. Several studies have reported sulfoximines as potential pharmacophores.[4] The pharmacokinetics of sulfoximine drug analogues have been studied extensively. A variety of molecules containing sulfoximines are now undergoing clinical trials in various disease areas. Recent clinical candidates are for example CDK9 inhibitors[40,41], serine/threonine kinase ATR inhibitors[42], or helicase-primase inhibitors[43] (Scheme 14).



Scheme 14. Structure of sulfoximines containing compounds with biological activity.

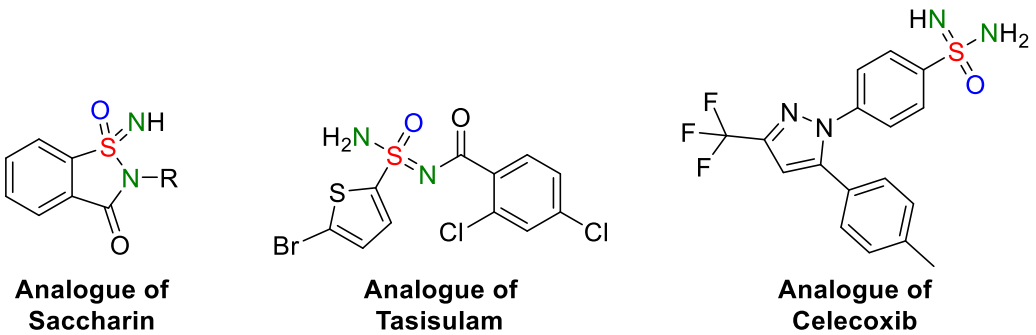
1.1.3.3 Sulfonimidamides

Compared to sulfonamides, the sulfonimidamide framework features an additional basic sp^2-hybridized nitrogen atom (imine), which provides a reactive site for chemical modifications. This flexibility can be utilized to fine-tune the physico-chemical and biological properties of the compound. When the sp^3-hybridized nitrogen atom (amide) is either primary or secondary, sulfonimidamide undergoes tautomerism due to proton transfer between the imine and amide nitrogen atoms.[44] The extent of this tautomerism depends on the R^2 and R^3 substituents (Scheme 15),[45] presenting valuable opportunities for drug design. These substituents may influence the molecule's acid/base characteristics.



Scheme 15. Tautomerism of sulfonimidamides.

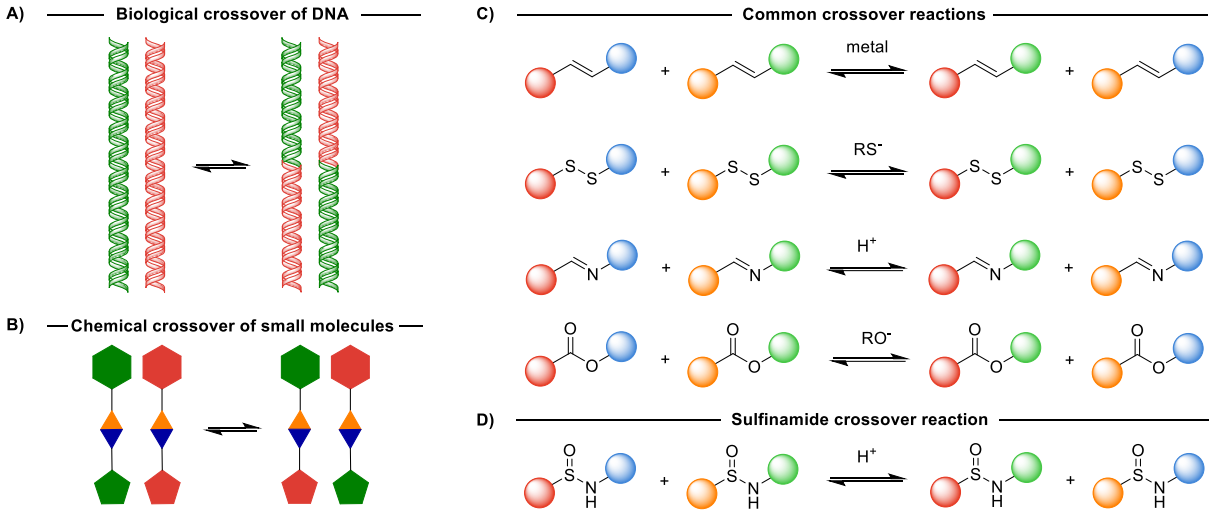
The free imine in sulfonimidamide can act as a hydrogen bond acceptor or donor, depending on its protonation state.[46] The sulfur atom in sulfinamide molecules has a tetrahedral shape and is typically stable, making the molecule chiral. This chirality is crucial in interactions with biomacromolecules, such as proteins and nucleic acids.[47] Based on these chemical properties, sulfonimidamides have started to come to the forefront of research into pharmacologically active compounds (Scheme 16),[8] this figure shows examples of bioactive sulfonimidamides, including a novel saccharin aza-bioisostere,[48] an analogue of the anticancer drug Tasisulam,[49] and non-steroidal anti-inflammatory drug Celecoxib.[50] Several methods for the synthesis of sulfonimidamides from sulfinamides have been described. The most common methods are those with a sulfonimidoyl chloride intermediate.[14,32,51]



Scheme 16. Structures of sulfonimidamide drug analogues.

1.2 Crossover Reaction

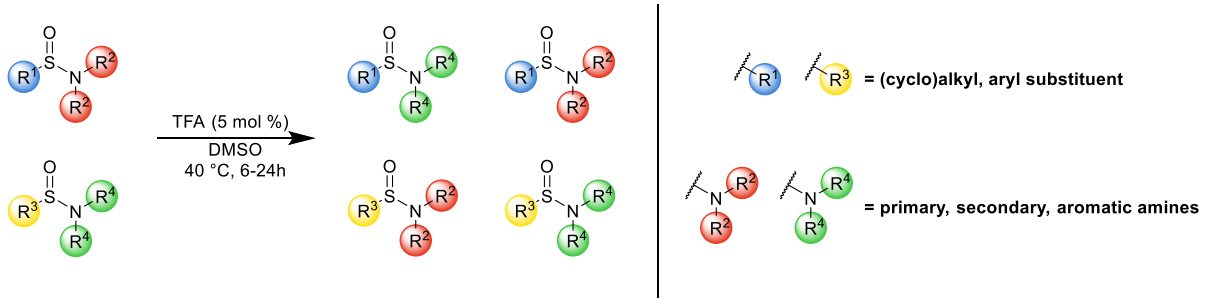
The concept of crossover is well known in biology. In sexually reproducing organisms, it is a source of diversity and thus, one of the drivers of evolution. Chromosomal crossover is the exchange of genetic information between non-sister chromatids of two homologous chromosomes during the meiotic division of gametes.[52] This introduces variability into the genetic information inherited from parents. Similarly to chromosomal crossover, under specific conditions small organic molecules can undergo chemical crossover. Examples of already known crossovers include metal-catalyzed alkene cross-metathesis, thiol-catalyzed disulfide exchange, acid-catalyzed imine exchange, acid or base-catalyzed transacetalation, and transesterification (Scheme 17).



Scheme 17. Schematic depiction of crossover reaction in biology (A) and chemistry (B). Common crossover reactions (C) and sulfinamide crossover (D).

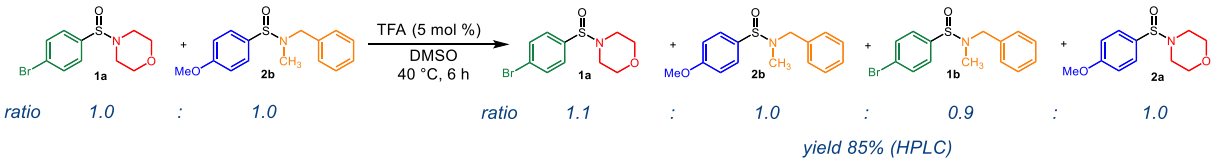
Several of the mentioned reactions play a key role in dynamic combinatorial libraries. These libraries can be applied in polymer chemistry, metal catalyst development, and especially in ligand-drug discovery and optimization in medicinal chemistry[53–58]. For these applications, functional groups like disulfides, acetals, and imines are limited by their dynamic nature, and groups such as alkenes and esters often lack the chemical properties required for drug-likeness. Tackling these issues frequently involves altering functional groups to block crossover, by e.g. reducing imines to amines. Moreover, searching for biomolecular ligands is usually complicated by functional group compatibility, limited structural complexity, and different thermodynamic stability of products under equilibrium. Therefore, enhancements in crossover reaction methodologies are highly valuable in discovery chemistry. Our lab has recently

discovered a new sulfinamide crossover reaction that overcomes several complications. It tolerates a wide variety of functional groups and can be easily oxidized to form a sulfonamide library, which is medicinally relevant (Scheme 18).[59]



Scheme 18. General scheme of crossover reaction and functional groups tolerance.

Sulfinamides were chosen for their favorable chemical properties, because unlike sulfonamides, feature a nitrogen atom with notable nucleophilicity. Combining nitrogen's nucleophilic nature with the electrophilic character of the sulfur-oxygen bond creates an ideal environment for a crossover reaction between two different sulfinamides. It was discovered by our lab that mildly acidic conditions facilitate a crossover reaction of sulfinamides with an almost quantitative yield.[59] As a model example, two tertiary sulfinamides, **1a** and **2b**, were chosen and mixed, initiating the crossover reaction by adding 5 mol % TFA in DMSO at 40 °C. The reaction mixture was analyzed after 6 hours and its crossover resulted in the formation of four sulfinamides (**1a**, **2b**, **1b**, **2a**) in nearly equimolar amounts (Scheme 19).[59]



Scheme 19. Model example of crossover reaction shows almost equimolar number of products.

It should be noted that the sulfinamide crossover reaction is not compatible with aqueous environment. Acidic aqueous environment leads to hydrolysis of sulfinamides and therefore, screening against biological targets requires stopping the crossover by neutral conditions or by oxidation to sulfonamides. Thus, the equimolar representation of the individual members of the crossover libraries are advantageous for the final pseudostatic libraries.

1.3 Library Screening

In the early stages of a drug discovery, identifying hits and leads for a target relies on efficient techniques to examine target-ligand interactions. In the past 30 years, the field of hit identification has been dominated by high-throughput screening (HTS), which is based on biochemical assays primarily utilizing fluorescence and luminescence detection. However, new approaches, such as fragment-based drug discovery (FBDD) and, more recently, DNA-encoded library (DEL) technology, have provided complementary options for exploring challenging targets.[60] The FBDD approach first identifies starting points: very small molecules (fragments) that are approximately half the size of typical drugs. These fragments are then expanded or linked together to generate drug leads.[61] In DEL technology, each small molecule in the library is chemically linked to a unique strand of DNA that acts as a molecular "barcode". DELs enable the screening of libraries containing billions of distinct compounds, and bound molecules are identified by sequencing the attached DNA.[62]

Biophysical methods based on mass spectrometry (MS) outputs have recently re-emerged as robust technologies for HTS in hit identification. As label-free methods, MS-based systems allow for a screening of molecules under conditions close to their native state, which can minimize the occurrence of false positives during screening.[60] MS-based methods can be divided into two categories based on ligand identification: the first focuses on detecting ligands after they are released from the target, inferring affinity from the amount of free ligands. The second category directly detects noncovalent ligand-target complexes in the gas phase. The affinity of the ligand-target interaction is determined from the abundance of the noncovalent complex.[63]

1.3.1 Affinity Selection Mass Spectrometry (AS-MS)

The development of AS-MS began alongside the growing interest in combinatorial chemistry. Both academic and industrial sectors were looking for methods that would allow the screening of large compound libraries. It was described that non-covalent receptor-ligand complexes can be analyzed in water using ion-spray-MS.[64] This discovery paved the way for screening libraries where neither the ligand nor the target needs to be modified. However, the challenge remained to find a way to separate the target–ligand complexes from the unbound compounds. In 1997, a method was developed based on the combination of ultrafiltration and electrospray MS, called pulsed ultrafiltration MS (PUF-MS).[65] In parallel, size exclusion chromatography (SEC) was used to physically isolate active components from the library,

followed by direct identification of the active compounds bound to the target molecule using MS.[66] This was followed by the development of the automated ligand identification system (ALIS), a high-throughput platform based on online SEC/liquid chromatography (LC)-MS.[67] Following ALIS, the affinity ultrafiltration-MS (AUF-MS or UF-MS) method was used in hit identification. In this approach, which can be performed on plates, an ultrafiltration membrane selectively concentrates the target–ligand complexes from the solution, separating them from unbound compounds.[68] Alternatively, the target can be immobilized on a solid support such as magnetic microbeads. After incubating the immobilized target with a compound mixture and washing away unbound compounds, the ligand-target complex is denatured, and the resulting filtrate is analyzed by MS to identify the ligand structures.[69] A key advantage of AS-MS is its ability to screen compounds in pools. The screening time and the amount of target material needed can be greatly reduced by enabling the simultaneous evaluation of multiple compounds. The number of compounds in each pool can vary widely, typically from 5 to over 2000, depending on the experimental setup. Because hit identification relies on exact mass detection, minimizing mass redundancy is crucial to prevent ambiguities in assigning hits.[64]

