Dynamic Sulfur Chemistry: Screening, Evaluation and Catalysis

Rémi Caraballo

KTH Chemical Science and Engineering

Doctoral Thesis

Stockholm 2010

Akademisk avhandling som med tillstånd av Kungl. Tekniska Högskolan i Stockholm framlägges till offentlig granskning för avläggande av licentiatexamen i kemi med inriktning mot organisk kemi onsdagen den 12 maj kl 10.00 i sal F3, KTH, Lindstedtvägen 26, Stockholm. Avhandlingen försvaras på engelska. Opponent är Professor Nicolas Giuseppone, Université de Strasbourg, Frankrike.
Abstract

This thesis deals with the design, formation and evaluation of dynamic systems constructed by means of sulfur-containing reversible reactions, in organic and aqueous media, and under mild conditions.

In a first part, the synthesis of thioglycoside derivatives, constituting the biologically relevant starting components of the dynamic systems, is described. In addition, the pD-profile of the mutarotation process in aqueous media for a series of 1-thioaldoses is reported and revealed an astonishing β-anomeric preference for all the carbohydrate analogs under acidic or neutral conditions.

In a second part, the phosphine-catalyzed or -mediated disulfide metathesis for dynamic system generation in organic or aqueous media is presented, respectively. The direct in situ $^1$H STD-NMR resolution of a dynamic carbohydrate system in the presence of a target protein (Concanavalin A) proved the suitability and compatibility of such disulfide metathesis protocols for the discovery of biologically relevant ligands.

In a third part, hemithioacetal formation is demonstrated as a new and efficient reversible reaction for the spontaneous generation of a dynamic system, despite a virtual character of the component associations in basic aqueous media. The direct in situ $^1$H STD-NMR identification of the best dynamic β-galactosidase inhibitors from the dynamic HTA system was performed and the results were confirmed by inhibition studies. Thus, the HTA product formed from the reaction between 1-thiogalactopyranose and a pyridine carboxaldehyde derivative provided the best dynamic inhibitor.

In a fourth and final part, a dynamic drug design strategy, where the best inhibitors from the aforementioned dynamic HTA system were used as model for the design of non-dynamic (or “static”) β-galactosidase inhibitors, is depicted. Inhibition studies disclosed potent leads among the set of ligands.

Keywords: Dynamic combinatorial chemistry; dynamic sulfur chemistry; dynamic drug discovery; thioglycoside; mutarotation; disulfide metathesis; hemithioacetal formation; $^1$H STD-NMR; inhibition; phosphine; Concanavalin A; β-galactosidase.
This thesis is based on the following papers, referred to in the text by their Roman numerals I-VI:

I. **Synthesis of Positional Thiol Analogs of β-D-Galactopyranose**  
Zhichao Pei, Hai Dong, Rémi Caraballo and Olof Ramström  

II. **pH-Dependent Mutarotation of 1-Thio-Aldoses in Water**  
Rémi Caraballo, Lingquan Deng, Luis Amorim, Tore Brinck and Olof Ramström  
*Submitted manuscript.*

III. **Phosphine-Catalyzed Disulfide Metathesis**  
Rémi Caraballo, Martin Rahm, Pornrapee Vongvilai, Tore Brinck and Olof Ramström  

IV. **Phosphine-Mediated Disulfide Metathesis in Aqueous Media**  
Rémi Caraballo, Morakot Sakulsombat and Olof Ramström  
*Submitted manuscript.*

V. **Direct STD NMR Identification of β-Galactosidase Inhibitors from a Virtual Dynamic Hemithioacetal System**  
Rémi Caraballo, Hai Dong, João P. Ribeiro, Jesús Jiménez-Barbero and Olof Ramström  

VI. **Towards Dynamic Drug Design: Identification and Optimization of β-Galactosidase Inhibitors from a Dynamic Hemithioacetal System**  
Rémi Caraballo, Morakot Sakulsombat and Olof Ramström  
*ChemBioChem*, Accepted manuscript.
# Table of Contents

Abstract

List of publications

Abbreviations

1 Introduction .................................................................................................................. 1  
1.1 Supramolecular chemistry .................................................................................... 2  
1.2 Dynamic combinatorial chemistry ....................................................................... 3  
1.3 Dynamic sulfur chemistry...................................................................................... 7  
1.4 The aim of this thesis .......................................................................................... 10  

2 Components for dynamic systems: syntheses and mutarotation properties of glycosyl thiols ................................................................. 13  
2.1 Introduction ............................................................................................................ 13  
2.2 Synthesis of sulfur-containing carbohydrate building blocks ....................... 14  
  2.2.1 Synthesis of thioglycosides ............................................................................... 14  
  2.2.2 Synthesis of glycosyl disulfides ......................................................................... 16  
2.3 Mutarotation of 1-thioglycosides in water ........................................................ 17  
  2.3.1 Introduction ....................................................................................................... 17  
  2.3.2 pH-Dependence in the mutarotation of 1-thioaldoses ....................................... 17  
  2.3.3 Reversibility of the mutarotation process .......................................................... 20  
  2.3.4 Mechanism of the mutarotation of 1-thioaldoses .............................................. 21  
  2.3.5 Solvation effects on the mutarotation of 1-thioaldoses ..................................... 21  

