Dynamic Systems: Evaluation, Screening and Synthetic Applications

Morakot Sakulsombat

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Abstract

The research work reported in the thesis deals with the development of dynamic covalent systems and their applications in evaluation and screening of protein-ligands and enzyme inhibitors, as well as in synthetic methodologies. The thesis is divided into four parts as described below.

In part one, synthetic methodologies to access 3-functionalized phthalides and 3-thioisoindolinones using the concept of cascade reactions are demonstrated. Efficient syntheses of the target products are designed and performed in one-pot process under mild reaction conditions.

In part two, phosphine-catalyzed disulfide metathesis for the generation of dynamic carbohydrate system in aqueous solution is demonstrated. In the presence of biological target (Concanavalin A), the optimal dynamic ligand is successfully identified in situ by the $^1$H STD-NMR spectroscopy.

In part three, lipase-catalyzed resolutions of dynamic reversible systems using reversible cyanohydrin and hemithioacetal reactions in one-pot processes are demonstrated. The dynamic systems are generated under thermodynamic control in organic solution and subsequently resolved by lipase-mediated resolution under kinetic control. The resolution processes resulted in the lipase-selected substrates with high structural and stereochemical specificities.

In the last part, dynamic fragment-based strategy is presented using β-galactosidase as a model target enzyme. Based on our previous study, the best dynamic inhibitor of β-galactosidase was identified using $^1$H STD-NMR technique from dynamic hemithioacetal systems. The structure of the dynamic inhibitor is tailored by fragment linking and optimization processes. The designed inhibitor structures are then synthesized and tested for inhibition activities against β-galactosidase.

**Keywords**: constitutional dynamic chemistry; dynamic combinatorial chemistry/resolution; dynamic fragment-based approach; dynamic kinetic resolution; dynamic reversible systems; $^1$H STD-NMR; multicomponent reaction; tandem reaction.
This thesis is based on the following papers, referred to in the text by their Roman numerals I-VI:

I. **Tandem Reversible Addition-Intramolecular Lactonization for the Synthesis of 3-Functionalized Phthalides**
   Morakot Sakulsombat¹, Marcus Angelin¹, Olof Ramström

II. **A Dynamic Multicomponent Approach for One-Pot Synthesis of 3-Thioisooindolinones**
    Morakot Sakulsombat, Marcus Angelin, Rémi Caraballo, Olof Ramström
    *Submitted for publication.*

III. **Phosphine-Mediated Disulfide Metathesis in Aqueous Media**
    Rémi Caraballo, Morakot Sakulsombat, Olof Ramström

IV. **In Situ Evaluation of Lipase Performances Through Dynamic Asymmetric Cyanohydrin Resolution**
    Morakot Sakulsombat, Pornrapee Vongvilai, Olof Ramström

V. **Dynamic Asymmetric Hemithioacetal Resolution by Lipase-Catalyzed \( \gamma \)-Lactonization: In Situ Tandem Formation of 1,3-Oxathiolan-5-one Derivatives**
    Morakot Sakulsombat, Yan Zhang, Olof Ramström
    *Submitted for publication.*

VI. **Towards Dynamic Drug Design: Identification and Optimization of \( \beta \)-Galactosidase Inhibitors from a Dynamic Hemithioacetal System**
    Rémi Caraballo, Morakot Sakulsombat, Olof Ramström

¹ Authors contributed equally to this work
Publication not included in this thesis:

Journal article

**Racemase Activity of *B. cepacia* Lipase Leads to Dual-Function Asymmetric Dynamic Kinetic Resolution of α-Aminonitriles**

Pornrapee Vongvilai, Mats Linder, Morakot Sakulsombat, Maria Svedendahl Humble, Per Berglund, Tore Brinck, Olof Ramström

*Angew. Chem., Int. Ed.* **2011**, *Accepted for publication.*
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<tr>
<td>Ac</td>
<td>acetyl group</td>
</tr>
<tr>
<td>Asp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>CAL-B</td>
<td><em>Pseudozyma</em> (formaly <em>Candida</em> antartica) lipase B</td>
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<tr>
<td>CDC</td>
<td>Constitutional Dynamic Chemistry</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate Recognition Domain</td>
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<tr>
<td>d</td>
<td>day(s)</td>
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<tr>
<td>d</td>
<td>deuterated</td>
</tr>
<tr>
<td>DCC</td>
<td>Dynamic Combinatorial Chemistry</td>
</tr>
<tr>
<td>DCR</td>
<td>Dynamic Combinatorial Resolution</td>
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<tr>
<td>dr</td>
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<td>dithiothreitol/ 1,4-bis(sulfanyl)butane-2,3-diol</td>
</tr>
<tr>
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<td>Enzyme Commission</td>
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<tr>
<td>ee</td>
<td>enantiomeric excess</td>
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</tr>
<tr>
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<td>Histidine</td>
</tr>
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<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
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<tr>
<td>NMR</td>
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<tr>
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<tr>
<td>PS</td>
<td><em>Burkholderia</em> (formaly <em>Pseudomonas</em>) cepacia</td>
</tr>
<tr>
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</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>S&lt;sub&gt;N&lt;/sub&gt;2</td>
<td>bimolecular nucleophilic substitution</td>
</tr>
<tr>
<td>STD</td>
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<tr>
<td>-------</td>
<td>--------------------------------</td>
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<tr>
<td>Thr</td>
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</tr>
<tr>
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<td>Tyrosine</td>
</tr>
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<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
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<tr>
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<tr>
<td>°C</td>
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1

Introduction

Organic chemistry is the branch of chemical science dealing with designing, synthesizing, and characterizing compounds as well as developing applications for compounds which contain carbon. In various chemical industries, organic chemistry is involved in key processes, for example in the production of food, medicine, plastics, fuels, cosmetics, detergents, and electronic devices. These chemical processes rely on the development and optimization of chemical reactions and their adaptation to production conditions. The goal of chemical synthesis is often the development of novel and efficient chemical reactions as well as the incorporation of catalysts or biocatalysts into known syntheses to streamline the synthesis of target chemicals and reduce the production cost.

This development is perhaps especially important in the pharmaceutical industry, where novel synthetic methodologies and techniques for identifying potential drug candidates have become a requirement for success. Historically, most drugs were discovered either by identification of active compounds from traditional remedies or in some particular cases by sheer serendipity. The identified compounds were tested against different biological targets and further synthetically modified in order to obtain compounds with high biological activities.

The necessity for new, fast and efficient drug production have however spawned novel drug discovery methodologies. Methodologies are now based on understanding disease at the molecular and physiological level, and utilizing the specificity of biological targets. The combination of organic synthetic chemistry, molecular biology and computational science has led to the unparalleled progress where in biologically active compounds have been identified, as well as designed and synthesized based on the knowledge of biological targets. In this context, some of the most important synthetic methodologies in the drug discovery process are based on combinatorial (high-throughput) and fragment-based strategies. In addition, dynamic chemistry, based on the application of reversible reactions, has emerged as a new technique for rapid identification of ligands for biological targets and inhibitors of enzymatic processes.

1.1. Reversible chemistry

Chemists, especially synthetic organic chemists, are trained to focus on efficient synthetic reactions in order to obtain high conversion, purity and selectivity of the
desired products. Although reversible chemical reactions have been known for more than two centuries, synthetic chemists generally avoid reversible reactions because they often do not reach completion, resulting in mixtures that may be difficult to purify.

Reversible chemical reactions were first proposed by C. L. Berthollet in 1803. Berthollet speculated that the formation of sodium carbonate crystals around the edges of a salt lake in Egypt might be a reversible process, having resulted from the high concentration of salt in the lake (Scheme 1).

\[
\begin{align*}
\text{Sodium carbonate formation at the edge of the salt lake} \\
2 \text{NaCl} + \text{CaCO}_3 & \rightarrow \text{Na}_2\text{CO}_3 + \text{CaCl}_2 \\
\text{The reverse reaction} \\
\text{Na}_2\text{CO}_3 + \text{CaCl}_2 & \rightarrow 2 \text{NaCl} + \text{CaCO}_3
\end{align*}
\]

Scheme 1. The formation of sodium carbonate and its reverse reaction.

In 1864, Berthollet’s observation was supported by P. Waage and C. M. Guldberg. They formulated the law of mass action – a mathematic model for predicting the behavior of the solution in dynamic equilibrium. The reversible reaction theory was subsequently settled in the chemical society when H. L. Le Châtelier, in parallel with J. H. van’t Hoff, further explained the behavior of reversible reactions. Their explanation has later been named “Le Châtelier’s principle”. In a closed system, a reversible reaction reaches its equilibrium when the rates of the forward and backward reactions are equal. If a reaction parameter changes, for example, temperature, pressure, volume, or concentration, the equilibrium of the reversible reaction will shift in a way to compensate for changed condition.

1.2. Applications of reversible reactions

Since these early discoveries, reversible reactions can be applied into several instances, from simply controlling the reaction conditions (using Le Châtelier’s principle), to combining reactions with other transformations, for example, in cascade reactions and dynamic kinetic resolutions. Recently, systems incorporating multiple exchange and interexchange reactions have been developed, often referred to as constitutional dynamic chemistry (CDC).
1.2.1. Cascade reactions

Following the emergence of synthetic organic chemistry, a wide range of synthetic methodologies have been developed. From simple single step reactions producing small organic compounds, the field has now advanced to encompass multi-step reactions producing complex molecules with high stereoselectivity. The awareness of reducing waste has also grown with the emphasis on the minimization of environmental harm. The ideal synthetic chemical reaction is regarded to be a multi-step reaction operated in a one-pot process in order to reduce the production time, cost and waste. With this idealistic objective in mind, a convincing breakthrough was the synthesis of tropinone (4), a natural bicyclic alkaloid compound, by R. Robinson in 1917. Several bonds could thus successfully be formed in a one-pot process using the reaction of succindialdehyde (1), methylamine, and an acetonedicarboxylic salt (2) in a double Mannich reaction, generating tropinone (4) (Scheme 2).

![Scheme 2. Robinson’s total synthesis of tropinone 4.](image)

Until now, these conceptual reactions are variably named as “cascade”, “domino”, “tandem”, “sequential”, or “consecutive” reactions to describe the process, in which two or more sequential transformations involving consecutive bond formation, are generated in one-pot process without isolating any intermediates. The advantages of cascade reaction has led to the exploration of many new combinations of consecutive reactions for the synthesis of natural product compounds, for example, (+)-progesterone, endiandric acid, and (+)-hirsutene. Since then, cascade reactions have been challenged for designing new synthetic procedures and exploring new complex reactions, based on several key reaction mechanisms; nucleophilic, electrophilic, radical, pericyclic, and transition metal-catalyzed reactions.

Multicomponent reactions, an important subcategory of cascade reactions, are defined as reactions containing three or more starting materials consecutively reacting in a one-pot process to provide complex products. Although, the concept of multicomponent reactions has been known for more than a century, it
has only witnessed its most extensive development during the last two decades, following the increased interests in high-throughput and medicinal chemistry. The most attractive advantage of multicomponent reactions is not only reducing the production cost but also allowing facile and rapid access to high diversity of complex products in one-pot reactions. By employing a multicomponent reaction, the substituents on the target product motif can be easily modified by changing the series of the starting materials. Increasing the number of starting materials will in principle raise the degree of product diversity.

Two famous and versatile multicomponent reactions, the Passerini and Ugi reactions, are commonly used in synthetic routes to access various natural product compounds and peptide fragments (Scheme 3).\textsuperscript{33, 48, 49} The Passerini three-component reaction is the nucleophilic addition reaction of acid 5, aldehyde or ketone 6, and isocyanide 7 resulting \(\alpha\)-acyloxy amide product 8 (Scheme 3a). In analogy, addition of one more component, primary amine 9, in the Ugi four-component reaction, results in at least four possible substituted modifications on the product motif (Scheme 3b). Furthermore, these two powerful reactions can also supply asymmetric products using prochiral substrates (aldehydes or ketones). These basic considerations of multicomponent reactions are gaining strong interest from chemists, especially in medicinal chemistry.

**Scheme 3.** Examples of multicomponent reactions. a) Passerini three-component reaction; b) Ugi four-component reaction.