Generally, AS-MS consists of three steps: i) an affinity selection part, where the biomolecule is incubated with ligands and forms a complex with any ligand capable of binding; ii) the resulting target-ligand complexes are separated from non-binding chemical molecules; iii) identification of dissociated ligands from the complexes by MS (Figure 1).[70]

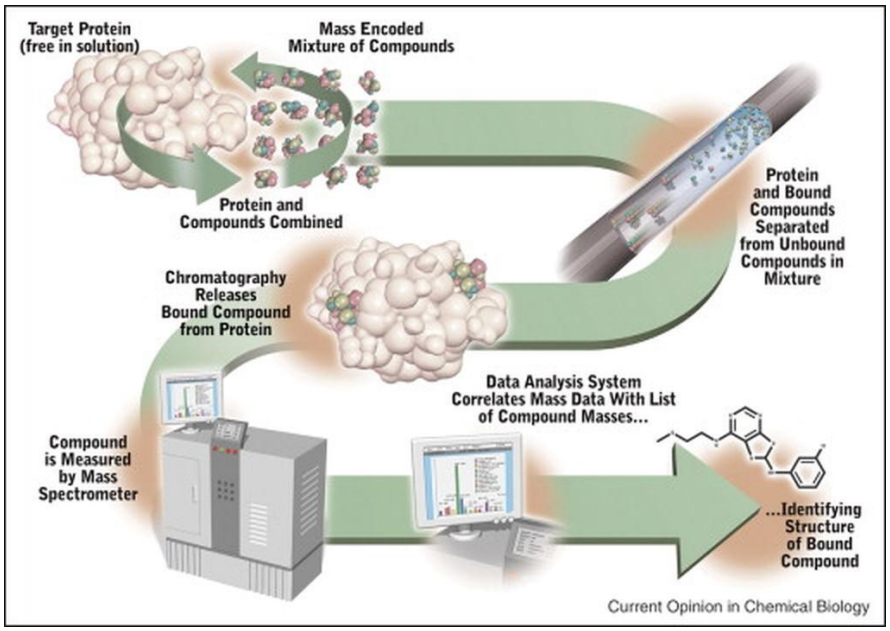


Figure 1. Schematic diagram of the AS-MS process (from Annis, 2007, p. 522).

1.4 Factor Xa

Factor Xa is a key serine protease in the coagulation cascade. It catalyzes the conversion of prothrombin into thrombin. Thrombin then facilitates the conversion of fibrinogen into fibrin, leading to the formation of a stable blood clot. Targeting factor Xa has become a promising approach in antithrombotic drug development because its inhibition can effectively block both the extrinsic and intrinsic coagulation pathways (Figure 2).[71]

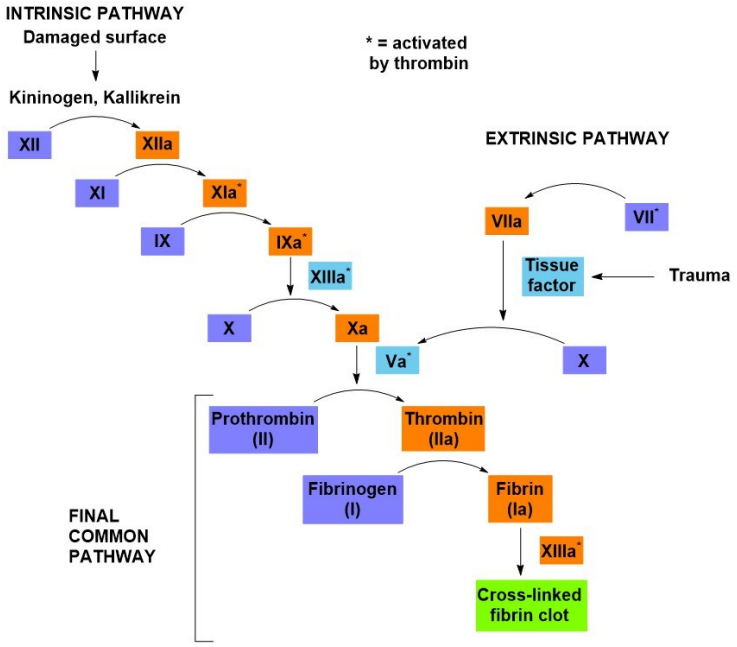
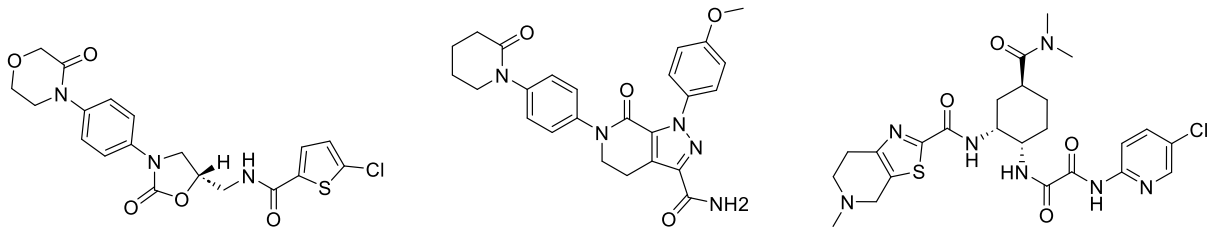


Figure 2. Diagram of coagulation cascade.

Inhibition of factor Xa interrupts the coagulation cascade upstream of thrombin, thereby preventing clot formation without directly affecting the level of existing thrombin. This results in a lower risk of bleeding compared to direct thrombin inhibitors. Several factor Xa inhibitors, including Rivaroxaban,[71] Apixaban,[72] and Edoxaban,[73] are approved for specific medical conditions (Scheme 20). Another studied group of factor Xa inhibitors are sulfonamides, which were utilized in this work.[74]



Scheme 20. Structures of approved factor Xa inhibitors.

2 Aims of the Thesis

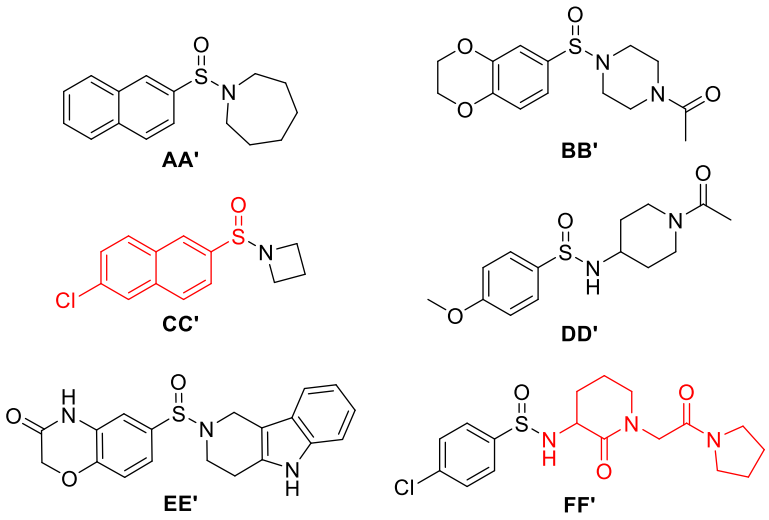
The aims of the thesis were as follows:

1. Synthesis a small set of structurally diverse sulfinamides through a one-pot reductive method.
2. Application of a sulfinamide crossover reaction to diversify the sulfinamide library.
3. Testing of the resulting libraries by the affinity selection mass spectrometry with coagulation factor Xa.

3 Results and Discussion

3.1 Design of the Sulfinamide Library

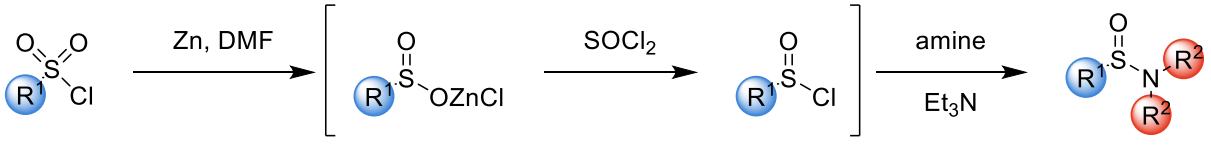
Starting materials for this reaction were selected based on their structural diversity and to avoid isobaric compounds in the crossover library. Also, Lipinski's rule of five was considered to keep ligand-like characteristics of the compounds in the final library. Crossover also works with aliphatic S-substituents, aromatic ones were chosen because crossover proceeds faster with them. N-substituents were chosen to form both secondary and tertiary sulfinamides. Additionally, S-substituent of compound **CC'** and N-substituent of compound **FF'** (Scheme 21 shown in red) constitute a precursor (sulfinamide) of known factor Xa sulfonamide inhibitor that should be present in the final library after the crossover reaction.[74]



Scheme 21. Sulfinamides for the crossover library generation. The combination of the red fragments forms the precursor of the factor Xa inhibitor.

3.2 Synthesis of Target Sulfinamides

A general and mild one-pot method, previously discovered in our group, was used for the preparation of sulfinamides from sulfonyl chlorides.[32] This method tolerates a wide range of functional groups. Sulfonyl chlorides are reduced to zinc sulfinates and further transformed into sulfinyl chlorides, which readily react with amines to form sulfinamides (Scheme 22).



Scheme 22. General method of sulfinamide synthesis.

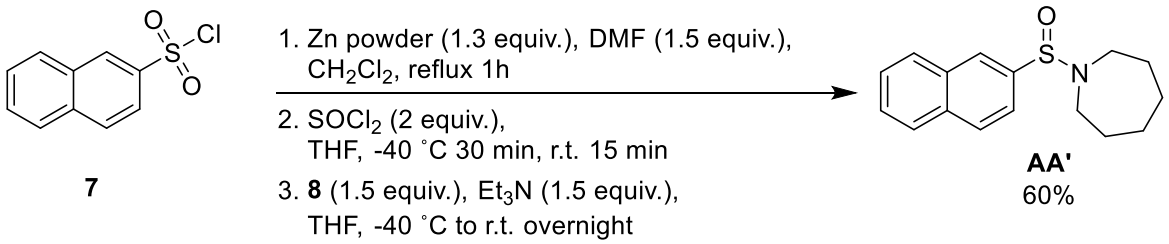
3.2.1 *1-(naphthalen-2-ylsulfinyl)azepane* (**AA'**)

Sulfinamide **AA'** was prepared using 2-naphthalenesulfonyl chloride **7** and azepane **8** as a starting material. However, the reported conditions[32] provided only a small amount of product based on a TLC analysis. Therefore, it was necessary to optimize the second step of the reaction which seemed to be the problematic one (Table 1).

Table 1. Optimization of reaction conditions for the synthesis of **AA'**.

Entry	$SOCl_2$	2. reaction time + temperature	under inert	yield
1	old	45 min at -40 °C	no	not purified
2	freshly distilled	45 min at r.t.	yes	27 %
3	freshly distilled	30 min at -40°C then 15 min at r.t.	yes	60 %

It turned out that the quality of thionyl chloride and temperature are crucial factors. Also, the reaction must take place in an inert atmosphere, otherwise the sulfinyl chloride is re-oxidized back to sulfonyl chloride **7**. Therefore, using freshly distilled thionyl chloride, keeping the reaction under inert atmosphere and optimizing the temperatures during the final addition of the amine led to an improvement in the yield of compound **AA'** to 60 % (Scheme 23).



Scheme 23. AA' synthesis.

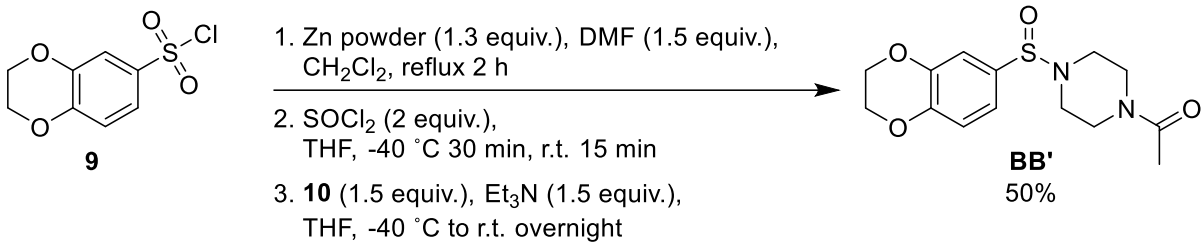
3.2.2 *1-(4-((2,3-dihydrobenzo[b][1,4]dioxin-6-yl)sulfinyl)piperazin-1-yl)ethan-1-one* (**BB'**)

Sulfinamide **BB'** was prepared using 2,3-dihydro-1,4-benzodioxine-6-sulfonyl chloride **9** and 1-acetylpiperazine **10** as a starting material. After the first reaction, in which the reduction with zinc took more than five hours, the zinc was freshly activated,[75] shortening the reaction time to 1.5 hours. Different thionyl chloride quality was tested (Table 2).

Table 2. Optimization of reaction conditions for the synthesis of **BB'**.

Entry	Zn	$SOCl_2$	yield
1	unactivated	old	13 %
2	activated	freshly distilled	26 %
3	activated	newly purchased	50 %

It turns out, that reaction time of the first step is considerably reduced by zinc activation. Combined with the use of newly purchased thionyl chloride instead of a distilled one, the yield of compound **BB'** increased to 50 % (Scheme 24).



Scheme 24. BB' synthesis.