3 Exploration of a new catalytic format for disulfide metathesis: the action of phosphine compounds on disulfides....................................................... 23  
3.1 Introduction ............................................................................................................ 23  
3.2 Phosphorous compounds in disulfide chemistry ............................................. 23  
  3.2.1 Reduction of disulfides ...................................................................................... 23  
  3.2.2 Desulfurization of disulfides .............................................................................. 24  
  3.2.3 Disulfide metathesis .......................................................................................... 24  
3.3 Experimental evaluation and scope of the phosphine-catalyzed disulfide metathesis ....................................................................................... 26  
  3.3.1 Early systems .................................................................................................... 26  
  3.3.2 Screening of the phosphine catalysts ............................................................... 26  
  3.3.3 Solvent effect on the disulfide metathesis rate .................................................. 28  
3.4 Exploration of phosphine-catalyzed disulfide metathesis in DCC protocols ............................................................................................. 29  
  3.4.1 System reversibility ........................................................................................... 29  
  3.4.2 First dynamic systems ....................................................................................... 30
3.4.3 Optimized dynamic disulfide system ................................................................. 31

3.5 Towards phosphine-catalyzed disulfide metathesis in aqueous systems . 32
  3.5.1 Low water-content systems ......................................................................... 32
  3.5.2 Biphasic systems .......................................................................................... 33

3.6 Exploration of phosphine-mediated disulfide metathesis in water ............. 33
  3.6.1 System optimization ..................................................................................... 33
  3.6.2 Phosphine concentration effect on the disulfide metathesis ....................... 36

3.7 Exploration of the system biocompatibility ...................................................... 37
  3.7.1 Target protein: Concanavalin A (Con A) ...................................................... 37
  3.7.2 Direct resolution of a dynamic carbohydrate system for the binding of Concanavalin A .................................................................................................................. 38

4 Direct resolution of a dynamic hemithioacetal system using $^1$H STD NMR: identification of β-galactosidase inhibitors .................................................. 41
  4.1 Introduction ........................................................................................................ 41

4.2 Target protein: E. coli β-Galactosidase (EC 3.2.1.23) .................................. 42
    4.2.1 Biological function and mechanism of β-galactosidase ................................ 42
    4.2.2 Inhibition of β-galactosidase .................................................................... 43
    4.2.3 The selection of β-galactosidase as target protein ................................... 44

4.3 Dynamic hemithioacetal system in aqueous media ........................................ 44
    4.3.1 Starting component design ........................................................................ 44
    4.3.2 Hemithioacetal formation in water: the unexpected virtuality ................. 45
    4.3.3 Dynamic hemiacetal system formation ..................................................... 47

4.4 Determination of the inhibitory potency of the component associations ...... 48
    4.4.1 $^1$H-NMR competitive inhibition evaluation and screening ..................... 48
    4.4.2 Dynamic hemithioacetal system for the in situ binding studies ............... 50

4.5 Binding studies: direct identification of the best β-galactosidase binders ...... 50
    4.5.1 Saturation transfer difference (STD) NMR ............................................... 50
    4.5.2 $^1$H STD-NMR resolution of the dynamic hemithioacetal system ............ 51
    4.5.3 Determination of the binding nature of the aldehyde derivatives ............. 53
    4.5.4 Binding evidences for the determination of the best ligands .................... 54

4.6 Confirmation of the structure of the best inhibitors ........................................ 56

5 Dynamic hemithioacetal system-based drug design of β-galactosidase inhibitors .................................................................................................................. 59
  5.1 Introduction ........................................................................................................ 59

5.2 The fragment-based strategy in drug discovery ............................................ 60
    5.2.1 Introduction to the fragment-based approach ......................................... 60
    5.2.2 Dynamic combinatorial chemistry: a fragment-based approach .......... 60

5.3 Design and synthesis of β-galactosidase inhibitors ..................................... 61
    5.3.1 The design strategy .................................................................................... 61
    5.3.2 Synthesis of the thioglycoside series ......................................................... 63
    5.3.3 Synthesis of the β-C-Glycoside series ..................................................... 63
5.4 Evaluation of the inhibitory potency of the static library components to β-galactosidase ....................................................................................................... 64
5.4.1 $^1$H-NMR competitive inhibition studies .............................................................. 64
5.4.2 UV-Vis competitive inhibition studies ................................................................ 66
5.5 Important fragments for β-galactosidase inhibition ............................................. 67