The ideal benefits of the strategy using a combination of multiple, consecutive reversible reactions together with an irreversible transformation are obtaining excellent conversion of desired product and avoiding the difficult purification of reaction mixture. Until now, the attention on cascade and multicomponent
reactions has focused on improving reaction efficiencies for production processes, applying a known reaction for synthesis of new interesting compound series, and exploring new and more sophisticated cascade reactions.

1.2.2. Dynamic kinetic resolution

The preparation of optically active drugs is one of the most important tasks for the pharmaceutical industry. For this reason, more facile and efficient methodologies for the preparation of chiral compounds have been extensively explored by both industrial and academic laboratories. Classical techniques, such as crystallization and solution-phase separation demand great skills and long production time to obtain optically active compounds on large scale. Another approach is to separate racemic mixtures, for example, through kinetic resolution (KR), where two enantiomers react at different reaction rates in a chemical transformation.\(^{50-56}\) In the presence of a chiral environment, one enantiomer of a substrate (\(S_R\)) may be transformed to its corresponding product (\(P_R\)) faster than the transformation of its enantiomer (\(S_S\)). In other words, the reaction rate of \(S_R\) is faster than \(S_S\) (\(k_R > k_S\)) as shown in Figure 1a. If the rate difference is large, one can obtain the transformed product \(P_R\) and the remaining substrate \(S_S\) in equal amounts. The different properties between \(P_R\) and \(S_S\) may subsequently allow for straight-forward separation of the final mixture.

![Conceptual schemes of dynamic kinetic resolution](image)

**Figure 1.** Conceptual schemes of a) kinetic resolution and b) dynamic kinetic resolution.

KR has been further developed to increase the efficiency of the reaction. By addition of a reversible reaction between two enantiomers, the unused enantiomer can be converted to the desired one. According to Le Châtelier’s principle, when the equilibrium between two enantiomers is disturbed by transformation of reactant \(S_R\) to product \(P_R\), the dynamic equilibrium of the reversible reaction will reproduce reactant \(S_R\) until both enantiomers of the starting compound are completely consumed. In an ideal case, the racemization rate \((k_{rac})\) is much faster than the resolution rate \((k_R)\) which allows the product \(P_R\) to be formed in 100% yield and
100% enantiomeric excess as shown in Figure 1b. The advantage of DKR is not only to reach full conversion of the optically pure product, but also to avoid a difficult separation process.

The increased demand of efficient asymmetric synthetic methods from the pharmaceutical industry has increased the expansion of diverse synthetic methodologies. Thus, new racemization techniques which are compatible with the kinetic resolution process have been extensively pursued. These techniques have mainly been developed from efficient reversible chemical reactions, as well as metal induced and enzymatic catalyzed racemization of prochiral starting materials. Furthermore, the asymmetric resolution process has also been developed using novel chiral metal catalysts, as well as new catalytic reactions of biocatalysts.

1.2.3. Constitutional dynamic chemistry (CDC)

The concept of CDC is based on chemical reversibility using both molecular and supramolecular interactions. The reversible reactions between components through covalent or non-covalent bonds are employed to generate a constitutional dynamic system under thermodynamic control, which can be used in a variety of applications. Based on Le Châtelier’s principle, the generated dynamic system is amenable to adaptive control from additional internal or external selection pressure. The most responsive constituent will be amplified from the dynamic system. The concept has led to the extensive exploration of new reversible reactions using both covalent and non-covalent bond formations. The applications of CDC can be categorized into three main topics, presented below.

Exploring the key features of constitutional dynamic chemistry

The key features of CDC are the generation of a dynamic reversible system and the study of the behavior of the formed dynamic system. The dynamic system consists of multiple components interacting with each other by either covalent or non-covalent interactions. The behavior of the dynamic system is the consequence of competing factors, which can arise from internal motivation (or internal selection) or external selection. For the former case, the dynamic system self-adapts to produce only the most stable constituent which is self-organized or self-assembled. For the latter case, when a reaction condition such as temperature or pH of the system is changed, or an external selection pressure, for example, electric field or metal ion, is applied, the dynamic system will adjust and produce the most responsive constituent.
Some of the earliest applications of CDC were developed in the groups of J. –M. Lehn and J. K. M. Sanders. In Lehn’s work, dynamic systems of circular helicates were generated by mixing tris-bipyridine ligands with an octahedral metal ion (such as Fe$^{2+}$). By applying different counterions, the helicate sizes could be changed from pentagons to hexagons. In the presence of a given ion, the dynamic system underwent adaptation to produce the most suitable size of helicates. In Sander’s group, the concept of macrocyclic receptor formation was used. Dynamic systems of macrocycles were generated and the specific size of certain macrocycles was amplified using specific guests.

**Generation of dynamic systems for biological matching**

This topic bears similarity to the concept of combinatorial chemistry, which involves screening of compound collections for specific ligands and potent inhibitors of various biological entities. Dynamic systems based on labile constituents have been generated by covalent bond formation under thermodynamic control. The fittest constituent, displaying the most favorable interaction with the target biomolecule, can then be identified under either thermodynamic or kinetic control using various identification techniques. For example, a dynamic covalent system was generated *in situ* under thermodynamic control in the presence of the target enzyme, and the best constituent was simultaneously kinetically resolved. This kinetic matching technique is called dynamic combinatorial resolution (DCR).

**Exploration of dynamic materials**

Based on the fundamental idea of statically synthetic materials, novel materials with desired properties can be designed and processed from dynamic systems through molecular or supramolecular bonds between the building blocks. Dynamic synthetic polymers were for example prepared from monomers bearing functional groups that can undergo interchange due to reversible covalent bonds. The resulting polymers possess the abilities to change in response to applied external stimulations, for example, heat, light, additives, etc.
1.3. Types of reversible reactions

Central to dynamic system generation is the reversible bond formation between the system components, and a multitude of interconnection types have been evaluated for this purpose. In principle, reversible chemical bonds can be classified into two types based on whether they are covalent or non-covalent formations (Figure 2).\textsuperscript{20, 22, 24} The reversible covalent bond formation can be divided into three different types, depending on atoms forming bonds, which are carbon-carbon, carbon-heteroatom, and heteroatom-heteroatom bond formations. In cascade reactions, reversible Diels-Alder and metathesis reaction and aldol formation are commonly used, while in DKR, the reversible reactions, involving in generation of a chiral center, such as imine, aldol and hemithioacetal formations, are often used.

In CDC, both covalent and non-covalent bond formations are used for generation of molecular and supramolecular dynamic systems, respectively. It should be noted that the reaction conditions for the reversible reaction should be compatible with the selection pressure and side products or side reactions that may interfere with the entire system should be avoided.
1 Covalent bond formation

a) Carbon-carbon bond formation

Diels-Alder reaction

\[ \text{CH} = \text{CH} + \text{CH} = \text{CH} \rightleftharpoons \text{CH} - \text{CH} - \text{CH} - \text{CH} \]

Metal thesis reaction

\[ R_1 \text{CH} = \text{CH} \rightleftharpoons R_1 \text{CH} - \text{CH}R_2 \]

Aldol formation

\[ \text{CHO} + \text{CHO} \rightleftharpoons \text{CHOH} - \text{CHO} \]

b) Carbon-heteroatom bond formation

Imine formation

\[ \text{CHO} + \text{H}_2\text{NR} \rightleftharpoons \text{CHR} \rightleftharpoons \text{H}_2\text{O} \]

Imine exchange

\[ \text{NH} + \text{H}_2\text{NR}_1 \rightleftharpoons \text{NH}_1 + \text{H}_2\text{NR} \]

Hemiacetal formation

\[ \text{CHO} + \text{HO}R_1 \rightleftharpoons \text{CHOH} - R_1 \]

Hemithioacetal formation

\[ \text{CHO} + \text{HS}R_1 \rightleftharpoons \text{CHO} - R_1 \]

Transtioesterification

\[ \text{COS}R + \text{HSR}_1 \rightleftharpoons \text{COS}R_1 + \text{HSR} \]

Michael addition

\[ \text{OCR} + \text{RSR}_2 \rightleftharpoons \text{OCR}_1 + \text{SR} \]

c) Heteroatom-heteroatom bond formation

Disulfide exchange

\[ \text{H}_2\text{SR}_2 + R_1\text{S} - R_4 \rightleftharpoons \text{H}_2\text{SR}_1 + R_1\text{S} - R_2 \]

Boronic ester formation

\[ \text{R} = \text{B(OH)}_2 + \text{HO}R_1 \rightleftharpoons \text{R} = \text{B(OH)}_2 \rightleftharpoons \text{R}_1 \]

2 Non-covalent bond formation

Metal coordination

\[ [\text{ML}_1]^{n+} + nL_2 \rightleftharpoons [\text{ML}_2]^{n+} + nL_1 \]

Hydrogen bonding

\[ \text{H} = \text{O} \rightleftharpoons \text{H} = \text{O} \]

\[ \text{H} \text{N} \rightleftharpoons \text{H} \text{N} \]

\[ \text{H} \text{N} \rightleftharpoons \text{H} \text{N} \]

\[ \text{H} \text{N} \rightleftharpoons \text{H} \text{N} \]

\[ \text{H} \text{N} \rightleftharpoons \text{H} \text{N} \]

Figure 2. Types of reversible reactions: 1) covalent bond formations with a) carbon-carbon; b) carbon-heteroatom; and c) heteroatom-heteroatom bond formations; 2) non-covalent bond formations.
1.4. The aim of this thesis

The overall aim of this work was to explore the use of reversible chemical reactions for the synthesis of selected target compounds, and the identification of the fittest ligands for biological molecules, utilizing the concept of CDC. First, reversible reactions were successfully applied in cascade and multicomponent reactions for facile synthesis of 3-functionalized phthalides and 3-thioisoindolinones in one-pot processes. Next, phosphine-catalyzed disulfide metathesis was used to generate dynamic carbohydrate systems, where the best constituent was identified in situ by $^1$H STD-NMR in the presence of a target biological molecule. Furthermore, multiple reversible covalent reactions, based on cyanohydrin reaction and hemithioacetal formations, were employed to generate complex dynamic systems. Enzyme-catalyzed asymmetric transformations were subsequently applied to kinetically resolve the fittest substrates from the dynamic systems. Finally, a dynamic fragment-based approach was used to design a series of inhibitors, mimicking the structure of the best labile inhibitor, identified by $^1$H STD-NMR from the previous study. The designed inhibitors were synthesized and inhibition activities tested against a target enzyme.
2

Designed cascade reactions for facile syntheses of 3-functionalized phthalides and 3-thioisoindolinone derivatives

(Papers I-II)

2.1. Introduction

From economic and practical points of view, the concept of cascade reactions in organic chemistry, performing consecutive multi-step reactions in one-pot processes, has rapidly gained interest during the past few decades. The studies have mainly been focused on the design and optimization of known reactions for highly efficient synthetic methodologies, as well as the exploration of novel and more sophisticated cascade reactions. The main advantages of cascade reactions in organic synthesis are that the final products, displaying efficient atom economy, are obtained without isolation of any intermediates. In order to demonstrate the application of reversible reactions in the cascade reaction concept, reversible chemical reactions were developed and combined with an irreversible reaction step to efficiently synthesize series of target compounds. Two cascade reactions, designed for concise access to known compounds from traditional multi-step reactions, were developed to prepare 3-functionalized phthalides and 3-thioisoindolinone derivatives, respectively, in one-pot reaction.

2.2. Tandem-driven reaction for synthesis of 3-functionalized phthalides

2.2.1. 3-Functionalized phthalides

3-Functionalized phthalides are important key precursors for the synthesis of quinone skeletons, common core structures in quinoid natural products\textsuperscript{71-73} and disperse anthraquinone dyes.\textsuperscript{74} There are many different synthetic routes for quinone synthesis.\textsuperscript{75-77} Among these methods, the Hauser-Kraus annulation provides an efficient preparation process using a one-pot reaction (Scheme 4). In 1978, this hydroquinone-forming reaction, based on Michael addition of a stabilized phthalide anion to an enone compound and subsequent Dieckmann condensation and aromatization, was independently discovered by F. M. Hauser and G. A. Kraus.\textsuperscript{78, 79} The reaction between phthalide 11 and Michael acceptor 12 in the presence of strong base thus generated naphthalene product 13.
The key steps in this annulation reaction are the formation of phthalide anion and its Michael addition to the enone compound. In order to obtain high product conversion, the Hauser-Kraus annulation has been improved using different phthalide derivatives, bearing various functional groups at the 3-position. The substituent at the 3-position of phthalide compound is essential for the annulation reaction because it acts as a stabilizing group for the formed benzylic anion and a leaving group for the annulation process. Although a series of 3-functionalized phthalides have been synthesized and employed in the Hauser-Kraus annulation, only phthalides 11a-11c are commonly used.