3.2.3 *1-((6-chloronaphthalen-2-yl)sulfinyl)azetidine* (**CC'**)

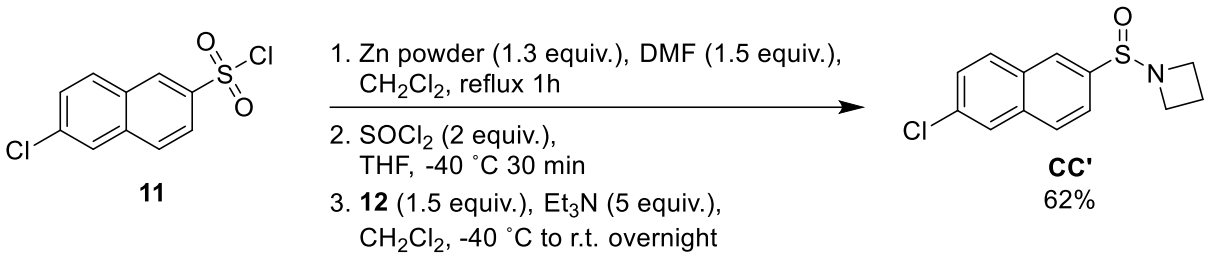
Sulfinamide **CC'** was prepared using 6-chloronaphthalene-2-sulfonyl chloride **11** and azetidine hydrochloride **12** as starting materials. Optimized conditions from the previous reactions give yields only up to 11 %. The potentially problematic second step was studied. Newly purchased undistilled thionyl chloride was used, and it was examined whether increasing the equiv. of thionyl chloride affects the yield. Additionally, the influence of reaction time and temperature was studied. For this screening of conditions, an amine azepane **8** that had performed well in a previous reaction was used. The reaction outcome was monitored by HPLC-MS (Table 3).

Table 3. Optimization of reaction conditions for the synthesis of **CC'**.

Entry	$SOCl_2$ (equiv.)	2. reaction time + temperature	amount of the product observed by HPLC-MS
1a	2	30 min at -40 °C	significant
1b		30 min at -40 °C + 15 min at -20 °C	low
1c		30 min at -40 °C + 15 min at -20 °C + 15 min at 7 °C	no
1d		30 min at -40 °C + 15 min at -20 °C + 15 min at 7 °C + 15 min at r.t.	no
2a	8	30 min at -40 °C	no
2b		30 min at -40 °C + 15 min at -20 °C	no
2c		30 min at -40 °C + 15 min at -20 °C + 15 min at 7 °C	no
2d		30 min at -40 °C + 15 min at -20 °C + 15 min at 7 °C + 15 min at r.t.	no

Increasing the amount of thionyl chloride or changes of reaction temperature and time of the second step did not increase the yield. A hitch arose in the third step of the reaction with the solubility of azetidine hydrochloride **12**. It was observed that replacing the solvent THF with CH_2Cl_2 and increasing the amount of Et3N to 5 equiv. improved solubility of azetidine

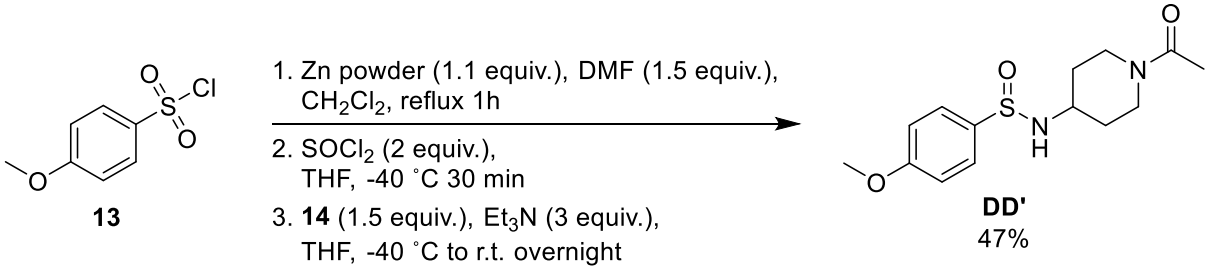
hydrochloride. Combining the optimized conditions, a yield of 62 % was achieved for compound **CC'** (Scheme 25).



Scheme 25. CC' synthesis.

3.2.4 *N-(1-acetylpiperidin-4-yl)-4-methoxybenzenesulfinamide* (**DD'**)

Sulfinamide **DD'** was prepared using 4-methoxybenzenesulfonyl chloride **15** and 1-acetyl-4-aminopiperidine hydrochloride **14** as starting materials. In this reaction, the already optimized conditions for step 2 were used. Activated zinc and newly purchased undistilled thionyl chloride were used. The amount of Et_3N was doubled due to the use of the amine in its hydrochloride form. A yield of 47 % was achieved for compound **DD'** (Scheme 26).

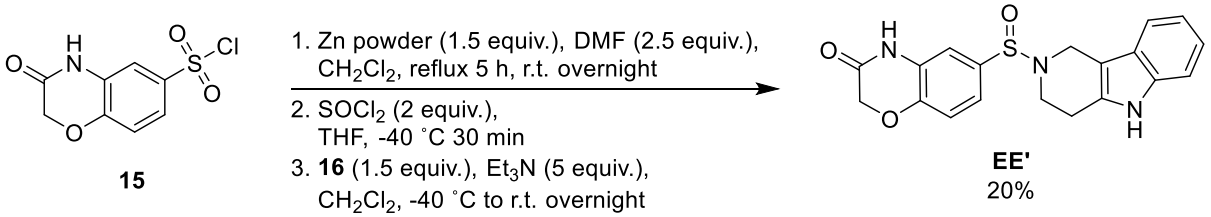


Scheme 26. DD' synthesis.

3.2.5 *6-((1,3,4,5-tetrahydro-2H-pyrido[4,3-b]indol-2-yl)sulfinyl)-2H-benzo[b][1,4]oxazin-3(4H)-one* (**EE'**)

Sulfinamide **EE'** was prepared using 3-oxo-3,4-dihydro-2H-1,4-benzoxazine-6-sulfonyl chloride **15** and 1H,2H,3H,4H,5H-pyrido[4,3-b]indole hydrochloride **16** as a starting material. The reduction with zinc was not completed after 5 hours of reflux of the reaction mixture, even though the zinc was activated. Reaction was left at room temperature overnight, and then the starting material was consumed. Another issue was the solubility of amine **16**, which could not be dissolved even under the optimized conditions for azetidine hydrochloride

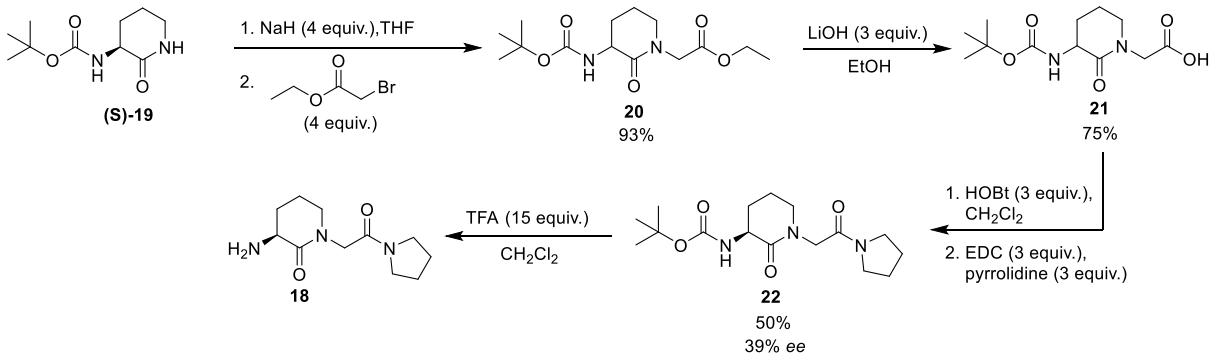
12. These issues contributed to the lower yield of the desired product. The resulting yield of the product **EE'** was 20 % (Scheme 27).



Scheme 27. EE' synthesis.

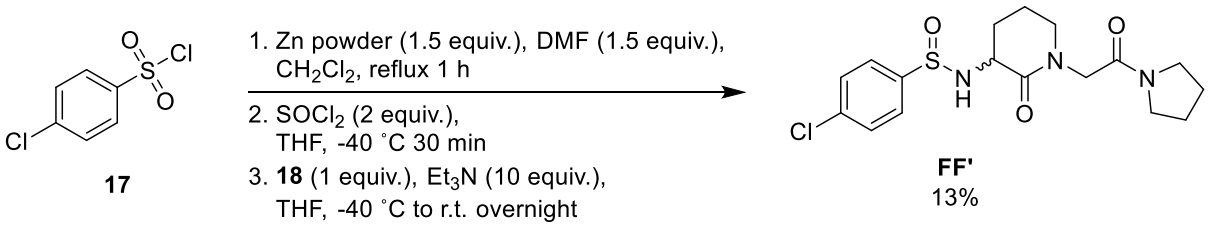
3.2.6 *4-chloro-N-(2-oxo-1-(2-oxo-2-(pyrrolidin-1-yl)ethyl)piperidin-3-yl)benzenesulfinamide* (**FF'**)

Sulfinamide **FF'** was prepared using 4-chlorobenzenesulfonyl chloride **17** and 3-amino-1-(2-oxo-2-(pyrrolidin-1-yl)ethyl)piperidin-2-one **18** as a starting material. First, amine **18** had to be prepared. Based on the reported synthesis from Munguía et al.[76], commercially available (S)-3-(Boc-amino)-2-piperidone **(S)-19** was alkylated with ethyl bromoacetate, and product **20** was isolated with 93 % yield. The second step was carried out according to Colobbio's protocol.[77] Ester **20** was hydrolyzed with LiOH and acid **21** was obtained in 75 % yield. In the next step, an EDC coupling was performed providing product **22** in 50 % yield. The enantiomeric excess of compound **(S)-22** was 39%. Amine **18** is the N-substituent of the factor Xa inhibitor, which is expected to form via a crossover reaction with the S-substituent of sulfinamide CC'. The factor Xa inhibitor is described as an enantiomerically pure compound. However, the synthesis of amine **18** is not described in the article on the factor Xa inhibitor.[74] Therefore, a different procedure was followed, which does not produce enantiomerically pure amine **18**. Compound **22** was deprotected with TFA (15 eq.) just before being used in the synthesis of sulfinamide **FF'** (Scheme 28).



Scheme 28. Amine **18** synthesis.

The reduction of compound **17** with zinc was completed within one hour. Thionyl chloride was used undistilled from a new bottle. Due to the small amount of amine **18**, the reaction was carried out on a small scale, and just 1 equiv. of compound **18** was added. Product **FF'** was isolated with a 13 % yield (Scheme 29). No significant side products were observed. Since this amount was sufficient to perform the crossover reaction, no optimization was performed.

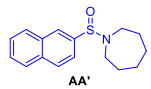
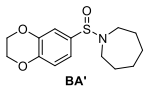
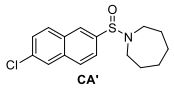
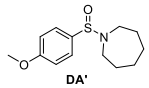
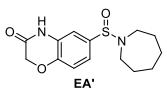
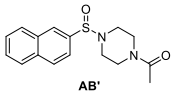
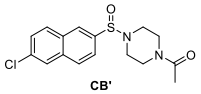
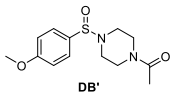
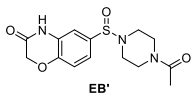
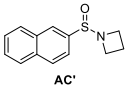
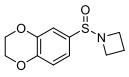
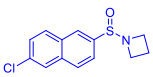
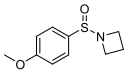
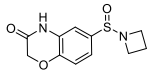
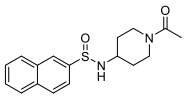
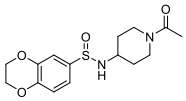
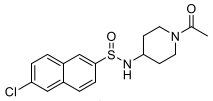
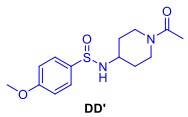
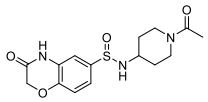
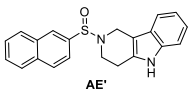
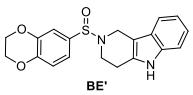
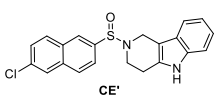
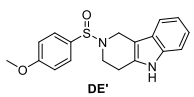
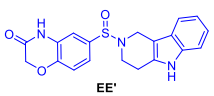


Scheme 29. FF' synthesis.