6 Summary of results and concluding remarks ....................................................... 69

Acknowledgements
Appendix
References
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac</td>
<td>Acetyl group</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRD</td>
<td>Carbohydrate recognition domain</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>d</td>
<td>deuterated</td>
</tr>
<tr>
<td>DCC</td>
<td>Dynamic combinatorial chemistry</td>
</tr>
<tr>
<td>DCL</td>
<td>Dynamic combinatorial library</td>
</tr>
<tr>
<td>DCR</td>
<td>Dynamic combinatorial resolution</td>
</tr>
<tr>
<td>DDD</td>
<td>Dynamic drug discovery</td>
</tr>
<tr>
<td>DFT</td>
<td>Density functional theory</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNC</td>
<td>Dynamic nitrogen chemistry</td>
</tr>
<tr>
<td>DSC</td>
<td>Dynamic sulfur chemistry</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol / 1,4-bis(sulfanyl)butane-2,3-diol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>eq./equiv.</td>
<td>equivalent</td>
</tr>
<tr>
<td>FBDD</td>
<td>Fragment-based drug discovery</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectroscopy</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamate / Glutamic acid</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HEPA</td>
<td>Hexaethylphosphorous triamide</td>
</tr>
<tr>
<td>HTA</td>
<td>Hemithioacetal</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Inh.</td>
<td>Inhibition</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin / degree Kelvin</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>Kₘ</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>ONP</td>
<td>o-Nitrophenol</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-Nitrophenyl-β-D-galactoside</td>
</tr>
<tr>
<td>OPC₃</td>
<td>Tricyclohexylphosphine oxide</td>
</tr>
<tr>
<td>PCM</td>
<td>Polarizable continuum model</td>
</tr>
<tr>
<td>PC₃</td>
<td>Tricyclohexylphosphine</td>
</tr>
<tr>
<td>PPh₃</td>
<td>Triphenylphosphine</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td>quant</td>
<td>quantitative</td>
</tr>
<tr>
<td>r.t.</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure activity relationship</td>
</tr>
<tr>
<td>S&lt;sub&gt;N&lt;/sub&gt;2</td>
<td>Bimolecular nucleophilic substitution</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>STD-NMR</td>
<td>Saturation transfer difference-nuclear magnetic resonance</td>
</tr>
<tr>
<td>TA</td>
<td>Thioacetal</td>
</tr>
<tr>
<td>TBASA&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Tetrabutylammonium thioacetate</td>
</tr>
<tr>
<td>Tf&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Trifluoromethane sulfonic anhydride</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>trNOE</td>
<td>transferred nuclear overhauser enhancement</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TS</td>
<td>Transition state</td>
</tr>
<tr>
<td>TsOH</td>
<td>p-Toluenesulfonic acid</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius / degree Celsius / degree centigrade</td>
</tr>
</tbody>
</table>
1 Introduction

Commonly described as the “Central Science” owing to its faculty to connect physical- with life- and applied sciences, Chemistry can be defined as the discipline studying matter and its transformations. Chemistry – a science which has always fascinated, intrigued, assembled or divided societies by the observations, laws and beliefs it generates – is ubiquitous in nature and has always been. However, on a manmade chemistry standpoint, its perpetual evolution over time, greatly influenced by the different religious, philosophical and political trends, has not been continuous. Thus, its blossoming only began in a recent past, with the birth of Modern Chemistry.

The increasing interest for general sciences and the quest for knowledge, originating from the “Scientific Revolution”, lead to the emergence of new scientific methods gathering the notions of experimental rigor, empirical evidence and reasoning.[1, 2] However, despite numerous and rapid progression across the sciences, the field of synthetic chemistry still remained somehow hampered in its infancy. A plausible reason for this slow evolution could rely on the belief in “Vitalism”, a doctrine that fully separates the substances from the living entities and these from inanimate bodies.[3-6] This doctrine indeed distinguished and classified two forms of matter: the inorganic (non-organic, inanimate) and the organic (living). According to vitalists, the presence of an “élan vital”, only existing in organic matter, was essential to life. In other words, organic materials could not be synthesized from inorganic ones, the latter being destitute of this divine substance. Therefore, the organic chemist had a limited molecular playground and his/her main contributions to Science were mostly confined to the observation, extraction and identification of natural products.

In 1828, for the first time, Friedrich Wöhler, while investigating isomerism, reported the synthesis of an “organic” compound – urea – starting exclusively from inorganic materials.[7, 8] His discovery, a veritable breakthrough, occasioned many controversies and skepticism around both Vitalism and the provenance of the crystallized urea (organic or inorganic).[5, 9-12] However, the scientific impact of this first organic synthesis should not be resolved into the fate of Vitalism or into the organic/inorganic nature of the product, but rather into the advent of new perspectives for modern organic chemistry.[12] Therefore, from this door unconsciously opened by Wöhler, where inorganic substances can react and produce organic matter, emerged a vast number of organic syntheses capable of constructing from simple to sophisticated compositions and structures as well as a new tool for human creativity.
Thus, since this major event, the field of organic chemistry rapidly developed and broadened to other areas. Meanwhile, the vitalistic belief endured and evolved towards the exclusive differentiation of living and non-living entities rather than towards the distinction between organic and inorganic matters. On a vitalistic standpoint, it is nowadays fully accepted that inorganic substances can react and produce an organic matter. However, these substances would never be able to form a living entity, life being the transformation of the organic into the organized (owing to a vital force). This definition of the synthesis of life – the chemist’s greatest challenge – is not without a striking similarity with the description of Supramolecular Chemistry, the science exploring the chemistry from individual molecular entities to self-organization. Naturally, it would be inappropriate to associate this latter concept to a vital force involved in the process of life; however, one can just easily observe the ability of supramolecular chemistry to study the “life” of molecules, capable of self-organizing into structured “societies” (systems).