2.2.2. Design strategy

The syntheses of phthalide 11a-11c were reported using stepwise reactions, providing only moderate yield of the target products. These shortcomings were addressed in the present study, where it was reasoned that tandem-driven reactions, based on the combination of reversible nucleophilic addition and irreversible intramolecular lactonization, could be applied to considerably improve the synthesis of phthalides 11 (Scheme 5).

In this route, the reversible reaction between methyl 2-formylbenzoate 14A and a nucleophile (NuH) in organic solvent generates alcohol intermediate 15. The target product 11 is subsequently irreversibly formed by intramolecular lactonization of intermediate 15. Following Le Châtelier’s principle, the formation of product 11...
displaces the dynamic reversible nucleophilic reaction in the previous step toward intermediate (15) formation. The reversible reaction will thus reproduce the acetal intermediate 15 until the starting material is completely consumed. Furthermore, addition of more equivalent of base can accelerate lactone formation.

2.2.3. Synthesis of phthalide derivatives

To probe the product diversity of the process, the reactions between aldehyde 14A and a series of thiols 16a-16g in the presence of triethylamine base were initially observed to form 3-thiophthalide products 18a-18g as shown in Scheme 6. Different structures of thiols, including linear (n-pentylthiol, 16a), branched (tert-butylthiol, 16b), aromatic (thiophenols, 16d-16f), and functionalized (benzylthiol, 16c and methyl thioglycolate, 16g) structures, were selected. The amount of triethylamine was also optimized to enhance the reaction efficiencies. At very low concentration of triethylamine, the formation of dithioacetal by-product was observed but increasing the amount of triethylamine led to diminish by-product formation and accelerate the target product formation. The reversible nucleophilic addition of thiol to benzaldehyde 14A generates hemithioacetal intermediate 17, which was subsequently lactonized to form phthalide product 18.

![Scheme 6. 3-Thiophthalide formation.](image)

In the presence of five equivalents of triethylamine, the 3-thiophthalide products 18a, 18c-18e and 18g, derived from their corresponding thiols 16a, 16c-16e and 16g, were formed at more than 95% conversion in less than 5 hours. For the more sterically hindered thiol 16b and electron deficient thiophenol 16f, the formation time of their corresponding products 18b and 18f was longer and reached completion in two days. These results indicate that the structure, size and nucleophilicity of the thiols influenced the formation of the 3-thiophthalide products.
The synthesis of 3-cyanophthalide 11c was also investigated using the same concept (Scheme 7). Acetone cyanohydrin 19 was used to generate the cyanide anion under slightly basic conditions. The reactions of benzaldehyde 14A and acetone cyanohydrin 19 in the presence of 0.1 equivalents of triethylamine at 0 °C generated cyanohydrin intermediate 20, which slowly formed 3-cyanophthalide product 11c in moderate yield.

![Scheme 7. 3-Cyanophthalide formation.](image)

In conclusion, the tandem reaction concept was successfully demonstrated as a means to optimize the reaction efficiency for the synthesis of 3-functionalized phthalides. The designed reaction is based on the tandem reversible addition-intramolecular lactonization process. The reactions between methyl 2-formylbenzoate and different nucleophiles in the presence of base provide easy access to phthalide products in a one-pot reaction. The reactions were performed under mild reaction conditions, generally providing high yields of the phthalide products.

2.3. Multicomponent reaction for synthesis of 3-thioisoindolinones

2.3.1. 3-Thioisoindolinones

Isoindolinone (21) is a common heterocyclic core structure, found in many natural products and biologically active compounds,85-92 which is of interest to medicinal chemists. The substituents on the isoindolinone structure at the 2- (N-atom) and 3-positions have been reported as essential for their binding activities. AKS 186 (22) and lennoxamine (23), for example, exhibit such activities as shown in Figure 3a.93, 94 3-Thioisoindolinone (24), known as an important intermediate for synthesis of isoindolinones, has recently been reported as a structural element in compounds with potential inhibitory activities toward non-nucleoside HIV reverse transcriptase (25), DNA gyrase (26), and HIV-1 integrase (27) (Figure 3b).88, 89, 91, 92
Figure 3. Structures of a) isoindolinone (21), b) 3-thioisoindolinone (24), and some biologically active derivatives.

Because of versatilities of the core structure, synthetic pathways of 3-thioisoindolinones have received an increasing interest. 3-Thioisoindolinones with different substituents on the core structure have been synthesized using both multi-step reactions, resulting in low to moderate conversions to the desired products. Therefore, the synthesis of 3-thioisoindolinones in one-pot reactions, which allow for diversification of the substituents incorporated, was the aim of this study.

2.3.2. Design strategy

The multicomponent reaction between methyl 2-formylbenzoate 14, primary amine 28, and thiol 16 was designed for synthesis of 3-thioisoindolinones (Scheme 8). The strategy provides easy access to the modification of four substituents (R, R1, R2 and R3) on the 3-thioisoindolinone structure in a one-pot procedure. The substituents on the aromatic parent of compound 24 can be changed using different methyl 2-formyl benzoates 14, while the substituents on 3-thioisoindolinone 24 at the N- and S-atoms (R1 and R) can be easily modified using a series of primary amines 28 and thiols 16.
The simple reaction procedure is another advantage of the designed synthetic reaction. The target product could be obtained by mixing three starting materials in organic solvent. Drying agent (magnesium sulfate) was added to the reaction vessel in order to remove water, which is produced during the product formation. Considering the reaction mechanism, reversible imine formation between benzaldehyde 14 and primary amine 28 generated imine 30 and water (Scheme 9). According to Le Châtelier’s principle, removing water from the reaction will displace the reversible reaction toward imine formation. Then, reversible nucleophilic addition of formed imine and thiol 16 provided thioaminal intermediate 31, which was subsequently irreversibly transformed to amide product 24. The formation of product 24 is the key step to remove the intermediate 31 from the reversible sequence. Therefore, the reversible reaction will reproduce the intermediate 31 until the imine 30 is completely consumed.

**Scheme 8.** Multicomponent reaction for synthesis of 3-thioisoindolinones.

**Scheme 9.** Proposed mechanism for the formation of 3-thioisoindolinone.
2.3.3. Synthesis of 3-thioisoindolinones

In order to demonstrate the efficiency of the synthetic strategy, two methyl 2-formylbenzoates 14A and 14B were selected to react with a combination of amines and thiols as shown in Table 1. A variety of amines: aliphatic (28a), aromatic (28b), benzyl (28c) and chiral (28d), were chosen to modify the substituent located on the nitrogen. The substituents of the target product at the S-center were modified by employing thiols: alkyl (16i), phenyl (16d), benzyl (16c), and esters with different lengths (16g and 16h).

For products from compound 14A, thioisoindoline 32 was obtained from n-butylamine 28a and thiolester 16g in dichloromethane at room temperature (Table 1, entry 1). With more sterically hindered amines 28b and 28c (Table 1, entries 2-4), products 33-35 were still obtained at room temperature from thiophenol 16d, thioester 16h and n-butylthiol 16i, respectively. Chiral amine 28d, (S)-1-phenylethylamine, was used in order to probe the potential stereocontrol at the 3-position of isoindolinone. When the reactions of chiral amine 28d were carried out at room temperature, the corresponding products were produced at low conversion. High product yields were however obtained when the reactions were performed in refluxing toluene, albeit with low diastereoselectivity. This indicates that although the chiral moiety of amine 28d affected the product formation, the reactions of benzaldehyde 14A and chiral amine 28d with thiophenol 16d and methyl 2-mercaptoacetate 16g (Table 1, entries 5 and 6) gave their corresponding diastereomeric products 36 and 37 in 1:1 diastereomeric ratio.

3-Thioisoindolinones 38-41 were obtained from methyl 2-formyl-3,5-dimethoxybenzoate 14B as shown in Table 1, entries 7-10. The reaction between n-butylamine 28a and benzylthiol 16c at room temperature produced the corresponding product 38 (Table 1, entry 7). The reactions of compound 14B with the more sterically congested amines 28b and 28c were still able to provide products 39 and 40, from thiols 16g and 16c, respectively, at room temperature (Table 1, entries 8-9). This indicated that the 3-methoxy group of compound 14B does not influence the formation of product. The reaction of compound 14B, chiral amine 28d, and thioester 16g was performed in refluxing toluene, resulting in diastereomer products 41 in a 1:1 ratio (Table 1, entry 10).
**Table 1.** Multicomponent reaction for syntheses of 3-thioisoindolinones.

| Entry | Starting materials | Product | % Yield
<table>
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<tr>
<td></td>
<td>Aromatic</td>
<td>Amine</td>
<td>Thiol</td>
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<tr>
<td>1</td>
<td>14A</td>
<td>28a</td>
<td>16g</td>
</tr>
<tr>
<td>2</td>
<td>14A</td>
<td>28b</td>
<td>16d</td>
</tr>
<tr>
<td>3</td>
<td>14A</td>
<td>28b</td>
<td>16h</td>
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<tr>
<td>10</td>
<td>14B</td>
<td>28d</td>
<td>16g</td>
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</table>

*\(^a\) % yield of isolated product. Reaction conditions: \(^b\) RT, CH\(_2\)Cl\(_2\), 12 h. \(^c\) Reflux, toluene, 24 h, 1:1 diastereomeric ratio.*

To further investigate the scope of the synthetic methodology, tricyclic γ-lactams, potential non-nucleosidic HIV reverse transcriptase inhibitors, were also synthesized using the same concept (Scheme 10). The reactions between methyl 2-formylbenzoates 14A and 14B with cysteamine hydrochloride 42 were performed in dichloromethane at room temperature. Two equivalents of triethylamine were added in this case to deprotonate the ammonium salt as well as to accelerate the process. The reactions efficiently provided tricyclic isoindolinones 43A and 43B, respectively.
Thus, the designed multicomponent reaction was successfully applied to the syntheses of 3-thioisoindolinones and tricyclic γ-lactams. For the 3-thioisoindolinone compounds, the substituents on the core structure could be easily modified in a one-pot reaction. The syntheses of 3-thioisoindolinones and tricyclic γ-lactams were easily performed under mild reaction conditions, providing high yield of the target products.

2.4. Conclusion

In this chapter, synthetic methodologies to 3-functionalized phthalides and 3-thioisoindolinones using the concept of cascade reactions have been successfully demonstrated. Efficient syntheses of the target products were designed and performed under mild reaction conditions. Using the same starting material (methyl 2-formylbenzoate), 3-functionalized phthalides and 3-thioisoindolinones were efficiently synthesized from the designed tandem reversible addition-intramolecular lactonization and multicomponent reaction, respectively.
Ligand identification of Concanavalin A from dynamic carbohydrate systems

(Paper III)

3.1. Introduction

An important application of constitutional dynamic chemistry continues to be the generation of dynamic covalent systems for the rapid identification of ligands for target biomolecules. Studies of protein-ligand recognition and interactions are central to the understanding of biological systems and the function of the target proteins, and constitute a major challenge in drug discovery. Numerous analytical techniques have been developed to facilitate these studies, of which \textit{in situ} methodologies are gaining increasing interest. One such approach is Saturation Transfer Difference (STD)-NMR spectroscopy, which has emerged as a powerful technique for efficient protein-ligand identification. This spectroscopy method was recently adapted to constitutional dynamic chemistry for the rapid identification of ligands for a target enzyme (Figure 4). The dynamic constitutional system was generated under mild reaction conditions, compatible with the biological systems under study. The optimal ligand (L3) was specifically bound by the target protein and could be directly identified by selective saturation of the target protein (P), allowing for detection by the subsequent attenuation of saturation, transferred from the protein to the bound ligand.

\textbf{Figure 4.} Representation of STD-NMR spectroscopy for ligand identification using the CDC concept.

In this chapter, the evaluation of protein-ligand interactions using a dynamic carbohydrate system was demonstrated by STD-NMR spectroscopy. The dynamic carbohydrate systems were generated through phosphine-catalyzed disulfide metathesis reactions in aqueous solution. In the presence of the target protein,
plant lectin Concanavalin A (Con A), the optimal carbohydrate constituent was identified from the dynamic carbohydrate system by $^1$H STD-NMR spectroscopy.