3.3 Crossover Reaction

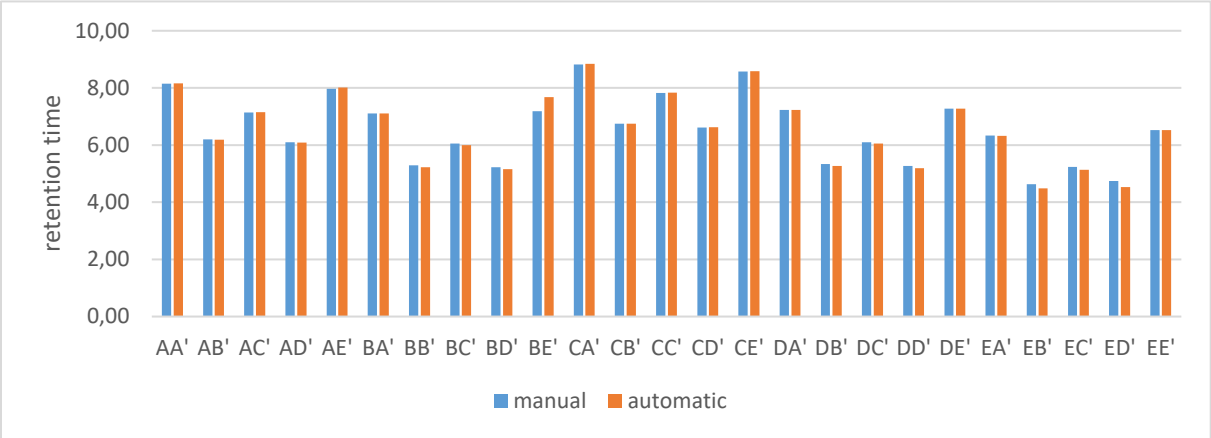
Five sulfinamides were selected for the initial crossover reaction **CROSS-1**. A previously described method developed in our laboratory was used.[59] It was assumed that N-substituents would be exchanged between all sulfinamides, resulting in a library of twenty-five different sulfinamides. Equimolar amounts (40 mM each, 200 mM final) of sulfinamides **AA'**, **BB'**, **CC'**, **DD'**, and **EE'** were mixed in DMSO and the reaction was initiated by the addition of 5 mol % of TFA. The reaction was incubated at 40 °C. After only two hours, twenty-five different sulfinamides could be detected in the reaction mixture (Table 4). Further prolongation of the crossover reaction time did not result in any change of the product ratios as indicated by HPLC-MS. This initial screening confirmed the applicability of the crossover reaction for the selected sulfinamides.

Table 4. Library of twenty-five sulfinamides formed after the crossover **CROSS-1**. Blue are the initial sulfinamides.

 AA'	 BA'	 CA'	 DA'	 EA'
 AB'	 BB'	 CB'	 DB'	 EB'
 AC'	 BC'	 CC'	 DC'	 EC'
 AD'	 BD'	 CD'	 DD'	 ED'
 AE'	 BE'	 CE'	 DE'	 EE'

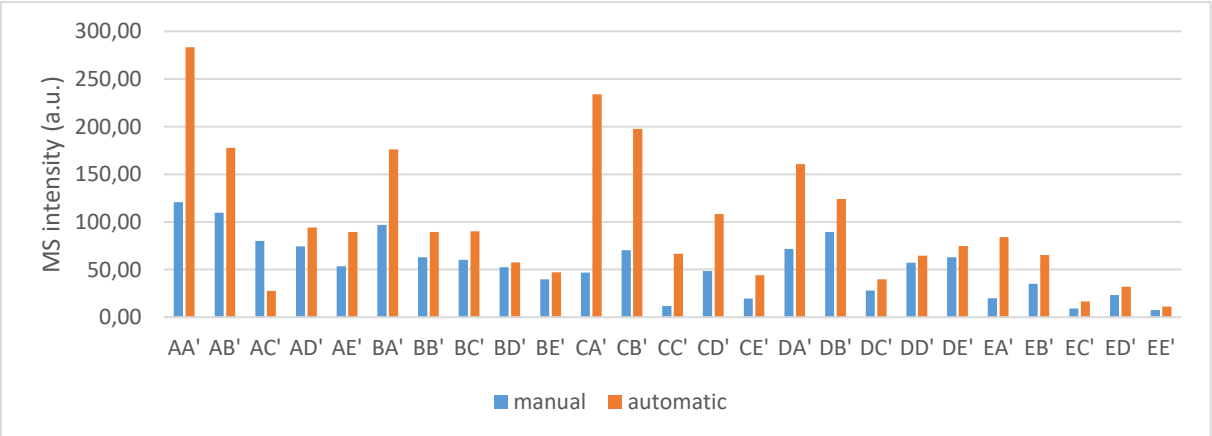
Detection was performed on an HPLC-MS instrument. Exact masses were found both manually and using MassHunter Qualitative Analysis software (Agilent). Retention times of the products determined manually match those found by automatic analysis (Graph 1).

Graph 1. Manual and automatic approach to the analysis of retention times.



Interestingly, automatic integration yielded higher values in most cases compared to manual approach (Graph 2). This is likely because the software can search for adducts with all ions simultaneously, whereas in the manual method only the peak for the H^+ adduct was integrated. Since the automatic method proved reliable, it was used exclusively for further analysis.

Graph 2. Manual and automatic approach to the analysis of MS intensities.



Additionally, two crossover reactions **CROSS-2** and **CROSS-3** were designed, each with five sulfinamides. In the **CROSS-2** reaction, sulfinamide **AA'** was substituted with **FF'**. Thus, the precursor **CF'** of the factor Xa inhibitor, described in the literature, was formed after the crossover reaction (Table 5). In the **CROSS-3** reaction, sulfinamide **CC'**, containing the S-substituent of the inhibitor, was substituted with **AA'** (Table 6).

Table 5. Library of twenty-five sulfinamides formed after the crossover **CROSS-2**. Blue are the initial sulfinamides. Red is the precursor of factor Xa inhibitor.

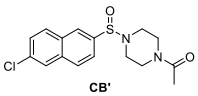
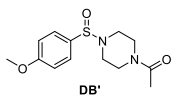
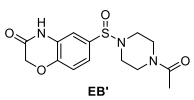
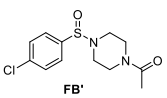
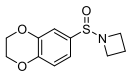
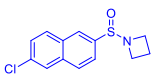
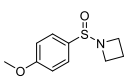
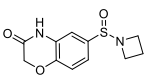
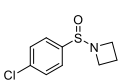
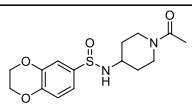
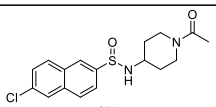
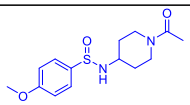
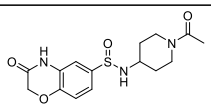
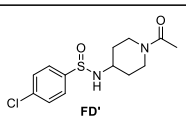
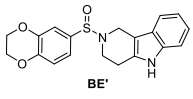
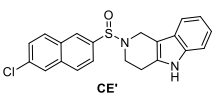
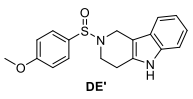
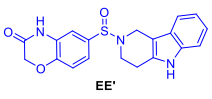
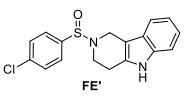
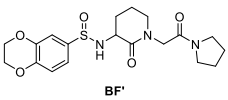
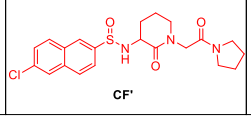
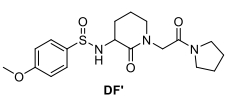
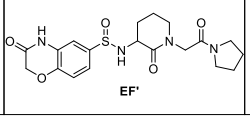
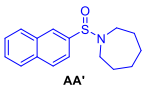
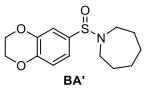
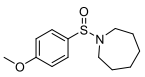
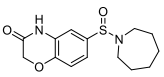
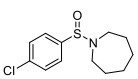
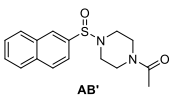
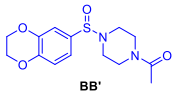
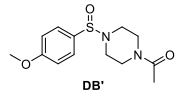
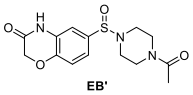
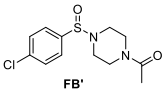
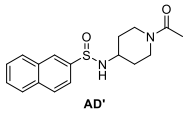
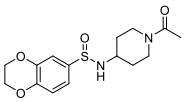
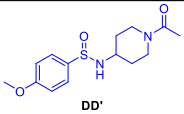
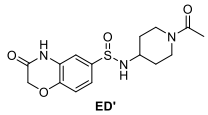
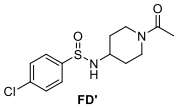
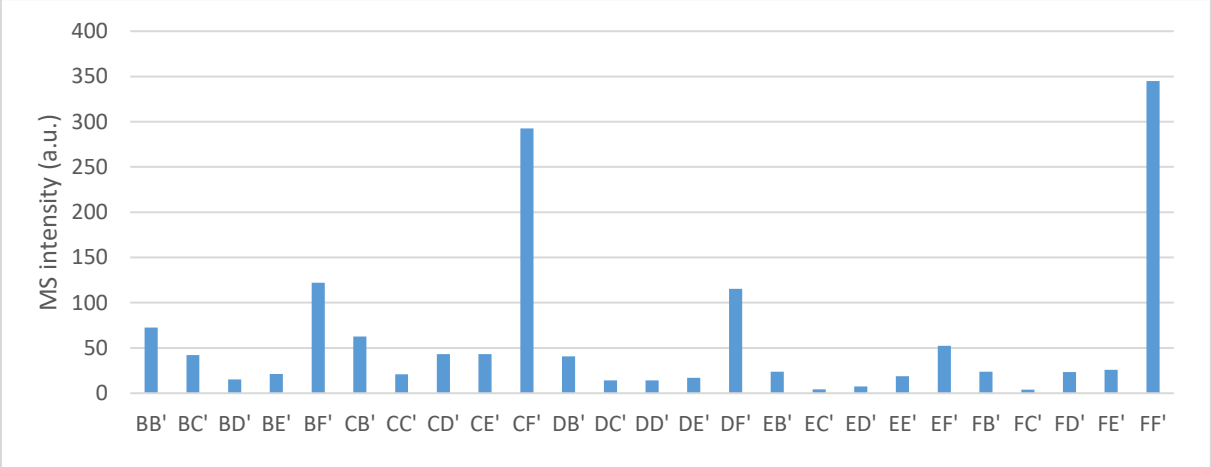
 BB'	 CB'	 DB'	 EB'	 FB'
 BC'	 CC'	 DC'	 EC'	 FC'
 BD'	 CD'	 DD'	 ED'	 FD'
 BE'	 CE'	 DE'	 EE'	 FE'
 BF'	 CF'	 DF'	 EF'	 FF'

Table 6. Library of twenty-five sulfinamides formed after the crossover **CROSS-3**. Blue are the initial sulfinamides.

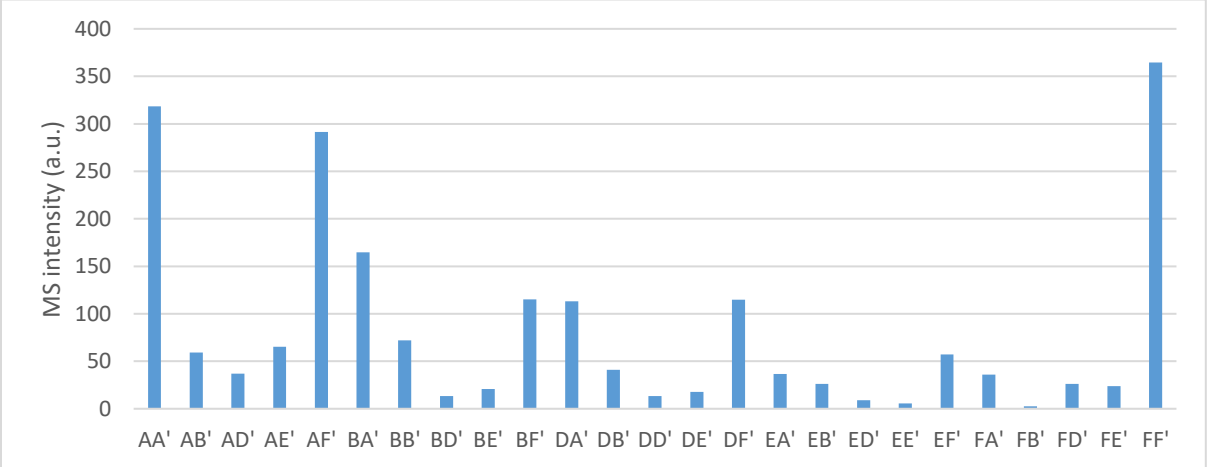
 AA'	 BA'	 DA'	 EA'	 FB'
 AB'	 BB'	 DB'	 EB'	 FB'
 AD'	 BD'	 DD'	 ED'	 FD'
AE'	BE'	DE'	EE'	FE'
AF'	BF'	DF'	EF'	FF'

Data from HPLC-MS were analyzed automatically. In both cases, twenty-five sulfinamides were found after two hours (Graph 3, Graph 4). MS intensities of the individual compounds in the crossover libraries varies. As it was shown in our lab, libraries of equimolar compounds can vary in their MS intensities, which is the effect of the propensity of compounds to electrospray ionization. Precise quantification of the library members would be difficult due to the library complexity. However, MS intensities of all the compounds are sufficient for testing the libraries in binding assays.

Graph 3. MS intensities of twenty-five sulfinamides after **CROSS-2** crossover.