1.1 Supramolecular chemistry

Supramolecular chemistry, strongly rooted in organic chemistry, describes the “chemistry beyond the molecule”.[13-16] In contrast to classical organic synthesis which rapidly developed methods to afford artificial or natural complex molecules through covalent bonds, supramolecular synthesis takes advantage of inter- and/or intramolecular non-covalent interactions to construct systems of even higher level complexity.[17-19]

Supramolecular chemistry originates from the 1960s and stems from different scientific discoveries, all providing an essential piece toward the final concept (Figure 1).[6] An early piece of the supramolecular puzzle was reported in 1873, by Johannes Diderik van der Waals and his publication on the existence of intermolecular forces either of attractive or repulsive nature.[20] At the end of the 19th century, Emil Fischer ingeniously described the substrate/enzyme interactions in a pictorial “lock and key” model and therefore respectively contributed to build up the basis of the field and concept, yet non-existing, of supramolecular chemistry and molecular recognition.[21] Furthermore, the existence of hydrogen bonds, demonstrated in 1920 by Wendell Latimer and Worth Rodebush, gave a more complete picture of the puzzle.[22] Besides, another remarkable discovery based on hydrogen bonding is also believed to have hastened the dawn of the supramolecular area: the establishment of the double helix structure of DNA.[23]

Thus, supramolecular chemistry was fully established with the successive studies and discoveries of Charles Pedersen, Jean-Marie Lehn and Donald Cram on crown-ethers, cryptands and synthetic host/guest complexes.[24-26] Since then, this interdisciplinary and highly Nature-inspired area, awarded
with a Nobel Prize in 1987, has continuously been in development and has become increasingly popular. From molecular recognition and molecular information to self-organized systems, supramolecular chemistry, over time, has supplied chemistry and life sciences with crucial understandings and new perspectives. It has also contributed to the blossoming of new approaches and concepts. [16, 27-29]

Figure 1. Basis of Supramolecular Chemistry: a) van der Waals forces represented by dipole-dipole interactions, b) Lock and key method for enzyme/substrate interactions and c) Double helix structure of DNA originated by hydrogen bonding.

1.2 Dynamic combinatorial chemistry

Dynamic combinatorial chemistry (DCC) is a recently explored area strongly connected to supramolecular chemistry and, more precisely, to the dynamic (labile) nature of the bonds and forces interconnecting the entities in all supramolecular systems. [30-39] A first approach towards dynamic chemistry corresponds to constitutional dynamics where a molecular entity, rather than a supramolecular assembly, incessantly changes in its constitution through dissociation and recombination of all the possible molecular associations. [28, 29, 40, 41] DCC, by studying the interconversion of molecular fragments (or molecules) through reversible covalent reactions or non-covalent interactions, is a tool providing changes in constitution.

A dynamic system (or dynamic combinatorial library (DCL)) of high diversity and complexity can be rapidly and conveniently obtained starting from a strategically designed pool of starting components (or building blocks) reacting with one another through reversible processes. Owing to its dynamic nature, the system self-adapts towards a thermodynamic minimum in which the starting components and the products are continuously communicating. The product distribution is governed by the intrinsic stability of the different
system components and, therefore, the component associations with the lowest energy are preferentially formed. However, the introduction of external stimuli or factors might displace this thermodynamic equilibrium. The system will subsequently respond to this stimulus by spontaneously adopting a new equilibrium and by leading to a redistribution of the product ratios.\textsuperscript{[42]}

This self-adaptive property has greatly contributed to the immediate success of DCC and constitutes one of its strengths over conventional combinatorial chemistry. The presence of an artificial or natural template/receptor in a dynamic system often results in an increased formation, commonly defined as “amplification” or “over-expression”, of the best component associations at the expense of the other species (Figure 2).\textsuperscript{[43-56]} Therefore, if a template operates as a molecular trap for one or several component associations the dynamic system will naturally re-adapt itself in order to produce more of the best binder(s).

Figure 2. Schematic representation of the dynamic combinatorial chemistry concept. A dynamic system is generated through reversible exchange of the starting components. The best binder(s) is (are) selected by a receptor and the dynamic system self-adapts to produce more of this entity.

The selection approaches

One of the DCC challenges relies on the alteration of the product distribution by external factors (e.g. amplification of the best component associations). Several approaches have been developed, for this purpose. The first approach involves the selection through molecular recognition of an external template and has been extensively used for the study of dynamic systems in the presence of biological targets.\textsuperscript{[31-34, 53-58]} The component associations can form either a host molecule (usually a macrocycle) for small entities or, as depicted
in Figure 2, a guest ligand susceptible to interaction with a receptor. Then, there is the possibility to induce a selection through self-templating.\textsuperscript{[31, 59, 60]} In this case, the component associations are self-selected through intermolecular and/or intramolecular interactions and the components exhibiting the greatest ability to associate and/or self-associate are amplified. In addition, the most stable component associations are produced in higher concentrations. Finally, the selection through external physical stimuli such as, pH, temperature and light can also be used to influence the mixture distribution at equilibrium.\textsuperscript{[61-66]}

\textit{The reversible reactions}

The first step to consider when designing a DCC system for a specific target is the determination of the selection approach. Then, the choice of the reversible chemistry, often the actual keystone of DCC, can be performed according to the conditions set by both the selection approach and the target. To date, a broad panel of chemical reactions covering a fair range of experimental conditions is available for DCC purposes (Figure 3).\textsuperscript{[30-33]} However, the choice of the reversible scaffold for a given system must follow specific criteria. The reaction must be reversible and provide the dynamic system with a reasonable time scale, relative to the selection process. Then, the bond-breaking/bond-repairing reaction should be compatible with and proceed under similar experimental conditions as the selection step, so that they can operate simultaneously. Importantly, the reversible scaffold should not be involved in other processes, so that side reactions within the system are avoided.