3.2. Target protein: Concanavalin A

3.2.1. Discovery of plant lectin Con A

Concanavalin A, a member of the large plant lectin family, can be isolated from the Jack bean (*Canavalia ensiformis*). Proteins from the Jack bean were purified and Concanavalin protein was identified for the first time by D. B. Jones and C. O. Johns in 1916. A few years later, J. B. Summer successfully crystalized two fractions of this protein mixture, which were called Concanavalin A and B, respectively. Fraction B, Con B, could be dissolved only in saturated salt solution, while fraction A, Con A, dissolved well in water. The biological activity and structure of Con A were revealed to have specific binding to saccharide molecules. Therefore, over the past two decades, the interaction between Con A and various saccharides has been increasingly explored, especially for the study of protein-ligand interactions, as well as for the development of biosensors and in drug discovery.

3.2.2. Structure and ligand binding interaction of Con A

Although the function of Con A in Jack bean is unknown, it has gained much attention in its specific binding to saccharides. These studies offered the perspective for an attractive source of new drugs, involving cell-type selective determinant for targeted drug delivery. In order to understand the activities of Con A, the physical properties of Con A, including determination of its structure by X-ray diffraction techniques, have been extensively studied. Con A forms a dimeric structure at below pH 6, while tetrameric Con A is formed at above pH 7. Each subunit contains 237 amino acid residues, forming two antiparallel beta sheets. The first sheet has six strands and the other contains seven strands. In the tetrameric form of Con A (Figure 5), four identical subunits are held together through the six-stranded sheets, whereas the seven-stranded sheets, containing the ligand binding sites, are pointing away from the six-stranded sheets.

Con A displays specific binding to $\alpha$-D-mannose and $\alpha$-D-glucose structures. X-ray crystallography revealed that each subunit of Con A contains one Mn$^{2+}$ ion, one Ca$^{2+}$ ion and a carbohydrate recognition domain (CRD) as shown in Figure 5. The metal ions are bound by amino acids that point away from the seven-stranded sheets. The existence of these metal ions in Con A is required for the binding of the
incoming saccharide molecules in the carbohydrate binding site. The carbohydrate recognition domain of Con A is not only specific to monosaccharide molecules but also oligo- and polysaccharides.

![Figure 5](image)

**Figure 5.** Structure of Con A. Left side: tetramer of Con A. Right side: enlarged area of a carbohydrate recognition domain (CRD). Image from the RCSB PDB of PDB ID: 2P2K with Jmol software.

3.3. Generation of dynamic carbohydrate systems

3.3.1. Thiol-disulfide interchange reactions

The studies of disulfide properties and formations are highly interesting. The disulfide exchange reaction can be generated using thiol-disulfide interchange and disulfide metathesis methodologies where both reactions are possessing similar reversibility characteristics. For thiol-disulfide interchange, different methods were used to create disulfide exchange reactions (Scheme 11). For example, in the presence of air in basic solution (Scheme 11a), thiol starting materials can be oxidized to form disulfide products. The formed disulfide can in this case undergo exchange with the thiolate anion, generated from deprotonation of the thiol starting compounds. Analogously, thiol-disulfide exchange can also be generated directly from disulfide and thiol in the presence of base as shown in Scheme 11b. Reducing conditions can also be used, and the addition of catalytic amounts of reducing
agent, e.g., dithiothreitol (DTT), into a mixture of disulfides initiates the disulfide exchange reaction (Scheme 11c).

\[
\begin{align*}
\text{a) } & R^1\text{-SH} + R^2\text{-SH} \xrightarrow{\text{O}_2, \text{base}} R^{1-}\text{S}_{\text{S-R}^1} + R^{2-}\text{S}_{\text{S-R}^2} + R^{1-}\text{S}_{\text{S-R}^2} \\
\text{b) } & R^1\text{-S}_{\text{S-R}^1} + R^2\text{-SH} \xrightarrow{\text{base}} R^{2-}\text{S}_{\text{S-R}^2} + R^{1-}\text{S}_{\text{S-R}^2} \\
\text{c) } & R^1\text{-S}_{\text{S-R}^1} + R^2\text{-S}_{\text{S-R}^2} \xrightarrow{\text{base/DTT}} R^{1-}\text{S}_{\text{S-R}^2}
\end{align*}
\]

Scheme 11. Thiol-disulfide interchange reactions.

In CDC, the thiol-disulfide interchange reaction was first implemented in organic media in 1998. Then, the generation of dynamic thiol-disulfide systems was further studied in aqueous solution where the reaction conditions are more compatible to biological systems. These challenges prompted us to explore new and potential dynamic disulfide metathesis systems, generated from the reactions between disulfides in the presence of catalyst under mild reaction conditions.

3.3.2. Disulfide-phosphine reactions

The chemical properties of phosphines have been extensively studied and phosphines became well known as good reducing agents for aromatic nitro compounds and aromatic sulfonyl chlorides in 1962. The reactions between phosphines and disulfides were subsequently studied. The aims of these reactions were mainly to produce thioether products from their corresponding disulfides; however, the results indicate that the reaction conditions and the phosphine structures affected the product formations (Scheme 12a-c). For example, the reaction between symmetric disulfides and trialkoxy phosphite in organic solvent proceeded via a Michaelis-Arbuzov reaction, providing unexpected asymmetric thioether product as shown in Scheme 12a. Hexaethylphosphorous triamide (HEPA), on the other hand, was successfully used to desulfurize symmetric disulfides, yielding the desired thioether products and the aminophosphine sulfide by-product (Scheme 12b).
24

Scheme 12. Phosphine-disulfide reactions: a) and b) desulfurization of disulfide; c) reduction of disulfide; d) disulfide metathesis.

The reaction of symmetric disulfide and tris(2-carboxyethyl)phosphine in aqueous solution generated phosphine oxide and thiol products (Scheme 12c).

Recently, disulfide metathesis reactions using a phosphine promoter in organic solvent were reported generating dynamic disulfide systems as shown in Scheme 12d. In addition, the product formations under these reaction conditions depended on the reaction between the formed phosphonium ion and the thiolate anion. Therefore, the phosphine and the reaction conditions are significant parameters in the generation of dynamic carbohydrate systems.

3.3.3. Generation of dynamic carbohydrate systems using the disulfide metathesis reaction

In order to identify the optimal ligand for the plant lectin Con A, three symmetric glycosyl disulfides, 1-thio-β-D-galactoside dimer 44, 1-thio-β-D-glucoside dimer 45 and 1-thio-α-D-mannoside dimer 46, were synthesized and constituted the dynamic carbohydrate systems as shown in Scheme 13. The dynamic systems were generated in d-buffer solution at pH 8 in order to follow the reactions by NMR spectroscopy. A range of phosphine compounds were evaluated as disulfide metathesis catalysts. First, tricyclohexylphosphine (PCy₃), known as disulfide metathesis catalyst, was applied. However, although several equivalents of phosphine were used; the expected asymmetric glycosyl disulfide products were only detected in small amounts. The more polar phosphine, HEPA, was subsequently assessed, and in this case, the dynamic carbohydrate systems were successfully generated when one equivalent of HEPA was applied.
From $^1$H NMR spectroscopy (Figure 6b), the signals from the anomeric proton of the symmetric disulfide starting materials were clearly observed in the enlarged area (4.4-5.7 ppm). After the dynamic system reached equilibrium, the corresponding asymmetric disulfides, 47, 48 and 49, were formed in comparable ratios compared to their starting disulfides as shown in Figure 6c. In order to increase the rate of the exchange reaction and phosphine solubility, 10% $d$-acetonitrile was added. Remarkably, the reaction conditions, used for generating the dynamic system, did not provide any side products, for example, thiol derivatives or the corresponding thioether products.

After the formed dynamic system had reached equilibrium, another symmetric disulfide, thiocholine dimer 50, was added in order to probe the reversibility of the dynamic system as well as to demonstrate the size and scaffold expansion of the dynamic system (from 6 to 10 different disulfides) as shown in Scheme 13. The dynamic system was reequilibrated and three new asymmetric disulfides, 51, 52 and 53, were formed as shown in Figure 6d. These new disulfide components were formed unequally, likely depending on the steric congestion at the anomeric center of the carbohydrate part. Comparison of these three new disulfides revealed that
disulfide 53, containing mannose and thiocholine moieties, was the least formed product and required the longest equilibration time, while disulfide 52, containing glucose and thiocholine moieties, showed the opposite trend.

Figure 6. $^1$H NMR spectra of dynamic carbohydrate system: a) full spectrum; b) enlarged area of symmetric disulfide starting materials; c) enlarged area after equilibration and d) enlarged area of system re-equilibration after addition of disulfide 50.

Next, the concentration of aminophosphine was optimized in order to enhance the rate of the reversible reaction. The symmetric disulfides 44, 45 and 46 (15 mM each) were used and the HEPA concentration was varied from 25 mol% to 200 mol% as shown in Figure 7. The formed disulfide 49 was monitored since the proton at the anomeric position of mannose in disulfide 49 could be clearly detected without overlap with the solvent peak in the NMR spectra. When 75, 100 and 200 mol% of HEPA were applied, the formation of disulfide 49 reached half-equilibration times ($t_{1/2}$) of 80, 59 and 35 minutes, respectively. Interestingly, 50 mol% loading of HEPA did not provide the same product distribution plateau, however, addition of disulfide 50 into the formed dynamic system still provided small amount of its corresponding exchanged disulfide products. Furthermore, when 25 mol% of HEPA was loaded, and only trace amounts of exchanged
disulfide products were detected. Therefore, 100 mol% of HEPA was chosen to generate the dynamic systems.

![Figure 7](image)

**Figure 7.** Formation of asymmetric glycosyl disulfide 49 at different concentrations of HEPA catalyst. Experimental conditions: 44, 45 and 46 (15 mM each) and specific concentration of HEPA, room temperature, d-buffer/d-acetonitrile (90:10), pH 8.  

a Conversion calculated relatively to the mannose anomeric proton of disulfide 49.  
b Half-equilibration time ($t_{1/2}$).  
c Trace amount of 49 was detected.

3.4. Direct identification of the best binder of Con A from dynamic carbohydrate systems

3.4.1. Saturation transfer difference (STD) NMR

To gain a deeper understanding of biological function, studies of protein-ligand interactions are of wide interest. Therefore, a rich variety of techniques for protein-ligand identification have been developed, for example, enzyme-linked immunosorbent assay (ELISA) and several new NMR spectroscopic techniques. Among these NMR techniques, STD-NMR has been recognized as a powerful and accurate technique for evaluating binding of ligand to target protein.

The binding activity of the ligand to the protein can be determined by selectively saturating the target protein and then detecting the energy transferred to the ligand by observing the decreasing intensity of the binding ligand signal. The advantage of the STD-NMR technique is free of isotope-labeling on the target protein, only requiring small amount of target enzyme (at least 0.1 nmol) and providing highly accurate determination of protein-ligand interaction.

The principle for analyzing protein-ligand interactions using STD-NMR is schematically shown in Figure 8.
ligand (L), a part of the ligand population is, in principle, bound to the protein (PL). When the protein is irradiated at a resonance where no ligand signal is present (on resonance, Figure 8a), the irradiated protein (P*) transfers the received energy, by spin diffusion, to the bound ligand (L*). This results in an attenuation of the intensity of the ligand signal upon release. Another irradiation (off resonance, Figure 8b) is then generated at a resonance where no signal of either the ligand or the protein is present. This off resonance spectrum will be fairly comparable to a normal NMR spectrum of the mixture of the protein and the ligand. Ultimately, subtraction of on and off resonance spectra provides the difference spectrum, where only signals from the bound ligand were attenuated by saturation transfer.

Figure 8. STD-NMR principle for analyzing protein-ligand interaction: a) on resonance irradiation and b) off resonance irradiation.