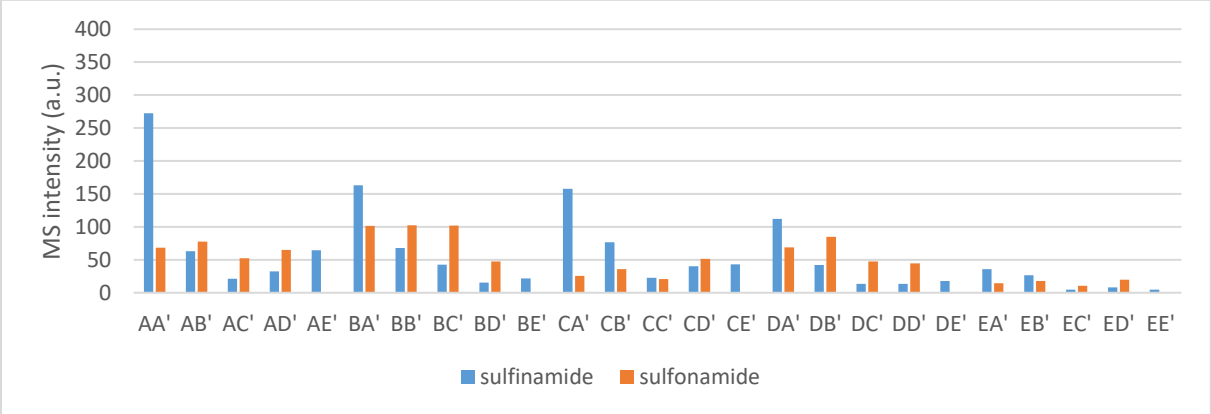
Graph 4. MS intensities of twenty-five sulfinamides after **CROSS-3** crossover.



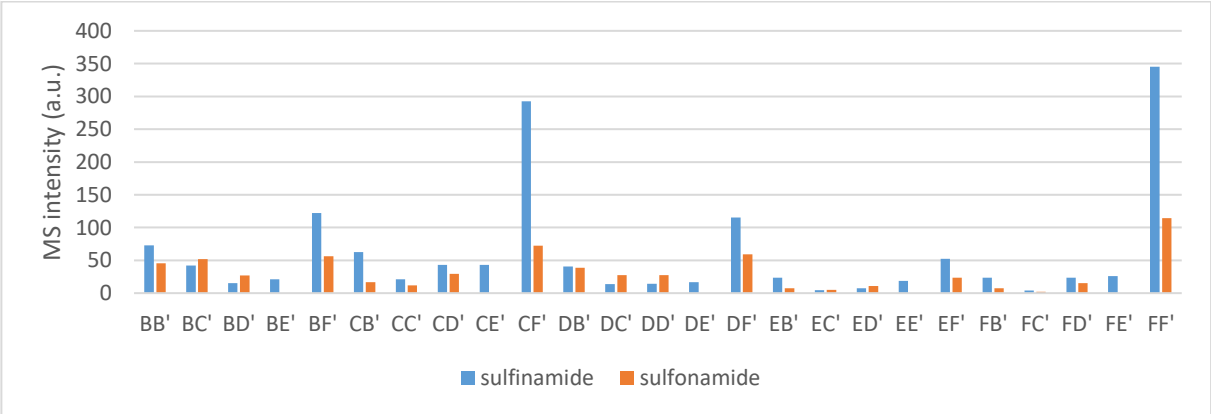
3.4 Oxidation of Sulfinamide Libraries after Crossover

The sulfinamide libraries generated after the **CROSS-1**, **CROSS-2,** and **CROSS-3** crossover were subsequently oxidized to the corresponding sulfonamide libraries **CROSS-1(o)**, **CROSS-2(o),** and **CROSS-3(o)**. The oxidation of the libraries was mainly used to generate the known sulfonamide inhibitor **CF'(o)**. Also, the oxidation stabilizes the libraries in the acidic aqueous environment. The libraries were oxidized using mCPBA (2 equiv.) in CH_2Cl_2 and analyzed by HPLC-MS (Graph 5, Graph 6, Graph 7).

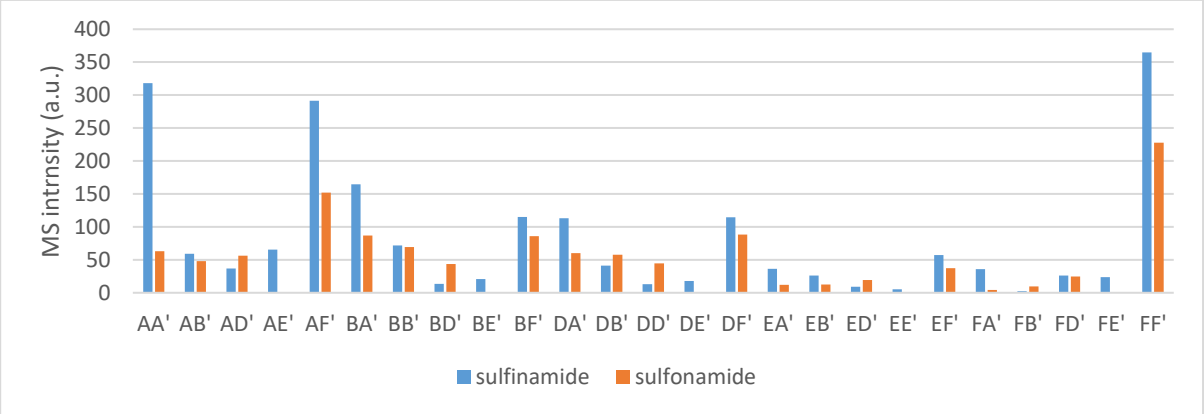
Graph 5. Comparison of MS intensities of sulfinamides and sulfonamides resulting from the **CROSS-1** crossover.



Graph 6. Comparison of MS intensities of sulfinamides and sulfonamides resulting from the **CROSS-2** crossover.



Graph 7. Comparison of MS intensities of sulfinamides and sulfonamides resulting from the **CROSS-3** crossover.

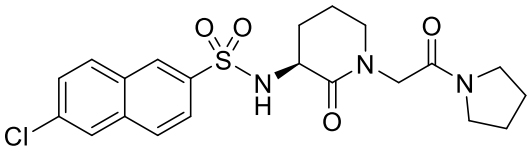


Notably, apart from the sulfonamides with N-substituent **E'**, all other sulfonamides were detected with good intensity. Electron rich indole substituent **E'** can probably undergo oxidation under the reaction conditions, which prevents the detection of the corresponding sulfonamides. The resulting side products were not identified, therefore, other oxidation conditions of sulfinamide **EE'** were tested as a model. Iodosobenzene diacetate, TCCA, and tert-butyl hydroperoxide were selected as test oxidants. According to the HPLC-MS analysis, iodosobenzene diacetate looks like a promising candidate. Two peaks with corresponding masses could be observed on HPLC-MS. This could mean that oxygen was attached to the molecule in two different ways. Other oxidants did not lead to the desired product. Further optimization of oxidation conditions was not performed. However, iodosobenzene diacetate seems to be milder and tolerant oxidant for the conversion of sulfinamides to sulfonamides.

3.5 AS-MS with Factor Xa

Factor Xa is an ideal target for small molecules due to its crucial role in blood coagulation and its well-characterized structure, making it a widely studied enzyme in drug discovery. All AS-MS assays were performed in factor Xa buffer. Factor Xa was incubated with different sulfonamide libraries for 1 hour at 25 °C. The concentration was estimated, so that each sulfonamide had similar molar concentration to factor Xa (1 μM). The resulting target-ligand complexes were separated from non-binding molecules by filtration through a 10 kDa membrane. The trapped molecules were eluted from the factor Xa by its denaturation with MeOH and separated from protein residues by filtration. In parallel with each experiment, a control assay (blank) without factor Xa was run to ensure reliability of the results. In the blank,

the same amount of buffer was added instead of factor Xa. The enrichment of factor Xa binders was evaluated by comparison of MS intensities with blank experiments. The factor Xa inhibitor **CF'(o)** with an IC_{50} of 32 nM was formed in library **CROSS-2(o)** (Scheme 30).[74] **CROSS-1(o)** and **CROSS-3(o)** libraries miss either the N-substituent or S-substituent of the inhibitor **CF'(o)**. In the first test of the assay, **CROSS-2(o)** library was used. These preliminary results showed that factor Xa inhibitor **CF'(o)** was indeed the only considerably enriched compound compared to blank (Graph 8). The other two libraries **CROSS-1(o)** and **CROSS-3(o)** contained only one part of the factor Xa inhibitor. No significant enrichment of sulfonamides was observed (Graph 9, Graph 10).

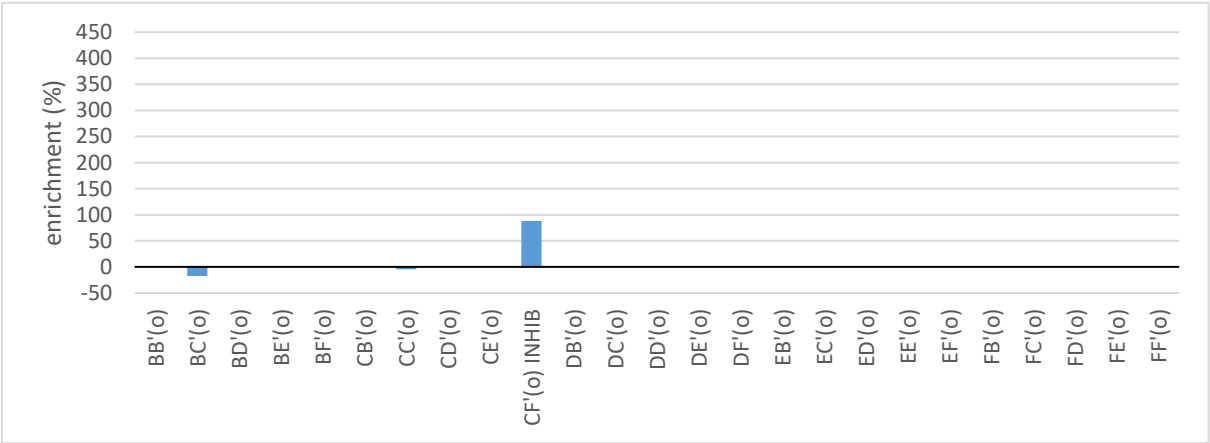


CF'(o) - enriched in assay

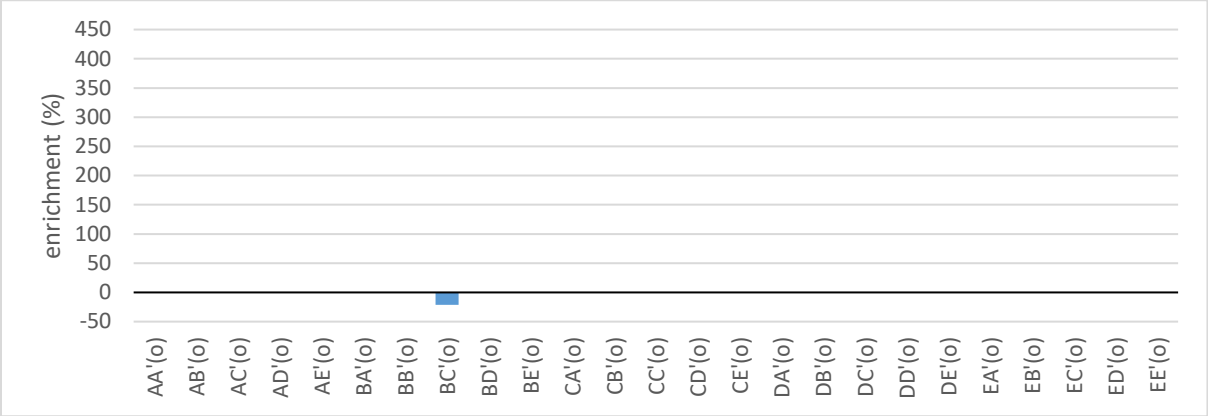
IC_{50} = 32 nM

Scheme 30. Factor Xa inhibitor **CF'(o)** enriched in assay with **CROSS-2(o)** library and its IC_{50} value.

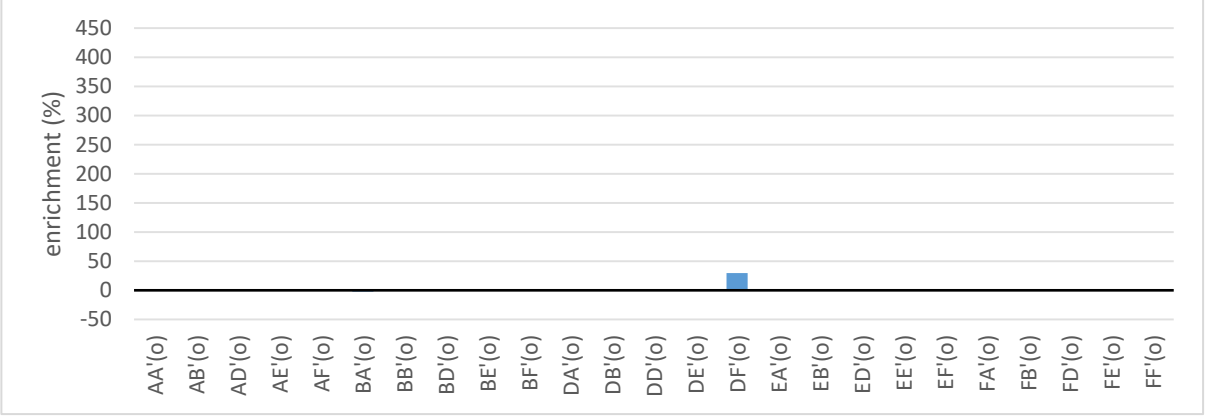
Graph 8. Enrichment of sulfonamides (**CROSS-2(o)** library) after elution in factor Xa assay compared to blank.