\textit{The design of the starting components}

The route to efficient dynamic systems is highly dependent on the reversible chemistry employed as well as on the design of the starting components. In an idealistic view of DCC, one can imagine a system composed of a vast number of randomly chosen constituents in which the addition of a template directs the formation of the best component association(s). It certainly contrasts with the reality where each step leading to the final system must be carefully controlled and designed. Thus, a strategically designed system may introduce a lower diversity but, on the other hand, it may raise the chances of successful hit(s) from one or several products with the target. The starting components should incorporate the suitable functional group(s) necessary for the reversible process(es); however, scaffolds interfering with the reversible reaction should be avoided. The structure and composition of the starting components have to be consistent with the experimental conditions and the selection process. For example, a dynamic system exclusively operating under aqueous conditions should necessarily be composed of water-soluble starting components. Finally, in absence of any external stimulus, non-statistical equilibrium, in which one or several component associations are favored and thus produced in higher
concentration, should be avoided. Therefore, the reacting building blocks should preferentially lead to the formation of isoenergetic products.

**Figure 3.** Examples of reversible non-covalent and covalent reactions used in dynamic combinatorial chemistry. [30-33]
DCC in drug discovery

Over the years, DCC has been evidenced as a multidisciplinary area. Examples of dynamic polymers (dynamers), self-replicating systems as well as the implementation of new reversible chemistries, multi-level DCC and new concepts have emerged. By taking great advantage of reversible reactions and equilibria, usually highly undesired in traditional organic synthesis, DCC provides an alternative to conventional combinatorial chemistry and therefore, offers a rather new, promising and powerful approach in drug discovery. In contrast to combinatorial chemistry which requires the individual characterization and biological evaluation of the library components, DCC allows the library formation, characterization and biological evaluation processes to be performed simultaneously. In addition, owing to its adaptive nature, DCC offers the unique possibility of an in situ generation of the best ligands and, therefore, a receptor can influence and drive the system constitution and distribution. As a result, during the past 15 years, DCC has proven very successful for the formation and identification of ligands of biological relevance as well as of enzyme inhibitors. Also, numerous reversible scaffolds have attractively demonstrated their biocompatibility and potency in generating dynamic systems from simple to high diversity.

1.3 Dynamic sulfur chemistry

Dynamic sulfur chemistry (DSC) refers to the use of sulfur-containing reversible reactions for the generation of dynamic systems and constitutes an important sub-category of DCC. The great advantage of scaffolds bearing one or more sulfur atoms are their compatibility and stability with numerous functional groups and biological systems. Since the mid-1990s and the first examples of DCC studies, DSC has become a convenient and efficient means for dynamic systems formation (Figure 4). Thus, together with the chemistry based on reversible nitrogen-containing scaffolds, arbitrarily defined as dynamic nitrogen chemistry (DNC), sulfur chemistry has been extensively explored in DCC. It provides the area with, to date, the most prolific approach to the formation of reversible reactions: the disulfide exchange reaction.

DSC offers a consequent set of reversible reactions available for dynamic system formation. Thus, thiol-disulfide interchange, disulfide metathesis, transthiosterification, thioacetal exchange, hemithioacetal formation and conjugate addition reactions have been unevenly and successfully applied in the formation of dynamic systems (Figure 5).
Figure 4. Number of DCC publications, until the end of 2008, grouped and classified by type of reversible covalent chemistry (data from reference 31).

The thiol-disulfide exchange and disulfide metathesis reactions

The thiol-disulfide exchange/interchange, involved in several biological processes, is a well-studied reaction. The first example of this chemistry for the generation of dynamic systems appeared in the late 1990s, a couple of years after the emergence of DCC. Since then, thiol-disulfide exchange has proven a compelling method for dynamic system formation.

Thiol-disulfide exchange and disulfide metathesis are of similar nature and may provide the same dynamic mixture. In a typical thiol-disulfide exchange reaction, a thiol nucleophile reacts with a disulfide derivative under basic conditions providing the formation of the different component associations. Under aerobic conditions, the thiol species slowly oxidize into their corresponding disulfides. The exchange process is also turned off by protonation of the active nucleophile. Thus, the system loses its dynamic properties upon oxidation and in acidic conditions. However, in basic conditions, the addition of a catalytic amount of reducing agent (e.g. dithiothreitol (DTT) or phosphine) or a small amount of thiol derivative can temporarily circumvent this problem.

In a disulfide-metathesis process the starting disulfides, also components of the final system, generate the component associations owing to an external promoter/catalyst. The latter initiates and/or catalyzes the exchange reaction through the transient formation of thiolate intermediates, acting as the active species.
Figure 5. Reversible reactions used in dynamic sulfur chemistry to date.

The transthioesterification reaction

The transthioesterification reaction is related to the transesterification process and, similarly, exhibits reversible properties. However, in contrast to the transesterification exchange which usually requires harsh conditions in organic media,\textsuperscript{[102-104]} the sulfur analog reaction readily occurs under slightly basic aqueous conditions.\textsuperscript{[57, 58]} The transthioesterification reaction has been successfully implemented as a convenient and efficient route to dynamic systems. It also provides a good alternative to dynamic disulfide chemistry. Mechanistically, it involves the base-catalyzed reaction between a thiol nucleophile and a thioester derivative.