3.4.2. $^1$H STD-NMR for direct ligand identification from a dynamic carbohydrate system

Following equilibration of a dynamic carbohydrate system containing symmetric disulfides 44, 45 and 46 and asymmetric disulfides 47, 48 and 49, the system was challenged with plant lectin Con A (0.1 mM). The $^1$H STD-NMR technique was used to identify the optimal carbohydrate ligand for Con A from the dynamic system. The on resonance $^1$H STD-NMR was set at 8 ppm where no signal from the dynamic carbohydrate system was present and off resonance was performed at 100 ppm, displaying no signals from either the dynamic system or Con A. The $^1$H STD-NMR spectra are displayed in Figure 9. The results clearly demonstrate the specific lectin-mannose binding interaction. Only glycosyl disulfides 46, 48 and 49, all containing the mannose species, showed binding interactions to the lectin. Furthermore, the phosphine-catalyzed disulfide metathesis methodology proved to be compatible with the target system under these conditions.
3.5. Conclusion

The disulfide metathesis method has been successfully demonstrated, where dynamic systems could be generated using phosphine mediation under mild reaction conditions in aqueous media. The dynamic systems were challenged with the plant lectin Con A, and the best glycosyl disulfide was successfully identified \textit{in situ} by the $^1$H STD-NMR technique. This combination of constitutional dynamic chemistry and STD-NMR spectroscopy proved very efficient, showing a high potential for discovery-oriented applications.
4

Lipase-catalyzed asymmetric resolution of dynamic covalent systems

(Papers IV-V)

4.1. Introduction

Dynamic combinatorial resolution (DCR) has emerged as an important application of constitutional dynamic chemistry, applying kinetic resolution pathways to dynamic covalent systems, in order to efficiently select and identify optimal system constituents. Different kinetic resolution strategies can be employed, of which enzymatic protocols have been shown to be particularly efficient (Figure 10). 

Dynamic systems were, in these cases, generated from reversible covalent bond formations of different starting components under thermodynamic control. The target enzyme was then applied to kinetically resolve the best binder (A₁B₃) from the dynamic system, resulting in its corresponding product (C₁) formation. Following Le Châtelier’s principle, when intermediate A₁B₃ was consumed by enzymatic transformation, the dynamic system was disturbed and forced to reproduce this intermediate until the reaction reached completion.

In order to forward the concept of dynamic combinatorial resolution further, new dynamic systems were generated from carbon-carbon bond forming, and carbon-sulfur bond forming reactions, respectively, under thermodynamic control. Thus, cyanohydrin and hemithioacetal systems were developed under mild reaction conditions compatible with enzymatic reactions. Lipases, recognized as efficient and highly stereospecific biocatalysts, were chosen as the target enzymes because of their high stabilities under many reaction conditions, and their ability to accept diverse substrate structures. In accordance with the DCR concept, the employment of kinetic lipase-catalyzed transformations to the dynamic systems will result in the

Figure 10. Concept of enzyme-catalyzed dynamic combinatorial resolution.

In order to forward the concept of dynamic combinatorial resolution further, new dynamic systems were generated from carbon-carbon bond forming, and carbon-sulfur bond forming reactions, respectively, under thermodynamic control. Thus, cyanohydrin and hemithioacetal systems were developed under mild reaction conditions compatible with enzymatic reactions. Lipases, recognized as efficient and highly stereospecific biocatalysts, were chosen as the target enzymes because of their high stabilities under many reaction conditions, and their ability to accept diverse substrate structures. In accordance with the DCR concept, the employment of kinetic lipase-catalyzed transformations to the dynamic systems will result in the
resolution of the optimal lipase substrates with high structural and stereochemical specificities. In a first study, lipase-catalyzed asymmetric transesterification and system optimization of dynamic cyanohydrin systems was designed and developed to reveal lipase performances in asymmetric cyanohydrin syntheses. Then, in a second study, lipase-catalyzed lactonization was further used to asymmetrically resolve the optimal substrates from dynamic hemithioacetal systems, resulting in the synthesis of optically active 1,3-oxathiolan-5-ones.

4.2. Target enzymes

4.2.1. Lipases

Enzymes, nowadays, are recognized as efficient catalysts for synthesis and have become widely used in both academic laboratories and industry because of their highly efficient catalytic reactions and influence on environmentally safe. Among all enzymes, lipases have gained much interest owing to their catalytic activities with high enantioselectivity, regioselectivity and broad substrate specificity. They are thus used in many important industrial processes, for example, food, pharmaceutical, cosmetics, and detergent productions.

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are important members of the hydrolase family. Lipases are recognized as versatile biocatalysts since they are robust, maintain their function under many reaction conditions, and do not require any cofactors for their catalytic activities. Many lipases are commercially available because lipases can be derived from many sources, for example, plants, animals, fungi and bacteria.

4.2.2. Biological function of lipases

As with hydrolase enzymes in general, lipases act on carboxylic ester bonds. The natural function of lipases is to hydrolyze triglyceride into diglyceride, monoglyceride, and glycerol, respectively, as shown in Scheme 14. Therefore, lipases, as efficient chiral catalysts, are commonly used to asymmetrically synthesize and resolve chiral alcohol and carboxylic acid derivatives.
Lipases from different sources have different size and number of amino acids in their primary sequences but the common feature of all lipases is that they contain three important amino acid residues, which are serine, histidine and aspartate (or glutamate) in a catalytic triad as shown in Scheme 15. The interaction of the negative charged residue of aspartate (Asp) allows the histidine (His) residue to act as a general base that removes the proton from the hydroxyl group of the serine (Ser) residue. The formed alkoxide anion on the serine residue is then available to attack the carbonyl group of the incoming ester substrate, forming the tetrahedral intermediate I. The acyl moiety of the ester substrate is attached to the serine residue through a covalent bond. The ester carbonyl function and the alkoxide anion of the intermediate are stabilized by the oxyanion hole, established by the involvement of two additional amino acid residues, glutamine (Gln) and threonine (Thr) residues, respectively. Then, the alcohol molecule is released and a water molecule enters the active site. A proton of water is abstracted by the histidine residue and the resulting hydroxide attacks the carbonyl group of the acyl enzyme. Tetrahedral intermediate II is then formed, again assisted by the oxyanion hole. Finally, the carboxylic acid product is formed and subsequently released from the active site. The catalytic triad of the lipase is then available for the next ester substrate.

Scheme 15. Catalytic mechanism of *Pseuodozyma antarctica* lipase.
In addition to carbon-oxygen bond breaking and forming reactions, lipases have
gained special attention for their promiscuity, as they can usually accept a broad
substrate scopes and carry out various carbon-nitrogen, carbon-sulfur and carbon-
carbon bond forming reactions.\textsuperscript{143} For example, lipases have recently been reported
to be active catalysts in amidation, thioesterification and Michael addition
reactions, demonstrating their catalytic promiscuity. Furthermore, the reaction
conditions, for example, the amount of lipase, the reaction solvent, the reaction
temperature and the acylating agent, also influence the lipase catalytic activities,
effects that are sometimes called condition promiscuity. The condition promiscuity
of lipases was for example explored by P. Rona \textit{et al}.\textsuperscript{144} In this case, a series of
experiments using different lipases in organic solvents for resolution of chiral
alcohols and esters demonstrated the effect of reaction conditions to the lipase
promiscuity.

Among the reaction parameters in lipase-catalyzed transforming reactions, the
reaction temperature plays a particularly important role for the enzyme activities,
not only for their stability but also their catalytic selectivities.\textsuperscript{145-149} The effect of
the reaction temperature on the lipase catalytic activity has been studied using both
experimental and molecular modeling approaches. These studies report that
although the lipase-catalyzed reactions proceeded at lower reaction rates at low
temperature, lipase enantioselectivities dramatically increased under these
conditions. This effect is proposed to be a consequence of the more favorable
enzyme conformations at low temperature.

Taken together these properties of the lipases prompted us to further employ them
as chiral catalysts for resolution of dynamic systems. Lipase-catalyzed transesterification and lactonization were thus developed to asymmetrically resolve
dynamic cyanohydrin and dynamic hemithioacetal systems, respectively. The
lipase performances were evaluated with respect to substrate structure and
enantiomeric selectivities by optimizing the reaction parameters.

4.3. Lipase-catalyzed asymmetric resolution of dynamic cyanohydrin systems

4.3.1. Cyanohydrin reaction

The cyanohydrin reaction, a versatile synthetic method in organic chemistry,
results from the cyanation of aldehydes or ketones.\textsuperscript{150-153} The reversible
nucleophilic addition of hydrogen cyanide to the carbonyl group generates a
racemic mixture of hydroxynitriles or cyanohydrin adducts (Figure 11a).\textsuperscript{152}
Optically active cyanohydrin compounds, found in many plants and insects, can be transformed into important functional motifs, for example, 1,2-amino alcohols, \( \alpha \)-azidonitriles and \( \alpha \)-hydroxy acids and amides (Figure 11b).\textsuperscript{153}

![Chemical Structures](image)

**Figure 11.** a) Reversible cyanohydrin reaction; b) possible transformations of the cyanohydrin adduct.

Optically active cyanohydrin compounds can be synthesized by both non-enzymatic and enzymatic procedures.\textsuperscript{153} In the enzymatic reactions, the oxynitrilase-catalyzed asymmetric cyanohydrin formation from aldehydes and cyanide sources, and lipase-catalyzed transformation of cyanohydrin compounds have been studied.\textsuperscript{152} Lipase-catalyzed transesterification was initially adopted to asymmetrically resolve cyanohydrin ester products by J. Oda and coworkers.\textsuperscript{154-157} This synthetic methodology has subsequently been developed to increase the product yield and optical purity, as well as to obtain broader structural diversity of the products.\textsuperscript{152, 158, 159} These studies have revealed that lipase-catalyzed resolution of cyanohydrin structures is efficient, but requires careful optimization of all reaction conditions in order to obtain high yields and high enantiomeric purities. This challenge makes the lipase-catalyzed resolution of reversible cyanohydrin systems an ideal candidate for the evaluation of lipase preferences of cyanohydrin structures using the DCR concept.
4.3.2. *In situ* lipase substrate evaluation of dynamic cyanohydrin systems

4.3.2.1. Generation of dynamic cyanohydrin systems

Although many cyanide sources can be used to produce cyanohydrin compounds, transhydrocyanation from acetone cyanohydrin (19) to aldehyde was selected to generate the dynamic cyanohydrin systems. In the presence of base, acetone cyanohydrin is decomposed to acetone and hydrogen cyanide under mild reaction conditions. Triethylamine was selected as the base to release hydrogen cyanide, and accelerate the rate of the cyanohydrin reactions. The amount of base was varied from catalytic amount to several equivalents, however, one equivalent of triethylamine was sufficient to establish the dynamic system during the enzymatic resolution. Then, five different aromatic aldehydes: 2-trifluoromethylbenzaldehyde (54a), 2-naphthaldehyde (54b), 2-methylbenzaldehyde (54d) and 4-methoxybenzaldehyde (54e), were chosen and investigated in the reversible cyanohydrin reactions. The dynamic cyanohydrin system was generated by mixing equimolar amounts of aldehydes 54a-54e, acetone cyanohydrin 19 and triethylamine in d-chloroform (Figure 12a). The dynamic system reached equilibration within 3 hours at room temperature, as monitored by $^1$H NMR spectroscopy (Figure 12b). Because of their similarity, the $\alpha$-protons of cyanohydrin intermediates 55a-55e were displayed in the same region (5.40-5.95 ppm). As can be seen, the intermediates were present at similar ratios, where cyanohydrin 55a was the most favored species and intermediate 55e was the least formed adduct.

4.3.2.2. Lipase-catalyzed resolution of the dynamic cyanohydrin systems

After the dynamic cyanohydrin system reached equilibrium, the enzymatic reaction was applied to resolve the dynamic system. Several reaction parameters were addressed including: lipase source, acylating agent, solvent and temperature. Different lipases from *Pseudomonas fluorescens*, *Candida rugosa*, *Pseuozyma antarctica*, and *Burkholderia cepacia* were probed for the resolution process. Among these lipases, lipase PS-C I, immobilized lipase from *Burkholderia cepacia*, provided the highest enzyme activities in both structural and enantiomeric selectivities.
In the lipase-catalyzed resolution of the dynamic cyanohydrin system, isopropenyl acetate 56 was chosen as the acylating agent because acetone, produced as by-product, did not interfere in either the dynamic system or the enzymatic reaction. In order to control the water activity, ground molecular sieve 4Å was added in the resolution process. Initially, the dynamic cyanohydrin system was resolved using 10 mg of lipase PS-C I in the presence of three equivalents of isopropenyl acetate.
The resolution process was followed by $^1$H NMR spectroscopy until full conversion of cyanohydrin ester products (6.30-6.75 ppm) was obtained (cf. Figure 12d). Interestingly, the major intermediate 55a in the dynamic system was disfavored by the lipase-catalyzed resolution, while ester product 57d from its corresponding intermediate 55d was preferentially produced. The resulting preference factors (see Figure 13 for definition) were calculated and displayed in Figure 13a (front row). Moderate lipase selectivities were obtained for intermediates 55b-55e ($F_p = 0.2-0.4$) and a negative preference factor for intermediate 55a ($F_p = -0.4$).