Graph 9. Enrichment of sulfonamides (**CROSS-1(o)** library) after elution in factor Xa assay compared to blank.

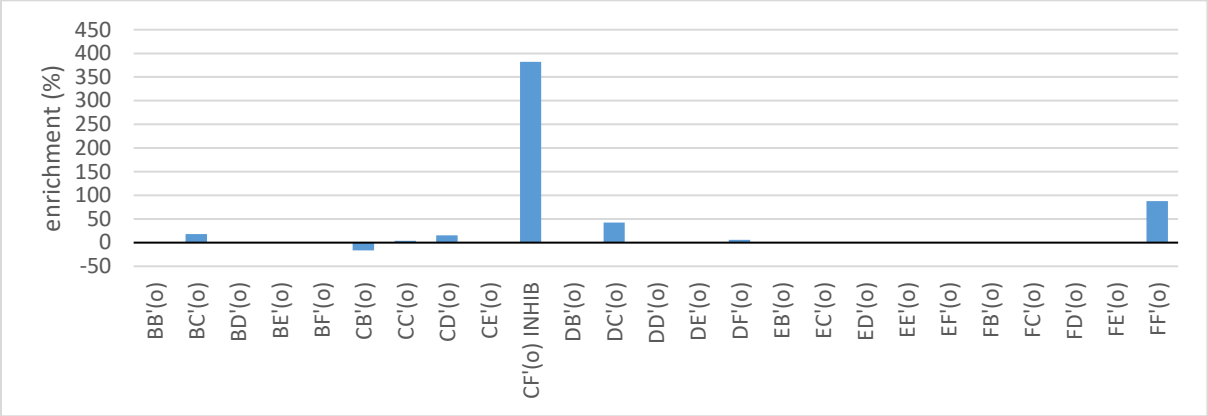


Graph 10. Enrichment of sulfonamides (**CROSS-3(o)** library) after elution in factor Xa assay compared to blank.

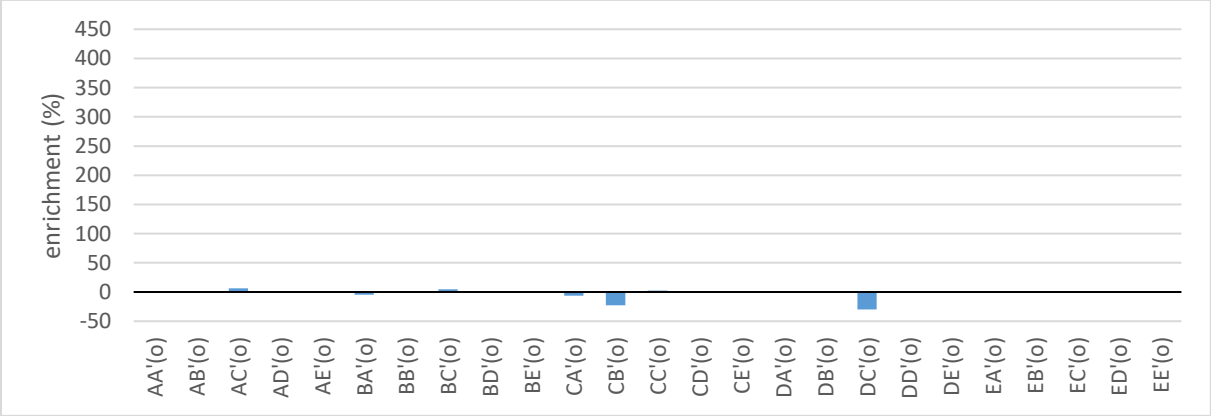


All detected sulfonamides were also present in similar amounts in the blank. The lipophilic nature of the membrane may have led to interaction with some sulfonamides. Therefore, an assay with indifferent BSA protein in the blank experiment was designed. The next steps of the experiment were carried out identically to the previous one. The BSA was intended to prevent non-specific interactions that may stem from the lipophilic nature of the filter membrane. This modification of the binding assay resulted in major improvement regarding the enrichment of the inhibitor **CF'(o)** with **CROSS-2(o)** library (Graph 11). Also, difference in more other compounds of the library were observed. In **CROSS-1(o)** library containing only S-substituent of the factor Xa inhibitor **CF'(o)**, only slight enrichment towards BSA protein was observed (Graph 12). BSA is a lipophilic protein able to bind wide range of small molecules. The significance of this observation remains to be determined. In **CROSS-3(o)** library containing only N-substituent of the factor Xa inhibitor **CF'(o)**, there was a significant enrichment of compounds **FF'(o)** and **AF'(o)** (Graph 13).

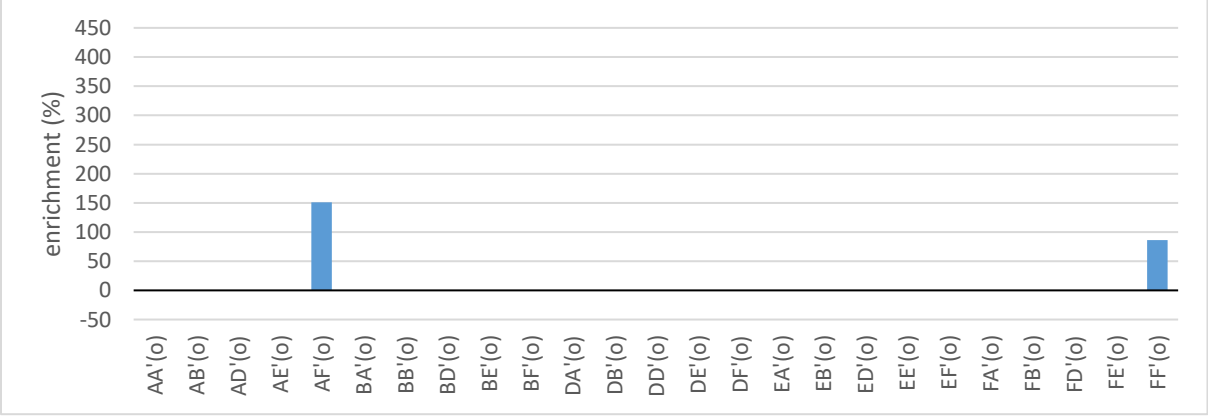
Graph 11. Enrichment of sulfonamides (**CROSS-2(o)** library) after elution in factor Xa assay compared to BSA assay.



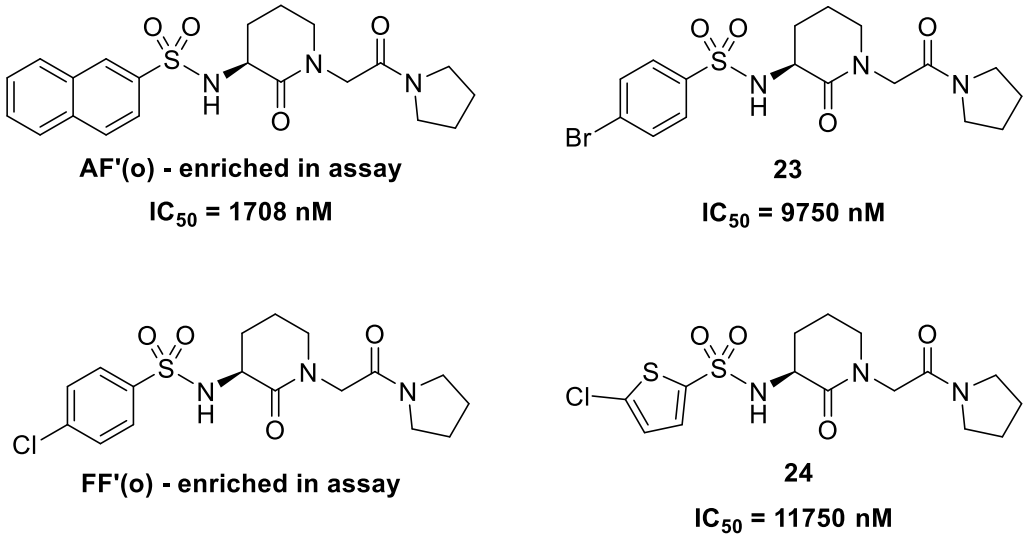
Graph 12. Enrichment of sulfonamides (**CROSS-1(o)** library) after elution in factor Xa assay compared to BSA assay.



Graph 13. Enrichment of sulfonamides (**CROSS-3(o)** library) after elution in factor Xa assay compared to BSA assay.



The compound **AF'(o)** was found in the literature to have inhibitory activity against factor Xa, while the **FF'(o)** compound, with its chemical structure, resembled other compounds **23** and **24** with described affinity for factor Xa (Scheme 31).[74]



Scheme 31. Factor Xa inhibitors including **AF'(o)** and their IC_{50} values and **FF'(o)** enriched in assay with **CROSS-3(o)** library.

This experiment indicates that in the absence of a strong binder, other compounds with much weaker micromolar binding constants can be captured by the assay. The upper limit of the binding constant values remains to be elucidated. This information will be useful for the design and size of the future crossover libraries.

4 Conclusion

This thesis deals with the synthesis of sulfinamides and their subsequent use for crossover reaction. The sulfinamide libraries generated by the crossover reaction were then oxidized and assayed against an enzyme of coagulation cascade factor Xa by affinity selection mass spectrometry.

First, the sulfinamides were synthesized using an optimized one pot reductive method developed in our laboratory. The quality of the thionyl chloride used seems to be a key aspect. Also, some less reactive sulfonyl chlorides required zinc activation prior to the reaction. Therefore, more reactive versions like Rieke zinc can be considered for the future preparation of sulfinamides from less reactive sulfonyl chlorides. The resulting sulfinamides were subjected to acid-catalyzed crossover reaction. Three different libraries starting with five sulfinamides were generated by the crossover reaction and all expected twenty-five sulfinamides were detected by HPLC-MS in all three libraries. These experiments confirmed the generality of the new crossover reaction that tolerates various ligand-like structural motifs and can provide complex libraries starting from a few building blocks. The latest experiments in the lab also indicates that generation of significantly more complex libraries is feasible. In the next step, the sulfinamide libraries were oxidized using mCPBA to a sulfonamide library in order to freeze the dynamic nature of sulfinamides and to generate known inhibitor in the library. HPLC-MS analysis showed that all sulfonamides are generated apart from the ones with an indole structural motif. Preliminary experiments with different oxidizing agents indicated that iodosobenzene diacetate is a milder oxidant that can tolerate also an indole structural motif. The sulfonamide libraries were used in the binding assay against an enzyme factor Xa. Affinity selection mass spectrometry provided an efficient tool to rapidly evaluate all libraries. The crossover library **Cross-2(o)** containing a known factor Xa inhibitor indeed show a strong enrichment of the inhibitor **CF'(o)** in the assay compared to a blank experiment. The **Cross-1(o)** library containing only the S-substituent of the known inhibitor provided no significant enrichment. On the contrary, **Cross-3(o)** containing only the N-substituent of the known inhibitor provided significant enrichment of two other substrates that is in agreement with the reported data. Interestingly, the reported inhibition concentrations (IC_{50}) of the captured sulfonamides range from 32 nM to ~10 μM. This aspect indicates that libraries with relatively weak binders can be captured by the AS-MS assay. These results confirm the potential of the sulfinamide crossover reaction for use in ligand discovery.