The thioacetal exchange and hemithioacetal formation

To date, the thioacetal (TA) and hemithioacetal (HTA) chemistries have not been thoroughly investigated in the DCC area and therefore remain at the proof-of-principle stage. However and similarly to the corresponding acetal and hemiacetal chemistries, TA and HTA are respectively accessed through the addition of two or one nucleophiles (thiols) at a carbonyl center (aldehyde or ketone). TA and, to a lower extent, HTA chemistries offer a convenient way for the induction of chirality and the generation of high diversity systems. In the former case, the possibility of interchanging two different scaffolds at the same carbon center exists whereas, in the latter case, the thiol addition to a carbonyl derivative leads to the formation of product bearing both vicinal hydroxyl and thioether functionalities. The HTA formation/dissociation process, in aqueous
media, can be catalyzed under both acidic and basic conditions.\[105-107]\] Recently, TA has demonstrated its capacity to undergo acid-catalyzed exchange processes in refluxing organic media.\[99]\]

**The conjugate addition reaction**

The reversible use of the conjugate addition of thiol derivatives reacting on an $\alpha,\beta$-unsaturated carbonyl compound (Michael acceptors) has been demonstrated. The component associations are rapidly and reversibly produced under neutral or slightly basic conditions.\[101]\]

**Orthogonal and tandem libraries**

In DCC, orthogonal and tandem approaches have proven to be compelling strategies for the introduction of increased diversity and complexity to dynamic systems.\[74-78]\] An orthogonal library is articulated around two different reversible reactions performing their respective exchange under different external conditions. DSC and more particularly disulfide chemistry has successfully demonstrated its compatibility and efficiency for dynamic orthogonal system generation. Thus, an example of disulfide interchange coupled with hydrazone exchange has been reported.\[108]\] In this particular case, disulfide and hydrazone chemistries, operating under different pH conditions, led to orthogonality of the system that could be controlled by a pH switch. In contrast, the tandem approach refers to the use of two different reversible chemistries under the same experimental conditions. Tandem dynamic systems involving DSC chemistries have been successfully generated. Examples of thiol-disulfide interchange coupled with the transthioesterification reaction have emerged.\[77]\] DSC has also proven to be an efficient approach towards the implementation of dynamic systems in other emerging concepts, such as, for example, dynamic combinatorial resolution (DCR).\[57, 58]\]

**1.4 The aim of this thesis**

In its entirety, this thesis outlines the original concept of dynamic combinatorial chemistry: from the design and synthesis of the starting components to the formation of dynamic systems and their application in drug discovery. In first place, thiocarbohydrate analogs, providing the biologically relevant ligands in the different studies, are prepared and their intrinsic properties evaluated; therefore broadening the overall interest to the glycochemistry area. Next, disulfide metathesis is targeted as an alternative reaction, in dynamic systems generation, to the widely used but rather slow thiol-disulfide exchange. New catalysts for this scrambling process are investigated and application in dynamic carbohydrate system generation in the presence of a biological receptor is challenged. Next, the implementation of
hemithioacetal formation as a reversible reaction for the formation of dynamic systems is proposed. In addition, a method for a direct and \textit{in situ} resolution of a dynamic system in the presence of a biological target is investigated. Finally, a dynamic drug design strategy is challenged for the discovery of potent leads.
2

Components for dynamic systems: syntheses and mutarotation properties of glycosyl thiols

(Papers I-II)

2.1 Introduction

Carbohydrates, constituting the most abundant class of biomolecules, are essential to many biological processes. Cell-cell interactions, cell communication and cell proliferation are examples of processes dependent on carbohydrate recognition.\cite{109-114} The synthesis of carbohydrate analogs and carbohydrate mimics, crucial for the study and understanding of such recognition processes, has therefore become of particular interest over the last decades. In this context, thiol analogs have been targeted owing to their biocompatibility and enhanced resistance to hydrolytic enzymes.\cite{115-117} In synthesis, thiol derivatives have several advantages. The combined nucleophilicity and chemical stability properties of a sulfhydryl group make thiol carbohydrate analogs excellent building blocks for glycosylation processes.\cite{118-122} Furthermore, the formation of dynamic carbohydrate systems has been demonstrated as an efficient approach for the discovery and study of protein-carbohydrate interactions.\cite{87-89, 123, 124} The presence of a sulfhydryl group in a carbohydrate structure enables the generation of dynamic systems through reversible sulfur chemistry. In this chapter, efficient syntheses of thiol carbohydrates building blocks for DCC purposes are reported. In addition, the mutarotation behavior of 1-thioglycosides has been investigated.

\[ \text{D- and L-galactose carbon numbering in Fisher projection and chair conformation.} \]
2.2 Synthesis of sulfur-containing carbohydrate building blocks

2.2.1 Synthesis of thioglycosides

The route to thiol analogs is highly dependent on the carbohydrate structure and on the reactivity of the different hydroxyl groups. Thus, chemical modifications at the anomeric carbon or at the sixth position of the carbohydrate ring are usually based on straightforward synthetic pathways. On the other hand, specific modifications at the C-2, C-3 or C-4 position of the carbohydrate ring require elaborated strategies due to the similar reactivity of the different hydroxyl groups.