To enhance this selectivity, the enzymatic reaction parameters were optimized. First, a range of acylating agents, for example, 2-naphthyl acetate and phenyl acetate and its derivatives, were applied to the enzymatic resolution process. The results indicate that these acylating agents only enhanced the rate of the enzymatic resolution, although, the lipase substrate selectivities remained unchanged. Then, the amount of lipase loading was optimized. When the amount of lipase was decreased from 10 mg to 3 mg, the reaction time was prolonged from 11 days to 20 days. The ratios of the ester products were monitored and used to calculate the substrate preference factors as shown in Figure 13b (second row). These results indicate that the amount of lipase in the enzymatic resolution process influence the resulting selectivities. The ester product 57d was the most amplified product. Its preference factor was increased from 0.4 to 0.9 with decreasing lipase loading to 3 mg per reaction. In contrast to the disfavored intermediates 55a and 55c, the
preference factor value of their corresponding products 57a and 57c was decreased from -0.4 to -0.6 and 0.2 to 0, respectively.

Next, the reaction temperature was decreased to further increase the lipase selectivities. The resolution process of the dynamic cyanohydrin system was performed using 3 mg lipase at 0 °C. The ratios of the ester products were monitored by 1H NMR spectroscopy until the reaction reached completion as shown in Figure 12d. The preference factors of the ester products were calculated and displayed in Figure 13c. The lipase substrate selectivity was clearly enhanced where the ester product 57d was the most amplified product ($F_p = 1.2$). The ester product 57a and 57c remained deselected by the lipase catalytic resolution. Interestingly, the lipase showed lower preference for cyanohydrin intermediates 55b ($F_p = 0.7$) and 55e ($F_p = 0.3$) at this reaction temperature.

To explore the lipase enantioselectivity, the ester products from the dynamic resolution process were subjected to chiral chromatography. The resulting enantiomeric ratios of individual esters in chloroform are shown in Table 2 (column 4). All ester products except the disfavored product 57a were asymmetrically resolved by the lipase-catalyzed transesterification. Interestingly, the most amplified product 57d provided the highest enantiomeric purity (83% ee). Furthermore, the reaction solvent was also changed to study the effect on the lipase selectivity. When toluene was used as reaction solvent, the resolution process showed less enantioselectivity of the ester products (Table 2, column 3).

**Table 2.** Enantiomeric excess of products 57a-57e from the lipase catalyzed resolution of dynamic cyanohydrin systems.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>% Enantiomeric excess$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>toluene</td>
</tr>
<tr>
<td>1</td>
<td>57a</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>57b</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>57c</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>57d</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
<td>57e</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$ The enantiomeric excess of ester products from lipase-catalyzed resolution was analyzed by HPLC at 200 nm using a combination of CPS cyano and OD-H chiral column; eluent: iso-propanol and hexane (1/99; v/v).
Our results demonstrated that lipase-catalyzed transesterification was successfully adapted to resolve dynamic cyanohydrin systems, providing amplification of the preferential product. Then, the reaction parameters were optimized to enhance the lipase selectivities. The lipase substrate selectivity could be enhanced by reducing the catalyst loading and decreasing the reaction temperature, resulting in increasing preference factors and amplification of the favored ester product.

4.4. Lipase-catalyzed asymmetric resolution of dynamic hemithioacetal systems

In order to expand the scope of the DCR concept, we explored new dynamic systems using different reversible covalent bond forming reactions and the formed dynamic systems were subsequently resolved with new enzymatic reactions. In this study, dynamic hemithioacetal systems were designed and generated using reversible hemithioacetal (HTA) formation in organic solvent. Then, lipase-catalyzed lactonization was applied to asymmetrically resolve the dynamic hemithioacetal system. The lipase-catalyzed resolution could be efficiently achieved in a one-pot process, resulting in the synthesis of optically active 1,3-oxathiolan-5-one products.

1,3-Oxathiolan-5-one derivatives are known as significant key intermediate in the preparation of oxathionyl-nucleosides, for example Emtricitabine (or Coviracil®) and Lamivudine (or 3TC™), which are potential nucleoside reverse transcriptase inhibitors (NRTI) as shown in Figure 14. For synthetic drug development, syntheses of these compound skeletons have gained much attention over the last decade; of particular interest is the control of the stereoselectivity of the substituent at 2-position. In order to prepare enantiomerically pure 2-substituted-1,3-oxathiolan-5-ones, chiral starting materials and biocatalyst-assisted indirect preparations have been utilized in many cases.

![Figure 14. Structures of 1,3-oxathiolan-5-one and oxathionyl nucleosides.](image-url)
4.4.1. Hemithioacetal reaction

Carbon-sulfur bond forming reactions play important roles in organic synthesis, in for example, the Pummerer rearrangement, Michael addition, thioesterification, as well as thioacetal and hemithioacetal formation. In addition, organosulfur compounds exhibit remarkable biological activities.\textsuperscript{168, 169} Although, other sulfur-containing reversible reactions, such as the thiol-disulfide interchange reaction and transthioesterification, have not been extensively explored in constitutional dynamic chemistry, the thioacetal and hemithioacetal formations have received much attention.

\[
\text{Scheme 16. Reversible hemithioacetal reaction.}
\]

The reversible hemithioacetal formation could be generated from nucleophilic addition of thiols to the carbonyl carbon of aldehydes, forming racemic mixtures of hemithioacetal intermediates as shown in Scheme 16.\textsuperscript{104} Recently, the reversible hemithioacetal reaction was reported by our group, resulting in generation of virtual dynamic hemithioacetal systems for the identification of β-galactosidase inhibitors in aqueous solution. Thus, this achievement prompted us to further the exploration of dynamic hemithioacetal systems in organic solvent.

4.4.2. \textit{In situ} lipase substrate evaluation of dynamic hemithioacetal systems

4.4.2.1. Generation of dynamic hemithioacetal systems

In generation of efficient dynamic hemithioacetal systems, controls of several reaction parameters are important to manage the outcome. To evaluate the dynamic resolution and the lipase substrate structure specificity, series of aldehydes and thiols were chosen to form a prototypic dynamic hemithioacetal system (Scheme 17). The aldehyde series contained aliphatic aldehydes \textbf{58} (\textit{iso}-butraldehyde) and \textbf{59} (\textit{n}-octylaldehyde), and aromatic aldehyde \textbf{60} (2-pyridinecarboxylaldehyde). The addition of selected thiols \textbf{16g} (methyl 2-mercaptopoacetate) and \textbf{16h} (methyl 3-mercaptopropionate), with appended ester groups and different lengths of the carbon chain, generated γ- and δ- hydroxyester intermediates \textbf{58g-60g} and \textbf{58h-60h}, respectively.
As can be expected, the reversibility of the hemithioacetal reaction depends on the polarity of the solvent used. Initially, the dynamic system was performed in \( d \)-solvent in order to optimize the reaction conditions and the reversibility was followed by \(^1\)H NMR spectroscopy. In nonpolar solvent such as \( d \)-chloroform and \( d \)-toluene, the equilibrium of the dynamic systems was shifted toward the reactants where the hemithioacetal intermediates were largely dissociated. Thus, different catalysts were added to accelerate rate of hemithioacetal formations. In the presence of acid catalysts, for example, zinc bromide, acetic acid and silica gel, dithioacetal by-products were irreversibly formed, while in the presence of organic base catalysts, such as pyridine and triethylamine, the dynamic system was displaced toward intermediate formation. Triethylamine base was thus selected because of its positive influence on the dynamic systems, its compatibility with the enzymatic reaction, and because it provided fast equilibration without the formation of any side products.

The dynamic hemithioacetal systems were studied by \(^1\)H NMR spectroscopy. Before addition of triethylamine, each starting compound, aldehydes 58-60 and thiols 16g and 16h, could be clearly discerned in the spectra and easily followed at different time intervals as shown in Figure 15a and 15b. In the presence of one equivalent of triethylamine, the dynamic hemithioacetal system was initiated and reached equilibration within three hours at room temperature (Figure 15c). As can be seen, all signals of the \( \alpha \)-protons of the six possible hemithioacetal intermediates (12 chiral intermediates) 58g-60g and 58h-60h were clearly displayed and present in similar ratios in the NMR spectrum.
Figure 15. $^1$H NMR spectra of dynamic hemithioacetal resolution: a) full spectrum with enlarged areas before dynamic system generation; b) enlarged areas before system generation; c) enlarged areas of dynamic system in $d$-toluene at equilibration; enlarged areas of lipase catalyzed resolution of dynamic system at -18 °C in the presence of d) 0.1 equivalent and e) 1 equivalent of triethylamine.

4.4.2.2. Lipase-catalyzed resolution of dynamic hemithioacetal systems

The formed dynamic system was combined with lipase-catalyzed resolution and the reaction conditions were subsequently optimized. First, different commercial lipases from *Burkholderia cepacia*, *Pseudomonas fluorescens*, *Candida rugosa* and
Pseudozyma antarctica, were screened under the same reaction conditions. The results indicate that the lipase from Pseudozyma antarctica (or Candida antarctica, CAL-B) proved superior to others in terms of catalytic lactonization activity and was thus chosen for further studies. The dynamic hemithioacetal system was subjected to the mixture of lipase CAL-B and molecular sieves 4Å at different reaction temperatures from -18°C to +40°C without stirring. The reaction mixtures were sampled and monitored by ¹H NMR spectra at different time intervals until one of the starting compounds was completely consumed.

Interestingly, the lipase-catalyzed resolution of the dynamic system resulted in the exclusive formation of lactone products 61g-63g, derived from γ-hydroxylester intermediates 58g-60g. The formations of other lactone products 61h-63h, derived from δ-hydroxyester intermediates 58h-60h, were not observed. These results indicate that the lipase could specifically catalyze the γ-lactone forming reaction. In addition, control experiments using bifunctional thiols 16g and 16h were performed in the presence of the lipase resolution process. The results indicate that none of the side lipase-catalyzed reactions, for example, transesterification and transthioesterification, of these thiols were detected.

In order to study the temperature effect, dynamic hemithioacetal systems were generated in the absence of triethylamine and applied to lipase-catalyzed resolution. Then, the resolution reactions were performed at different reaction temperatures. As can be expected, the lipase-catalyzed resolution was completed in shorter time at higher temperature, however simultaneously resulting in lower lipase selectivity, especially regarding enantioselectivity. Table 3 shows the results from the lipase-catalyzed resolutions in term of % conversion and % enantiomeric excess of lactone products 61g-63g at different reaction temperature. At room temperature (Table 3, column 2), product 61g was formed as the major product at 50% conversion and products 62g and 63g were produced at 29% and 1% conversion, respectively. Low enantiomeric excess (measured by chiral HPLC) of lactone product 63g was recorded at 53% ee, while racemic mixture of products 61g and 62g were obtained.

To enhance the lipase enantioselectivity, the low reaction temperature strategy was applied in the present study. At 0 °C (Table 3, column 3), similar conversions of the products were obtained. The enantiomeric excesses of products 61g-63g were obtained in 39%, 25%, and 72% ee, respectively. At similar reaction time, low conversion of products was obtained, however, the improvement of the enantiomeric ratios of products 61g (47% ee), 62g (49% ee) and 63g (95% ee) were recorded when the reaction was kept at -18 °C (Table 3, column 4). These
studies revealed the temperature dependency of the lipase on substrate and enantioselectivities. As mentioned above, at lower reaction temperature the enzyme movement is slowed down and its conformation becomes more rigid to the incoming substrates, hypothetically resulting in higher specificity for the substrate structure. Disfavored substrates, with longer or larger substituents, would be more difficult to accommodate in the enzyme pocket. In analogy, the activation entropy between the enantiomers in the enzyme catalyzed reaction was differentiated at lower reaction temperature, leading to the enhancement of lipase enantioselectivity. Temperature-induced restriction of one substrate enantiomer in the enzyme active site thus improved the overall resolution.