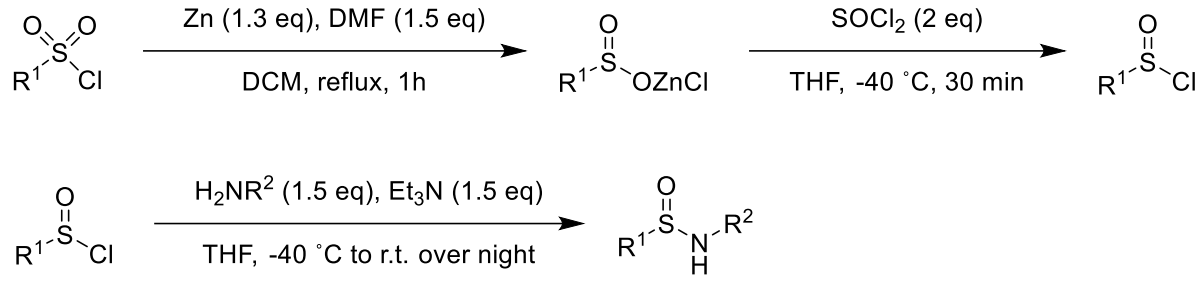
5 Experimental section

5.1 General Procedures and Chemicals

All starting reagents were commercially available and of analytical purity, and were used without further treatment unless otherwise stated. Thionyl chloride was freshly distilled at 76 °C – 78 °C under an inert atmosphere. Anhydrous THF was obtained by reflux with sodium/benzophenone and followed by distillation under a nitrogen atmosphere. CH_2Cl_2 was stored over 4 Å molecular sieves. Zinc powder was activated using a standard method.[75] The pH was analyzed using universal pH indicator strips (from Lach-Ner). Analytical TLC was performed using a pre-coated silica gel 60 Å F_{254} plates (0.2 mm thickness) and visualized by irradiation with UV light at 254 nm and by dipping the plate into $KMnO_4$ solution followed by heating with a heat gun. The $KMnO_4$ solution was prepared as follows: dissolve 1.5 g of $KMnO_4$, 10 g K_2CO_3, and 1.25 mL 10 % NaOH were dissolved in 200 mL of distilled water. Preparative column chromatography was carried out using silica gel 60 Å (particle size 0.063–0.200 mm). Enantiomeric excesses were determined using a SHIMADZU HPLC instrument with a SPD-M20A spectrophotometric detector using columns with a chiral stationary phase Daicel Chiralpak® IA. The detailed conditions are given at the characterization part of the products. The absolute configurations of the products were determined by the comparison of chiral HPLC retention times with the literature. ^{1}H NMR spectra were recorded at 400 MHz. ^{13}C NMR spectra were recorded at 101 MHz and were ^{1}H decoupled. Chemical shifts (δ/ppm) are referenced to the residual $CDCl_3$ signal (^{1}H, δ = 7.26; ^{13}C, δ = 77.0). Coupling constants J are given in Hz, multiplicity is defined as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Accurate mass measurements (HRMS) were obtained by ESI on the Agilent 6530 Q-TOF MS spectrometer. Analytical HPLC was performed under the following conditions: Agilent Eclipse plus C18 column (3.5 µL, 4.6×100 mm); UV/Vis detection at λ_{obs} = 254 nm; flow rate 0.4 mL/min; and gradient elution method (0.1% aqueous formic acid – CH_3CN from 95:5 to 0:100 in 13 min). Infrared spectra were recorded on the Nicolet Avatar 370 FT-IR ATR (thin film). IR absorptions are given in wavenumbers as cm^{-1}. Crossover reactions were carried at 40 °C in the MyTEMPMini incubator without mixing. The pH was measured with HI-2020 edge Hybrid Multiparameter Meter. Factor Xa assay was incubated in the Biosan Thermo-Shaker TS-100C and filtered through the Microcon-10 kDa centrifugal filter with the Ultracel PL-10 membrane. Centrifugation was performed in the Eppendorf Centrifuge 5418 R. The factor Xa and BSA were purchased from New England Biolabs.

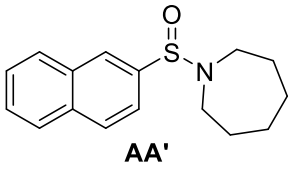
5.2 Synthesis of Sulfinamides

General procedure:



The compounds were prepared by a modified reported procedure.[32] Sulfonyl chloride (0.5 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (3 mL) in 25 mL round-bottom flask and DMF (58 µL, 0.75 mmol, 1.5 equiv.) was added. The resulting mixture was stirred for 10 min. Powdered zinc (42.5 mg, 0.65 mmol, 1.3 equiv.) was added, and the mixture was refluxed under a nitrogen atmosphere until all the starting sulfonyl chloride was consumed (typically 1 h). Then volatiles were evaporated under reduced pressure, the residue dissolved in THF (3 mL) and $SOCl_2$ (72.5 µL, 1 mmol, 2 equiv.) was added dropwise at -40 °C. The resulting mixture was stirred for 30 min at -40 °C under a nitrogen atmosphere. Then, volatiles were evaporated under reduced pressure, the residue dissolved in THF (2 mL), and was added to the solution of Et_3N (104.5 µL, 0.75 mmol, 1.5 equiv.) and amine (0.75 mmol, 1.5 equiv.) in THF (2 ml) at -40 °C. The reaction mixture was stirred at room temperature overnight under nitrogen atmosphere, then diluted with CH_2Cl_2 (10 mL), and washed with saturated aq. $NaHCO_3$ solution (20 mL). The water phase was extracted three times with CH_2Cl_2 (10 mL). The combined organic phases were washed with brine (20 mL), dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. Purification with column chromatography on silica gel afforded the product.

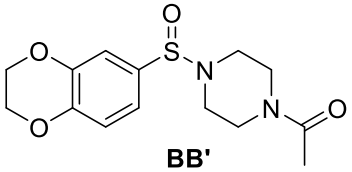
1-(naphthalen-2-ylsulfinyl)azepane (AA')


AA'

Prepared according to the general procedure using 2-naphthalenesulfonyl chloride (227 mg, 1 mmol) and azepane (169 µL, 1.5 mmol). In the first step, the sulfonyl chloride was consumed after 1 hour (TLC CH_2Cl_2:cyclohexane = 1:1). In the second step reaction proceeded for 30 min at -40 °C, then it was allowed to warm up to room temperature for 15 min. Purification by column chromatography (25 % of EtOAc in cyclohexane) afforded the product as a yellow oil, which turns into a yellow crystalline substance upon standing (163 mg,

60 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.31 (s, 1H), 8.04 – 7.88 (m, 3H), 7.65 – 7.55 (m, 3H), 3.40 – 3.16 (m, 4H), 1.75 – 1.51 (m, 8H); **^{13}C{^{1}H} NMR** (101 MHz, $CDCl_3$) δ 141.42, 134.22, 132.93, 128.85, 128.66, 127.85, 127.64, 126.97, 126.87, 122.36, 48.79 (2×), 29.59 (2×), 27.22 (2×); **HRMS** (ESI): *m/z* $[M+H]^+$ calcd for $C_{16}H_{20}NOS^+$ 274.1260, found 274.1263; **IR** (ν_{max}/cm^{-1}) 3043, 3014, 2924, 2854, 1589, 1502, 1452, 1165, 1068, 1049, 758.

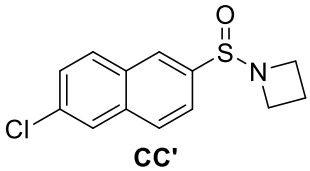
1-(4-((2,3-dihydrobenzo[*b*][1,4]dioxin-6-yl)sulfinyl)piperazin-1-yl)ethan-1-one (BB')



BB'

Prepared according to the general procedure using 2,3-dihydro-1,4-benzodioxine-6-sulfonyl chloride (121 mg, 0.5 mmol) and 1-acetylpiperazine (101 mg, 0.75 mmol). In the first step, the sulfonyl chloride was consumed after 1.5 hours (TLC CH_2Cl_2:cyclohexane = 1:1). Purification by column chromatography (CH_2Cl_2:MeOH = 40:1) afforded the product as a yellow oil (77 mg, 50 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.19 (s, 1H), 7.14 (d, J = 9.3 Hz, 1H), 7.01 (d, J = 8.5 Hz, 1H), 4.33 (s, 4H), 3.75 – 3.62 (m, 2H), 3.58 – 3.46 (m, 2H), 3.21 – 3.10 (m, 2H), 3.05 – 2.96 (m, 2H), 2.10 (s, 3H); **^{13}C{^{1}H} NMR** (101 MHz, $CDCl_3$) δ 169.00, 146.20, 144.05, 134.60, 119.22, 117.90, 115.31, 64.45, 64.26, 46.55, 45.64, 45.58, 41.65, 21.32; **HRMS** (ESI): *m/z* $[M+H]^+$ calcd for $C_{14}H_{19}N_2O_4S^+$ 311.1066, found 311.1084; **IR** (ν_{max}/cm^{-1}) 3066, 2999, 2960, 2925, 2871, 1616, 1579, 1487, 1234, 1178, 1080, 1039.

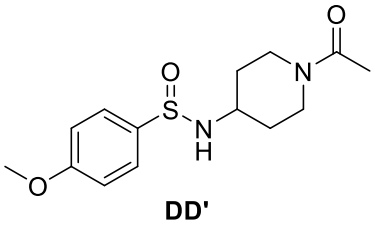
1-((6-chloronaphthalen-2-yl)sulfinyl)azetidine (CC')



CC'

Prepared according to the general procedure using 6-chloronaphthalene-2-sulfonyl chloride (137 mg, 0.5 mmol) and azetidine hydrochloride (74 mg, 0.75 mmol). In the first step, the sulfonyl chloride was consumed after 45 min (TLC CH_2Cl_2:cyclohexane = 1:1). In the third step, CH_2Cl_2 (11 mL) was used as a solvent and 5 equiv. of Et_3N (350 µL, 2.5 mmol). Purification by column chromatography (CH_2Cl_2:MeOH = 100:1) afforded the product as a yellowish oil, which solidified on standing to a yellowish amorphous solid (82 mg, 62 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.24 (s, 1H), 7.91 (d, J = 8.7 Hz, 1H), 7.89 (d, J = 2.1 Hz, 1H), 7.84 (d, J = 8.6 Hz, 1H), 7.65 (dd, J = 8.6, 1.7 Hz, 1H), 7.53 (dd, J = 8.7, 2.1 Hz, 1H), 3.96 (q, J = 7.7 Hz, 2H), 3.53 (q, J = 7.6 Hz, 2H), 2.25 (p, J = 7.7 Hz, 2H); **^{13}C{^{1}H} NMR** (101 MHz, $CDCl_3$) δ 140.67, 134.90, 133.64, 131.03, 130.28, 127.98, 127.92, 126.67, 126.01, 123.14, 45.35 (2×), 16.80; **HRMS** (ESI): *m/z* $[M+H]^+$ calcd for $C_{13}H_{13}ClNOS^+$ 266.0400; found 266.0391; **IR** (ν_{max}/cm^{-1}) 3396, 3053, 2924, 2852, 1597, 1491, 1281, 1250, 1092, 1053, 870.

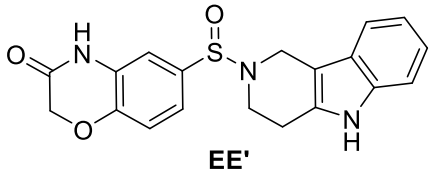
N-(1-acetylpiperidin-4-yl)-4-methoxybenzenesulfinamide (DD')



DD'

Prepared according to the general procedure using 4-methoxybenzenesulfonyl chloride (103 mg, 0.5 mmol) and 1-acetyl-4-aminopiperidine hydrochloride (111 mg, 0.75 mmol). In the first step, the sulfonyl chloride was consumed after 40 min (TLC CH_2Cl_2:cyclohexane = 1:1). In the third step, 3 equiv. of Et_3N (210 µL, 1.5 mmol) were used. Purification by column chromatography (CH_2Cl_2:MeOH = 20:1) afforded the product as a yellowish oil, which solidified on standing to a yellowish amorphous solid (71 mg, 47%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.64 (dd, J = 8.8, 1.0 Hz, 2H), 7.03 (dd, J = 8.8, 1.7 Hz, 2H), 4.52 – 4.34 (m, 1H), 3.99 (dd, J = 21.6, 6.6 Hz, 1H), 3.88 (s, 3H), 3.84 – 3.67 (m, 1H), 3.59 – 3.40 (m, 1H), 3.21 – 3.04 (m, 1H), 2.87 – 2.67 (m, 1H), 2.09 (d, J = 6.8 Hz, 3H), 1.93 – 1.73 (m, 1H), 1.63 – 1.31 (m, 2H); **^{13}C{^{1}H} NMR** (101 MHz, $CDCl_3$) δ 168.85, 161.91, 135.79, 127.40 (2×), 114.34 (2×), 55.54, 50.70, 44.89, 40.13, 34.66, 33.57, 21.42; **HRMS** (ESI): *m/z* $[M+Na]^+$ calcd for $C_{14}H_{20}N_2NaO_3S^+$ 319.1087; found 319.1082; **IR** (ν_{max}/cm^{-1}) 3450, 3192, 3005, 2925, 2858, 1622, 1593, 1441, 1248, 1086, 1047, 831.

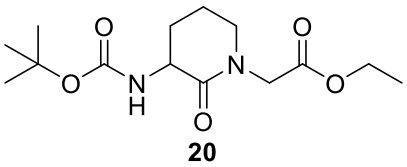
6-((1,3,4,5-tetrahydro-2H-pyrido[4,3-*b*]indol-2-yl)sulfinyl)-2H-benzo[*b*][1,4]oxazin-3(4H)-one (EE')



EE'

Prepared according to the general procedure using 3-oxo-3,4-dihydro-2H-1,4-benzoxazine-6-sulfonyl chloride (130 mg, 0.5 mmol) and 1H,2H,3H,4H,5H-pyrido[4,3-b]indole hydrochloride (164 mg, 0.75 mmol). In the first step, 2.5 equiv. of DMF (100 µL, 1.25 mmol) was used. The sulfonyl chloride was consumed after 5 hours of reflux and overnight at room temperature (TLC CH_2Cl_2:MeOH = 20:1). In the third step, CH_2Cl_2 (15 mL) was used as a solvent and 5 equiv. of Et_3N (350 µL, 2.5 mmol). Purification by column chromatography (CH_2Cl_2:MeOH = 20:1) afforded the product as a white solid (37 mg, 20%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.64 (s, 1H), 8.2 (s, 1H), 7.53 (d, *J* = 2.1 Hz, 1H), 7.28 – 7.21 (m, 2H), 7.17 – 7.04 (m, 2H), 7.03 – 6.92 (m, 2H), 4.57 (d, *J* = 2.0 Hz, 2H), 4.39 (d, *J* = 14.4 Hz, 1H), 4.11 (d, *J* = 14.4 Hz, 1H), 3.72 – 3.54 (m, 2H), 2.96 – 2.87 (m, 2H); **^{13}C{^{1}H} NMR** (101 MHz, $CDCl_3$) δ 164.54, 145.62, 136.51, 135.79, 131.53, 127.26, 125.40, 121.70, 121.65, 119.52, 117.44, 117.19, 114.71, 110.79, 106.99, 67.13, 45.81, 41.01, 24.55; **HRMS** (ESI): *m/z* $[M+Na]^+$ calcd for $C_{19}H_{17}N_3NaO_3S^+$ 390.0883; found 390.0882; **IR** (ν_{max}/cm^{-1}) 3390, 3190, 3045, 2924, 2852, 1689, 1601, 1491, 1375, 1072, 1036.