A comparison between the synthetic routes to 1-thio-β-D-galactopyranoside (1) and 2-thio-β-D-galactopyranoside (2) perfectly illustrates the regioselectivity differences within the same core structure (Scheme 1 and Scheme 2). Indeed, compound 1 was easily and rapidly accessed in good yields in two to four synthetic steps, whereas compound 2 was obtained in nine steps and 20% overall yield. The sulfur insertion at the anomeric carbon (compounds 5 and 7) was performed through the bromo derivative 4 or directly through the peracetylated galactose 3. Subsequent deprotection yielded the desired thiogalactoside 1 in up to 85% overall yield.

![Scheme 1. Synthesis of 1-thio-β-D-galactopyranoside (1); (a) HOAc-HBr, CH₂Cl₂, r.t., overnight, 70%; (b) thiourea, DMF or acetone, 60 °C, 4 h, 69%; (c) ethanolamine, acetone, 60 °C, 2 h, 81%; (d) NaOMe, MeOH, r.t., 2 h, 95%; (e) i: TBASAc, toluene, r.t., 2h or, ii: KSAc, acetone, r.t., 2 h, 88%; (f) BF₃·O(Et)₂, HSAc, CH₂Cl₂, 0 °C to r.t., 24 h, 89%.](image)

In contrast, the sulfur insertion at the second position of the galactose ring proved more challenging. An inversion strategy was designed and after modification and optimization of the reaction pathway compound 2 could be obtained via epimerization of a taloside derivative 15.
Scheme 2. Synthesis of 2-thio-β-D-galactopyranoside (2): (a) PhCH(OMe)$_2$, TsOH, DMF, r.t., 16 h, 70%; (b) PhCONCO, CH$_2$Cl$_2$, -30 to 0 °C, 3 h, 60%; (c) Tf$_2$O, pyridine, CH$_2$Cl$_2$, -30 to 0 °C, 2 h; (d) NaH, THF, 0 °C to r.t., 3 h; (e) NaOH, THF, r.t., 12 h, then 80 °C, 3 h, 82% for 2 steps; (f) i: Bu$_2$SnO, dry MeOH, reflux, 2 h; ii: Ac$_2$O, DMF, r.t, 2 h, 72%; (g) TBASAc, toluene, r.t., 2 h, 68%; (h) NaOMe, MeOH, 2 h.

In order to further expand the scope of thiol carbohydrate analogs for the generation of dynamic carbohydrate systems, other 1-thioglycosides were synthesized. Starting from their corresponding commercially available aldopyranose or peracetylated aldopyranose, 1-thio-α-D-mannopyranoside (17), 1-thio-β-D-glucopyranoside (18), 1-thio-α-D-altropyranoside (19) and 1-thio-β-L-fucopyranoside (20) were successively and conveniently accessed in good overall yields using the “BF$_3$·O(Et)$_2$” method depicted in Scheme 3.[87, 88, 125]

Scheme 3. Synthesis of a) 1-thioglycopyranosides 17-19 and b) 1-thio-β-L-fucopyranoside (20): (a) i: Ac$_2$O, pyridine, DMAP or ii: I$_2$, Ac$_2$O, 0 °C to r.t., 1h, quant.; (b) BF$_3$·O(Et)$_2$, HSAc, CH$_2$Cl$_2$, 0 °C to r.t., 24 h, 76-89%; (c) NaOMe, MeOH, r.t., 2 h, < 90%; (d) i: LiOH, MeOH, H$_2$O, r.t., 4 h; ii: H$^+$ exchange resin, 92%.
Efficient syntheses yielding 1-thio-\(\alpha\)-L-fucopyranoside (24) have not been reported in the literature. The thiofucoside derivative 24 could however be accessed despite an overall low yield through a straightforward strategy (Scheme 4).\(^{126}\) Formation of the thioacetate derivative 25 prior to the peracetylation resulted in 30\% of the desired \(\alpha\)-anomer 26. Subsequent deacetylation initiated by lithium hydroxide in a water-methanol mixture provided compound 24.

Scheme 4. Synthesis of 1-thio-\(\alpha\)-L-fucopyranoside (24): (a) HSAc-HCl; (b) Ac\(_2\)O, pyridine, DMAP, 30\% over two steps; (c) i: LiOH, MeOH, H\(_2\)O, r.t., 4 h; ii: \(H^+\) exchange resin, 90\%.

2.2.2 Synthesis of glycosyl disulfides

1-Thioglycoside dimers (glycosyl disulfides) were readily accessed by oxidation of their corresponding 1-thioglycosides. Two oxidative methods were tested for efficiency comparison (Scheme 5). First, the oxidation was performed by addition of hydrogen peroxide in aliquots (0.75 equiv.) over a period of two days, under neutral or slightly basic aqueous conditions.\(^{[88]}\) A second method was based on thiol oxidation by molecular iodine.\(^{[121]}\) The experimental procedure consisted in the colorimetric titration of an aqueous solution of thioglycoside derivative with a saturated ethanolic iodine solution. Subsequent evaporation of the solvents followed by recrystallization from ethanol or precipitation by the addition of excess ethyl acetate in methanol provided the pure glycosyl disulfides in good yields. The iodine method proved rapid, more convenient and, importantly, no side products were formed.