In the present case, the equilibration rates of the dynamic systems also decrease, and therefore, the amount of triethylamine was varied to enhance the chemical reversibility in order to attain a pseudo-equilibrium system, where equilibration proceed faster than the enzymatic reaction. In the presence of 0.1 equivalent of triethylamine, the products were formed at more than 70% conversion (Figure 15d). Product 61g (43%) was formed at a relatively higher rate than product 62g (29%), while product 63g was produced at only 2% conversion (Table 3, column 5). Chiral HPLC analysis of the lactone products provided reasonable enantiomeric excesses for compound 61g (57% ee) and compound 62g (67% ee), and high enantiomeric excess for compound 63g (>99% ee). These results indicate that the reversibility of dynamic hemithioacetal system is insufficient, and that equilibration proceeds at a lower rate than the enzymatic transformation.

**Table 3.** Effect of reaction temperature and amount of triethylamine on enhancing lipase catalyzed resolution of dynamic hemithioacetal systems. 

<table>
<thead>
<tr>
<th>Product</th>
<th>% conversion (% ee)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>RT 0 °C</td>
</tr>
<tr>
<td></td>
<td>0 eq. NEt&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>61g</td>
<td>50 (4)</td>
</tr>
<tr>
<td>62g</td>
<td>29 (0)</td>
</tr>
<tr>
<td>63g</td>
<td>1 (53)</td>
</tr>
</tbody>
</table>

*Reaction conditions: Solutions of dynamic hemithioacetal systems and specific amounts of triethylamine were added into a mixture of lipase CAL-B and molecular sieve 4Å. The reactions were kept at different reaction temperature without stirring. *b* The enantiomeric excesses of the lactone products were analyzed by HPLC at 200 nm; eluent: 1% of *iso-*propanol in hexane for 60 min and 5% *iso-*propanol in hexane for 70 min.
To circumvent this effect, the amount of triethylamine was further increased. When 0.5 equivalents of triethylamine were applied, higher enantiomeric excesses of product \textit{61g} (77\% ee), \textit{62g} (83\% ee), and \textit{63g} (>99\% ee) were recorded in Table 3, column 6. An equimolar amount of triethylamine was finally found to be optimal for the dynamic system, and the lipase resolution process thus resulted in more than 80\% total conversion (Figure 15e and Table 3, column 7). Lactone product \textit{61g} (55\%) was the most amplified product under these conditions at the expense of lactone product \textit{62g} (25\%), the formation of which was decreased. Formation of the aromatic lactone product \textit{63g} on the other hand still remained at 2\% conversion. By chiral HPLC analysis, good enantiomeric excesses of products \textit{61g}-\textit{63g} could be recorded, resulting in 89\%, 88\%, and >99\% ee, respectively.

From these results, dynamic hemithioacetal systems were successfully generated in organic solvent and challenged with enzymatic resolution processes. The reaction conditions were subsequently optimized by lowering the reaction temperature and equimolar base loading to enhance the lipase specificities in both substrate structural and enantiomeric selectivities. The lipase-catalyzed asymmetric resolution of dynamic hemithioacetal system was profitably performed in one-pot evaluation processes, resulting in the synthesis of optically active 1,3-oxathiolan-5-one products.

4.5. Conclusion

In summary, we have successfully demonstrated the enzymatic resolutions of dynamic reversible systems in one-pot processes. First, dynamic cyanohydrin systems, based on carbon-carbon bond forming reactions, were asymmetrically resolved by lipase-catalyzed transesterification and the reaction conditions were optimized for efficient evaluation of lipase performances in asymmetric cyanohydrin syntheses. Then, the scope of dynamic combinatorial resolution was extended. Dynamic hemithioacetal systems, based on carbon-sulfur bond forming reactions, were further studied using lipase-catalyzed lactonization. The successful resolution of dynamic hemithioacetal system was performed by decreasing the reaction temperature and optimizing the base loading, resulting in the synthesis of optically active 1,3-oxathiolan-5-ones.
5

A dynamic fragment-based approach
towards the identification of potent β-galactosidase inhibitors

Paper VI

5.1. Introduction

Drug discovery is a truly challenging process, and high societal demands coupled to huge investments are forcing both the pharmaceutical industry and academic organizations to develop faster and more efficient strategies. As a result, new drug leads are increasingly required to allow the release of more potent drugs to the market. However, conventional screening approaches, including random screening for new drugs and high-throughput screening methodologies, are not able to meet this demand rate with respect to both time and production costs. This has prompted the development of new strategies, and fragment-based approaches have thus emerged as promising, potent, and powerful alternatives to the more conventional methods. Fragment-based approaches have been adopted into the field of exploring new enzyme inhibitors as well as novel protein ligands, both of which subjected to key focus in the early stages of drug discovery. Over the last decade, several compounds have been discovered and tested in pre-clinical development using fragment-based approaches.

Fragment-based approaches are techniques using low molecular weight chemical fragments and the study of their binding abilities to, or their inhibition activities against, specific biological targets. The combined action of these fragments are subsequently merged into active substances possessing all the individual functionalities. These methodologies, converting fragments into highly potent inhibitors, can be accomplished following three strategies: fragment optimization, fragment merging, and dynamic fragment linking, respectively, as shown in Figure 1. The fragment optimization strategy (Figure 1a) is similar to the traditional drug discovery phase where a known inhibitor (H), normally with low binding affinity to the target enzyme, is further modified with various fragment substituents in order to both improve the affinity and other properties, such as solubility. Using another tactic, called fragment merging (Figure 1b), inhibitor (H) can also be chemically synthesized by connecting small fragments that have been shown to individually bind to the target at different parts of the binding site. Finally, based on constitutional dynamic chemistry, the target can be used as a template for the dynamic assembly/synthesis of the inhibitor from the fragments,
and subsequently transformed into a non-dynamic analog (Figure 16c). In situ fragment identification is in this case applied to screen the optimal inhibitor from a dynamic system using the target for thermodynamic resolution. The fragments of the optimal inhibitor are then modified and linked together with irreversible bond formation, providing the inhibitor (H). In addition, the inhibitors (H) from the fragment merging and dynamic linking strategies are normally further modified using fragment optimization to obtain more potent inhibitor.

**Figure 16.** Fragment-based approaches for the discovery of potent enzyme inhibitors.

In the present chapter, the dynamic linking strategy is further presented, using β-galactosidase as a model target enzyme. Based on our previous study, the optimal inhibitor component from a dynamic hemithioacetal library for β-galactosidase was identified using $^1$H STD-NMR technique. The structure of the dynamic inhibitor was then studied to determine potent inhibitors for β-galactosidase using fragment-based approaches. The optimal inhibitor was tailored by fragment linking and optimization processes. The designed inhibitor structures were then synthesized and tested for inhibition activities against β-galactosidase.

5.2. Target enzyme

5.2.1. β-Galactosidase

Glycosidases are members of the carbohydrate-converting enzymes in the family of hydrolases. Glycosidases, including β-galactosidase, are involved in various
metabolic disorders and diseases, for example, diabetes, cancer, viral (HIV, influenza) and bacterial infections. During the last decade, extensive studies of the chemistry and pharmacology of glycosides have been carried out to delineate their inhibitory activities. Numerous glycosidase inhibitors which often saccharide analogs and carbohydrate mimics, such as azasugars and carbasugars have been designed and synthesized.

β-Galactosidases can be found in many sources, such as plants, animals, yeasts, bacteria and fungi. Enzymes derived from different sources show variances in their properties although the catalytic activities of the enzymes remain the same. β-D-Galactosidase, sometimes also called lactase, is often used in industrial applications in the hydrolysis of lactose and lactose containing foods, such as sweets and whey.

5.2.2. Biological function of β-galactosidase

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is an enzyme that hydrolyzes disaccharide lactose into galactose and glucose, and also isomerizes lactose to allolactose, another disaccharide as shown in Scheme 18. β-galactosidase has a high specificity for the galactosyl scaffold whereas the aglycone moiety can be varied. Thus, it can also hydrolyze various β-D-galactopyranosides with different aglycone moieties.

β-Galactosidase hydrolyzes galactopyranosides with overall retention of the anomeric configuration by a double displacement mechanism as shown in Scheme 19. The incoming glycoside substrate first forms hydrogen bonds with three amino acid residues, two glutamic acids (Glu537 and Glu461) and one tyrosine (Tyr503). The oxocarbenium ion-like transition, in a half-chair conformation with a partial positive charge along the bond between the anomeric carbon and the endocyclic oxygen atoms, is formed. Subsequently, the glucose or aglycone moiety (product I) is released and the covalent galactosyl intermediate is formed at glutamic acid residue (Glu537). Then, a water molecule enters the active site and becomes activated through proton abstraction by the pendant tyrosine residue. The resulting hydroxy anion attacks at the anomeric carbon of galactosyl intermediate,
forming oxocarbenium ion-like transition state II. The monosaccharide galactose (product II) is then released from the enzyme active site.

**Scheme 19.** Proposed catalytic mechanism of β-galactosidase.

Benefitting from the structural promiscuity of β-D-galactosidase, chromogenic substrates, such as 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and o-nitrophenyl β-D-galactopyranoside (ONPG), are widely used to determine the activity of β-galactosidase. This has enabled efficient colorimetric assays resulting in detailed investigations of the catalytic effects of the enzyme, and allows us to demonstrate the fragment-based dynamic linking approach toward the identification of potent β-D-galactosidase inhibitors.
5.3. Design and synthesis of inhibitor collection

5.3.1. Design strategy

Recently, we successfully used $^1$H STD-NMR to identify the optimal inhibitor component for β-galactosidase from dynamic hemithioacetal systems.\textsuperscript{104} These results were further adopted in this study, where fragment-based screening was applied to design and synthesize a series of β-galactosidase inhibitors, based on the structure of the optimal reversibly formed inhibitor from our previous study. The optimal hemithioacetal constituent was thus tailored into three separate parts: the glycone, the linker, and the aglycone (R) group (Figure 17).

First, to transform the dynamic hemithioacetal component (i.e. a labile compound) into a “static” compound, the hemithioacetal scaffold was displaced with an irreversible linker (Figure 17a). The linker groups are important not only for linking the glycone and aglycone moieties together but are also required as synthetic equivalents to the hemithioacetal functional group. Therefore, thioglycosides containing thioether bond linker and C-glycoside derivatives with hydroxyl and ketone analogs (as R$^1$ and R$^2$) were selected to mimic the hemithioacetal element. Secondly, in order to underline the enzyme specificity, the glycone moiety was varied using galactose derivatives (expected inhibitor) and glucose derivatives (expected noninhibitor) as shown in Figure 17b. Finally, as fragment optimization (Figure 17c), the R group was modified from alkyl to aryl groups in order to study the binding abilities of these fragments to the target enzyme.

Figure 17. Design of β-galactosidase inhibitors: a) linker; and b) glycone and c) aglycone (R-group) modifications.

The designed β-galactosidase inhibitors can be categorized into four different glycoside series as shown in Figure 18. The thiogalactoside and thioglucoside compounds 64a-64c and 65a-65c have different appending R-groups: iso-butyl,
phenyl and 4-pyridine groups, respectively. The designed inhibitors 64a (R = iso-butyl) and 64c (R = 4-pyridine) were designed to mimic our optimal hemithioacetal inhibitors from the previous results,\textsuperscript{104} whereas compound 64b (R = phenyl) was chosen because of its known inhibitory activity against β-galactosidase.\textsuperscript{182} The C-galactoside and C-glucoside series (compounds 66a-66b and 67a-67b) contain hydroxyl and ketone functional groups at R\textsuperscript{1} and R\textsuperscript{2} groups to mimic the hemithioacetal scaffold.

![Figure 18. Designed β-galactosidase inhibitors.](image)

5.3.2. Synthesis of inhibitor collection

**Synthesis of thioglycoside series**

Thioglycosides can be synthesized using two different synthetic methodologies as shown in Scheme 20.\textsuperscript{183-185} In the first route (Scheme 20a), the Lewis acid-mediated coupling method between peracetylated D-aldopyranose 68 or 69 and a thiol derivative 16j or 16c was used to synthesize tetraacetylated thioglycosides 70j, 70c, 71j and 71c. Hydrolysis of these tetraacetylated glycosides resulted in the desired thioglycoside products 64a-64b and 65a-65b, respectively. For the second route, 1-thio-β-aldopyranose 72 or 73 was treated with bromopyridine derivative 74 in the presence of a base generating thioglycosides 64c or 65c as shown in Scheme 20b.
Scheme 20. Syntheses of thioglycoside inhibitors. Reaction conditions: i. BF$_3$OEt$_2$, CHCl$_3$, RT, 15 min, 90-98%; ii. NaOMe, MeOH, RT, 3 h, quantitative yield; iii. KOH, MeOH, RT, 6 h, 69-77%.