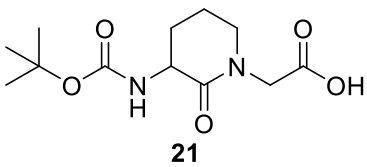
Ethyl 2-(2-((tert-butoxicarbonyl)amine)-*δ*-valerolactam-N-yl)acetate (20)



20

Compound **20** was prepared according to a published procedure.[76] A solution of 3-(Boc-amino)-2-piperidone (1.125 g, 5 mmol) and NaH (20 mmol, 672 mg of 60% oil dispersion, 4 equiv.) in THF (35 ml) was cooled to 0 °C and stirred at that temperature for 30 min. Ethyl bromoacetate (2.28 mL, 20 mmol, 4 equiv.) was added, brought to room temperature and allowed to react for 20 hours. The reaction mixture was quenched with saturated aq. $NaHCO_3$ solution (30 mL) and extracted with EtOAc (3 × 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography (50 % of EtOAc in cyclohexane) afforded the product as a colorless oil (1.40 g, 93%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.44 (br s, 1H), 4.30 – 4.08 (m, 5H), 3.91 (d, J = 17.3 Hz, 1H), 3.48 – 3.34 (m, 2H), 2.58 – 2.46 (m, 1H), 2.03 – 1.94 (m, 2H), 1.71 – 1.61 (m, 1H), 1.46 (s, 9H), 1.29 (t, J = 7.2 Hz, 3H); **HRMS** (ESI): *m/z* $[M+Na]^+$ calcd for $C_{14}H_{24}N_2NaO_5^+$ 323.1577; found 323.1538. The spectra were in agreement with the reported data.[76]

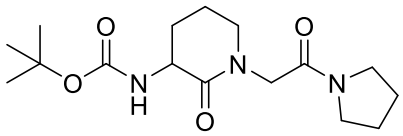
2-(2-tert-butoxicarbonylamine-*δ*-valerolactam-N-yl)acetic acid (21)



21

Compound **21** was prepared according to a modified published procedure.[77] A stirred solution of compound **20** (301 mg, 1 mmol) in EtOH (1.2 mL) was cooled at 0 °C. $LiOH{\cdot}H_2O$ solution (2.5 M, 1.2 mL, 3 equiv.) was added. The mixture was stirred at room temperature for 90 min. The reaction was quenched with saturated aq. NH_4Cl solution (3 mL), and pH of the reaction mixture was adjusted to 3 with concentrated HCl. The aqueous phase was extracted with EtOAc (7 × 5 mL). The combined organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. The crude product was dried in vacuo for 6 hours. The product was afforded as an amorphous white solid (205 mg, 75 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.57 (br s, 1H), 4.31 (dd, J = 17.3, 2.7 Hz, 1H), 4.20 – 4.06 (m, 1H), 3.95 – 3.81 (m, 1H), 3.52 – 3.32 (m, 2H), 2.41 (s, 1H), 2.02 – 1.90 (m, 2H), 1.80 – 1.65 (m, 1H), 1.44 (s, 9H); **HRMS** (ESI): *m/z* $[M-H]^-$ calcd for $C_{12}H_{19}N_2O_5^-$ 271.1300; found 271.1327. The spectra were in agreement with the reported data.[77]

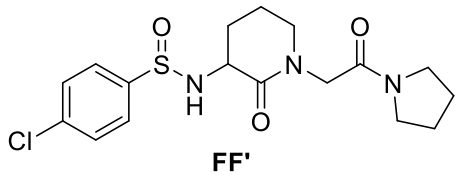
1,1-dimethylethyl [(3S)-1-[2-oxo-2-(1-pyrrolidinyl)ethyl]-2-oxo-3-piperidinyl]carbamate (22)



22

Compound **22** (160 mg, 0.587 mmol) was dissolved in CH_2Cl_2 (5 mL) and HOBt (270 mg, 1.76 mmol, 3 equiv.) was added. After 10 min of stirring under a nitrogen atmosphere pyrrolidine (147 µL, 1.76 mmol, 3 equiv.) and 1-ethyl-(3-(3-dimethylamino)propyl)-carbodiimide (317 µL, 1.76 mmol, 3 equiv.) were added. The mixture was stirred at room temperature overnight. The reaction was dissolved in CH_2Cl_2 (40 mL) and washed with saturated aq. NH_4Cl solution (3 × 20 mL), then saturated aq. $NaHCO_3$ solution (20 mL), and brine (20 mL). The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. Purification by column chromatography (CH_2Cl_2:MeOH = 20:1) afforded the product as a colorless oil (96 mg, 50 %). 39 % *ee*, the enantiomeric excess (*ee*) was determined by HPLC (IA chiralpak: heptane/propan-2-ol (80:20); flow rate 1 mL/min; 25 °C; 210 nm; t_R = 11.8 min (minor enan.), t_R = 15.3 min (major enan.); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 5.45 (br s, 1H), 4.30 (d, J = 16.0 Hz, 1H), 4.15 (p, J = 5.6 Hz, 1H), 3.84 (d, J = 16.1 Hz, 1H), 3.57 – 3.37 (m, 6H), 2.53 – 2.43 (m, 1H), 2.06 – 1.92 (m, 4H), 1.93 – 1.82 (m, 2H), 1.80 – 1.67 (m, 1H), 1.46 (s, 9H). **^{13}C{^{1}H} NMR** (101 MHz, $CDCl_3$) δ 170.43, 165.88, 155.98, 79.43, 51.80, 49.58, 49.00, 45.94, 45.72, 28.37 (3×), 27.92, 26.15, 24.07, 20.89. **HRMS** (ESI): *m/z* $[M+H]^+$ calcd for $C_{16}H_{28}N_3O_4^+$ 326.2074; found 326.2074; **IR** (ν_{max}/cm^{-1}) 3410, 3307, 2972, 2873, 1707, 1635, 1444, 1163.

4-chloro-N-(2-oxo-1-(2-oxo-2-(pyrrolidin-1-yl)ethyl)piperidin-3-yl)benzenesulfinamide (FF')



FF'

Prepared according to the general procedure using 4-chlorobenzenesulfonyl chloride (62 mg, 0,3 mmol) and compound **22** (96 mg, 0.3 mmol) that was first deprotected according to the following procedure. Compound **22** was dissolved in CH_2Cl_2 (5 mL). While stirring under nitrogen atmosphere TFA (339 µL, 4.43 mmol, 15 equiv.) was added. The mixture was stirred at room temperature for 2 hours. The solvent was evaporated under reduced pressure and the crude product was used for the coupling with sulfinyl chloride. In the first step, the sulfonyl chloride was consumed after 1 hour (TLC CH_2Cl_2:cyclohexane = 1:1). In the third step, 10 equiv. of Et_3N (411 µL, 3 mmol) were used. Purification by column chromatography (CH_2Cl_2:MeOH = 15:1) afforded the product as a yellow oil (15 mg, 13 %). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.74 – 7.68 (m, 2H), 7.50 – 7.46 (m, 2H), 4.36 – 4.23 (m, 1H), 4.15 – 3.92 (m, 1H), 3.87 – 3.76 (m, 1H), 3.54 – 3.35 (m, 6H), 2.61 – 2.47 (m, 1H), 2.07 – 1.95 (m, 4H), 1.90 – 1.84 (m, 2H), 1.62 – 1.45 (m, 1H);

$^{13}C\{^1H\}$ NMR (101 MHz, $CDCl_3$) δ 170.38, 169.33, 165.59, 143.99, 143.12, 137.31, 129.05, 127.50, 49.71, 49.32, 48.92, 45.97, 45.69, 28.47, 26.14, 24.07, 20.73; **HRMS** (ESI): *m/z* $[M+H]^+$ calcd for $C_{17}H_{23}ClN_3O_3S^+$ 384.1149; found 384.1138; **IR** (ν_{max}/cm^{-1}) 3492, 3431, 3234, 3080, 2954, 2922, 2873, 1628, 1446, 1050, 1012.

5.3 Crossover Reaction

250 mM stock solutions of individual sulfinamides were prepared in DMSO and dried with 4Å MS overnight. For the crossover reaction of five sulfinamides, 8 µL of the stock solution of each sulfinamide was placed into 1.5 mL glass vial with a screwing cap, followed by 9.5 µL of DMSO. The reaction started by adding 0.5 µL of freshly prepared 1M stock solution of trifluoroacetic acid (TFA) in DMSO (final concentration of sulfinamides 200 mM, final concentration of TFA 10 mM). The reaction vial was filled with argon, parafilmed, and incubated at 40 °C. The progress of the reaction was monitored by analytical HPLC-MS. Aliquots (1 µL) of the mixtures were diluted with Et_3N in CH_3CN (110 mM, 999 µL) and analyzed by HPLC-MS.

5.4 Oxidation of Sulfinamide Crossover Libraries

The sulfinamides libraries generated in the crossover reaction were subsequently oxidized. The crossover reaction (46 µL, 9.2 µmol) was quenched with Et_3N (2.5 µL, 18.4 µmol, 2 equiv.) and the mixture was lyophilized. The obtained oily residue was dissolved in CH_2Cl_2 (250 µL) and 3-chloroperoxybenzoic acid (<77%, 4.65 mg, 20.4 µmol, 2.2 equiv.) was added in one portion and the reaction mixture was stirred under argon atmosphere for 1 hour at room temperature. Then, 1 µL aliquot of the reaction mixture was diluted with Et_3N solution in CH_3CN (110 mM, 999 µL) and analyzed by HPLC-MS.

5.4.1 Test of Reagents for Oxidation of a Model Substrate **EE'**

From the prepared stock solution of sulfinamide **EE'** (250 mM in DMSO) 4 µL (1 µmol) were placed into a 1.5 mL glass vial with a screwing cap, followed by 8.6 µL of DMSO, and 1.4 µL of freshly prepared 100 mM stock solution of TFA (final concentration of sulfinamide 71.4 mM, and TFA 10 mM). Then Et_3N (0.6 µL, 4 µmol) was added, and the mixture was lyophilized. The obtained oily residue was dissolved in CH_2Cl_2 (200 µL) and equally divided into four 1.5 mL glass vials with a screwing cap (50 µL, 0.25 µmol of sulfinamide in each vial). TCCA (2 µL of 250 mM stock solution in EtOAc, 0.5 µmol), iodosobenzene diacetate (2 µL of 250 mM stock solution in CH_2Cl_2, 0.5 µmol), tert-butyl hydroperoxide (2 µL of 250 mM stock solution

in CH_2Cl_2, 0.5 µmol) and mCPBA (2 µL of 250 mM stock solution in CH_2Cl_2, 0.5 µmol) were each added to the different vials and stirred under argon atmosphere for 1 hour (final concentration of EE' 5 mM, oxidant 9.6 mM). Then, 10 µL aliquot of the reaction mixture was diluted with Et_3N solution in CH_3CN (110 mM, 90 µL) and analyzed by HPLC-MS.

5.5 Binding Assay

A 1.5 mL Eppendorf tube was charged with sulfonamide crossover library (100 µL of 1 µM stock solution) in factor Xa buffer (20mM TRIS-HCl, 100 mM NaCl, 2 mM $CaCl_2$, pH = 8) and factor Xa (0.85 µL of 117 µM, final concentration 1µM) in factor Xa buffer. At the same time another 1.5 mL Eppendorf tube was charged with sulfonamide crossover library (100 µL of 1 µM stock solution in factor Xa buffer) and 0.86 µL of factor Xa buffer was added. Both samples were incubated for 1 hour at 25 °C in a shaker (250 RPM). Samples were transferred to the Microcon-10 kDa centrifugal filter with the Ultracel PL-10 membrane and centrifuged for 15 min at 25 °C (14000 RPM). Samples were washed with 2×100 µL of factor Xa buffer, and after each wash samples were centrifuged (15 min, 25 °C, 14000 RPM). The combined filtrate was collected for HPLC-MS analysis. Samples were eluted with 3×100 µL of methanol, and after each elution samples were centrifuged (15 min, 25 °C, 14000 RPM). The combined filtrate was collected for HPLC-MS analysis. In another experiment, BSA protein (0.66 µL of 150 µM, final concentration 1 µM) was used instead of factor Xa buffer for the control experiment.

6 Use of Artificial Intelligence in This Work

Artificial intelligence, ChatGPT (OpenAI) and Grammarly (Grammarly, Inc.), was employed as a supplementary tool during the preparation of this thesis. It was utilized for providing linguistic corrections, stylistic suggestions, and improving clarity in English and for shortening sections and eliminating redundancy. All AI-generated outputs were carefully reviewed, edited, and adapted by the author to ensure accuracy and compliance with academic standards.The use of AI aligns with ethical practices and does not substitute for the author's critical analysis and independent research.

7 References

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