Synthesis of the $\beta$-C-glycoside series

The syntheses of $\beta$-ketoglycosides 66a and 67a can be accessed in one-pot reaction as shown in Scheme 21.\cite{186-188} In the presence of a base, $\beta$-diketone 77 was transformed into its corresponding carbanion, subsequently attacking the reduced form of the carbohydrate starting material 75 or 76. The resulting intermediate 78 or 79 then underwent elimination and formed its corresponding product 80 or 81, respectively, and intramolecular 1,4-addition, followed by retro-Claisen aldolization of compound 80 or 81, produced the corresponding ketone products 66a or 67a. Reduction of these $\beta$-ketoglycosides with NaBH$_4$ in methanol then generated $\beta$-hydroxyglycoside 66b or 67b, respectively. Due to their similar polarities, diastereomeric mixtures of compounds 66b and 67b were obtained in 3:1 and 1:1 diastereomeric ratios, respectively, after purification.
Scheme 21. Syntheses of C-glycosides. Reaction conditions: i. NaHCO$_3$, EtOH/H$_2$O (4:1), reflux, 12 h, 38-64%; ii. NaBH$_4$, MeOH, RT, 1 h, 78-86%.

5.4. Evaluation of designed inhibitors to β-galactosidase

5.4.1. ¹H-NMR competitive inhibition studies

To study the inhibition activity of the synthesized glycosides against β-galactosidase, the well-studied chromogenic substrate ONPG was used during the inhibition studies. The inhibition experiments were performed by mixing ONPG and an inhibitor in 1:1 ratio in d-buffer and the inhibitory effect was determined as the reaction time ratio as defined in Table 4. The reaction rates were followed by ¹H NMR spectroscopy through the formation of ONP (hydrolysis product of ONPG) at different reaction time intervals.

Table 4 shows the inhibitory effects of the glycoside inhibitors against β-galactosidase. The results reveal high specificity of β-galactosidase for most of the galactoside inhibitors. For thioglycoside inhibitors 64a-64c and 65a-65c, although they are bearing the same thioether linker and R group, thioglucosides 65a-65c exhibited weaker inhibitory effect against the target enzyme with values 1.3, 1.1 and 1.1, respectively (Table 4, entries 7-9).
Table 4. Evaluation of the inhibitory effects of the designed glycoside inhibitors.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Galactosides</th>
<th>Inhibitory effect&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Entry</th>
<th>Glucosides</th>
<th>Inhibitory effect&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td>1</td>
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<td>7</td>
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<tr>
<td>2</td>
<td>64b</td>
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<td>8</td>
<td>65b</td>
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</tr>
<tr>
<td>3</td>
<td>64c</td>
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<td>9</td>
<td>65c</td>
<td>1.1</td>
</tr>
<tr>
<td>4</td>
<td>66a</td>
<td>1.5</td>
<td>10</td>
<td>67a</td>
<td>1.1</td>
</tr>
<tr>
<td>5</td>
<td>66b&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9</td>
<td>11</td>
<td>67b</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>66b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7.4</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> Inhibition = \( \frac{t_{1/2}(\text{inh})}{t_{1/2}(\text{blank})} \), for which \( t_{1/2} \) = time for 50% ONPG hydrolysis. Diastereomeric ratio of \( b \) 3:1 and \( c \) 1:2.

In the thiogalactoside series, the results indicate a residual binding effect of the R group (Table 4, entries 1-3). As can be seen, the aromatic thiogalactosides 64<sup>b</sup> and 64<sup>c</sup> provided higher values for the inhibitory effects than alkyl thiogalactoside 64<sup>a</sup> (inhibition effect ~ 4.9 times). For comparison, inhibitor 64<sup>c</sup> with heteroaromatic group (4-pyridine group) expressed a lower value of the inhibitory effect than the known thiogalactoside 64<sup>b</sup> carrying a phenyl group (12.4 and 32.9 times, respectively). These results imply a hydrophobic preference in the enzyme active site.

The C-glycoside series also provided the expected enzyme specificity for the β-galactosidase inhibitors (Table 4, entries 4-6 and 10-11). For ketoglycosides 66<sup>a</sup> and 67<sup>a</sup>, the inhibitory effect differences between the galactoside and glucoside are unclear with values 1.5 and 1.1, respectively. In contrast to hydroxyglycosides 66<sup>b</sup> and 67<sup>b</sup>, these effects are relatively small. The inhibitory effect for glucoside 67<sup>b</sup> is only 1.6, whereas the galactoside 66<sup>b</sup> provided higher values at 4.9 and 7.4 times for 3:1 and 1:2 diastereomeric ratios, respectively. These results indicate a slight difference in inhibitory effect for the different diastereomers.

5.4.2. UV-Vis competitive inhibition studies

In addition to the \(^1\)H NMR results, the inhibitory effect of the potential β-galactosidase inhibitors 64<sup>a</sup>-64<sup>c</sup> and 66<sup>a</sup>-66<sup>b</sup> were further quantified in terms of inhibitory concentration (IC\(_{50}\)) and inhibition constant value (K\(_i\)) using UV-Vis spectroscopy. A colorimetric assay for measuring the β-galactosidase activity using
ONPG as substrate was adopted. The inhibition reactions were performed by mixing ONPG and an inhibitor in phosphate buffer and the rate of ONPG hydrolysis was followed by detecting the formation of ONP at 420 nm ($\lambda_{\text{max}}$) at different time intervals. The concentration dependence between the rate of ONPG hydrolysis and concentration of inhibitor was used to calculate the IC$_{50}$ values of the individual inhibitors 64a-64c and 66a-66b as shown in Figure 19a and 19b. From these values, the K$_i$ value of each inhibitor was calculated according to the Cheng-Prusoff equation as shown in Figure 19c. The Michaelis constant (K$_M$), defined as the concentration of substrate providing half the maximum activity of the enzyme, was set at 14.6 μM.

![Graph](image)

**Figure 19.** UV-Vis competitive inhibition studies of the static galactosides: a) IC$_{50}$ determination (normalized rate of ONPG hydrolysis); b) IC$_{50}$ and K$_i$ values for the set of inhibitors, K$_M$ = 14.6 μM; c) Cheng-Prusoff equation for the conversion of IC$_{50}$ into K$_i$ values. Diastereomeric ratios of $^a$ 3:1 and $^b$ 1:2. Reaction conditions: 25 °C, pH = 7.4.

The results demonstrated the same trend as observed in the $^1$H NMR studies. For the thiogalactoside series, the aromatic compounds 64b and 64c provided the most potent inhibitors with K$_i$ values of 4.6 μM and 17.6 μM, respectively (Figure 19b, entries 2-3), while thiogalactoside 64a, bearing an alkyl group, showed a lower value at 78.2 μM (Figure 19b, entry 1). In the C-galactoside series, the ketogalactoside 66a afforded the lowest inhibition character with a K$_i$ value of 214 μM (Figure 19b, entry 4). The different diastereomeric ratios (with dr 3:1 and 1:2) of hydroxygalactoside 66b showed different K$_i$ values of 62.9 and 43.7 μM, respectively (Figure 19b, entries 5-6).
5.5. Conclusion

In summary, we have successfully demonstrated a dynamic fragment-based screening methodology for identification of potent β-galactosidase inhibitors. Based on our previous studies, the optimal inhibitor constituent, identified from dynamic hemithioacetal systems by $^1$H STD-NMR spectroscopy, was tailored by fragment linking and optimization strategies to design static inhibitor series. Mimicking of the hemithioacetal scaffold element led to the syntheses of thioglycosides and C-glycosides in which the glycone and aglycone fragments were irreversibly linked with thioether and carbon bonds, respectively. The glycone and aglycone fragments were modified to study the enzyme specificity and structural binding ability, respectively. The designed glycoside inhibitors were synthesized and their inhibition activities against β-galactosidase were tested using $^1$H NMR and UV-Vis spectroscopy. The inhibition studies demonstrated that the aromatic thiogalactosides provided the strongest inhibitory effects in the designed glycoside series.
Concluding Remarks

The development of new synthetic methodologies for different target molecules and novel protocols for biological evaluation, based on the applications of reversible covalent reactions, constitute the achievements reported in this thesis.

Synthesis

The concept of cascade reactions, based on the consecutive multi-step reactions in one-pot processes, has been successfully demonstrated to yield concise access to syntheses of different target molecules. The construction of 3-functionalized phthalides, key precursors in the synthesis of quinone skeletons, can thus be performed in one-pot procedures using the tandem reaction of reversible nucleophilic addition and lactonization. In addition, the syntheses of 3-thioisoindolinones and tricyclic γ-lactams efficiently diversify the structures using one-pot multicomponent reactions.

Biological Evaluation

As an advantage of the constitutional dynamic chemistry concept, dynamic reversible systems are used to rapidly identify optimal ligands for target biomolecules. To study ligand interactions with the plant lectin Con A, dynamic carbohydrate systems were generated through a phosphine-catalyzed disulfide metathesis reaction in aqueous solution. In the presence of Con A, the best glycosyl disulfide was successfully identified in situ by the $^1$H STD-NMR technique. This combination of constitutional dynamic chemistry and STD-NMR spectroscopy proved very efficient, showing a high potential for discovery-oriented applications.

Then, the fragment-based screening methodology was adopted for identification of potent β-galactosidase inhibitors. Based on our previous studies, the optimal inhibitor constituent, identified from dynamic hemithioacetal systems by $^1$H STD-NMR spectroscopy, was tailored by fragment linking and optimization strategies to design different static inhibitor series. The designed glycoside inhibitors were synthesized and their inhibitory activities against β-galactosidase evaluated using $^1$H NMR and UV-Vis spectroscopy. Potent inhibitors were identified from the designed glycoside series, showing the potential of the dynamic drug discovery concept.
Another important application of constitutional dynamic chemistry is the concept of dynamic combinatorial resolution. Enzymatic resolution has been applied to dynamic covalent systems in order to efficiently select and identify optimal system constituents. In this context, dynamic covalent systems, using carbon-carbon bond forming and carbon-sulfur bond forming reactions, were generated under thermodynamic control and subsequently applied to enzymatic resolution. Thus, cyanohydrin and hemithioacetal systems, respectively, were developed under mild reaction conditions compatible with enzymatic reactions. In accordance with the DCR concept, the employment of kinetic lipase-catalyzed transformations to the dynamic systems will result in the resolution of the optimal lipase substrates with high structural and stereochemical specificities. In a first study, lipase-catalyzed asymmetric transesterification and system optimization of dynamic cyanohydrin systems were designed and developed to reveal lipase performances in asymmetric cyanohydrin syntheses. Then, in a second study, lipase-catalyzed lactonization was further used to asymmetrically resolve the optimal substrates from dynamic hemithioacetal systems, resulting in the synthesis of optically active 1,3-oxathiolan-5-ones.
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To Taey, Thank you so much for talking to me every day, for being who you are (for four years), and for encouraging me.
Appendix

The following is a description of my contribution to Publications I to VI, as requested by KTH.

Paper I: I contributed to the formulation of the research problems and shared the experimental work and wrote the manuscript.

Paper II: I contributed to the formulation of the research problems and performed the majority of the experimental work and wrote the manuscript.

Paper III: I contributed to the formulation of the research problems and performed part of the experimental work.

Paper IV: I contributed to the formulation of the research problems and performed the majority of the experimental work and wrote the manuscript.

Paper V: I contributed to the formulation of the research problems and performed the majority of the experimental work and wrote the manuscript.

Paper VI: I contributed to the formulation of the research problems and performed part of the experimental work.
11. Z. Lixin, A. L. Demain (Eds.), *Natural Product: Drug Discovery and Therapeutic Medicine*. **2005**.
115. The Protein Data Bank (http://www.rcsb.org/).
129. R. Caraballo, M. Rahm, P. Vongvilai, T. Brinck, O. Ramström, *Chem. Commun.* **2008**, *6603-6605.*