Scheme 5. Synthesis of glycosyl disulfides 27, 28 and 29, (a) H\(_2\)O\(_2\), H\(_2\)O, r.t., 2 d, full conversion; (b) I\(_2\)/EtOH, H\(_2\)O, r.t., 10 min, full conversion.
2.3 Mutarotation of 1-thioglycosides in water

2.3.1 Introduction

In 1846, Augustin-Pierre Dubrunfaut first reported the mutarotation of glucose; a phenomenon that he originally named “birotation” consequent to the observation of a change in the optical rotation to about half its original value in freshly prepared glucose solutions. [127] Thus, mutarotation corresponds to the phenomenon describing the change in specific rotation of carbohydrate derivatives. [128-132] In other words, it defines the equilibration process between α- and β-carbohydrate anomers in solutions (Figure 6). The final composition of a carbohydrate mixture is likely to be governed by a combination of steric hindrance, stereoelectronic and solvent effects. [133] For these reasons, 150 years after its discovery, the mutarotation of carbohydrate anomers still remains a relatively unpredictable phenomenon.

![Figure 6. Mutarotation of glucose in water.](image)

Aldopyranose analogs, where the endocyclic oxygen and/or the glycosidic oxygen atom have been replaced by another heteroatom, also undergo mutarotation. [128, 129, 134, 135] Therefore, the observation of the anomerization behavior of 1-thioglycosides in aqueous solution and in methanol was somewhat unsurprising. However, the phenomenon had never been investigated in detail, [136-138] and thus, we decided to explore the mutarotation of 1-thioglycosides in aqueous solutions.

2.3.2 pD-Dependence in the mutarotation of 1-thioaldoses

Investigations on the mutarotation process of stereochemically pure 1-thioglycosides were initiated under acidic (pD 4), neutral (pD 7) and basic (pD 9) buffer conditions, so that any influence of the pD on the final anomeric mixture composition could be demonstrated. The kinetics of anomerization of thioglycosides 1, 17, 18 and 24 was therefore successively and conveniently followed by 1H-NMR spectroscopy (Figure 7). The results were highly conspicuous and under acidic or neutral conditions the mutarotation of 1-
thioaldoses readily occurred. Galacto- (1), gluco- (18), and fuco- (24) derivatives yielded anomeric mixtures composed of slightly increased amounts of β-anomer (78.1, 74.1 and 74.3% of β-anomer at pD 4, respectively) compared to their corresponding hydroxyaldoses. Intriguingly, 1-thio-α-D-mannopyranose (17) preferentially yielded the β-anomer under acidic conditions as well (66.2% of β-anomer). Its anomeric ratio was however expected to be essentially composed of the α-anomer, in analogy to mannopyranose (66.6% of α-anomer, measured under the same experimental conditions).

Figure 7. ¹H-NMR kinetic studies: fraction of β-anomer formed from 1-thio-β-D-galactopyranose (1), 1-thio-α-D-mannopyranose (17), 1-thio-β-D-glucopyranose (18) and 1-thio-α-L-fucopyranose (24) under acidic conditions.

In comparison, the anomeric mixtures formed from the set of experiments performed under basic conditions provided entirely different α-/β-compositions at equilibrium. The anomerization of thiol analogs 1, 18 and 24 proved almost exclusively restricted at pD 9 (> 95, 94 and < 5% of β-anomer, respectively). A pD-dependence for the mutarotation process of 1-thio-aldoses was therefore clearly demonstrated. A control experiment based on the evaluation of the mutarotation of 1-thio-β-L-fucopyranose (20) undoubtedly confirmed this trend (Figure 8). Indeed, while compound 20 reached a similar anomeric composition (80% of β-anomer) as its corresponding α-anomer (24) under acidic or neutral conditions, anomerization did not occur at higher pD.
On the other hand, the mannose derivative (17) demonstrated significant mutarotation behavior (Figure 9). However, in contrast to the observations made under acidic and neutral conditions, the α-anomer proved more stable under basic conditions (24% of β-anomer).

Further investigations were aiming to evaluate the predictability of the mutarotation behavior of 1-thioaldoses. Structural analysis of the different 1-thioaldoses provided crucial information for the prediction of the anomeric composition at equilibrium. After analysis, it was hypothesized that the presence of an axial hydroxyl group at the C-2 position of the carbohydrate ring causes significant β-anomer and α-anomer preferences of thiol analog 17 under acidic/neutral and basic conditions, respectively (Figure 10).
Investigations on the mutarotation of 1-thioaltropyranose (19) were performed under acidic, neutral and basic conditions. The results proved fully consistent with the predictions. Interestingly, full conversions from $\alpha$- to $\beta$-anomer were immediately recorded at pD 4 and at pD 7 (100% $\beta$-anomer) whereas, no anomerization was observed under basic conditions within the same time frame (pD 9, 100% $\alpha$-anomer).

### 2.3.3 Reversibility of the mutarotation process

The mutarotation of carbohydrate anomers is a reversible phenomenon. Subsequent experiments were therefore performed to establish the pD-profile of 1-thio-mannopyranose (Figure 11) and 1-thio-altropyranose species.

For 1-thio-D-mannopyranose species, the pD was tuned from acidic to basic conditions and vice versa for several cycles. Consequently, the anomeric mixture composition responded to this pD switch by yielding more of the $\beta$- or $\alpha$-anomer, respectively. Similar experiments were conducted with thiol analog 19 and a pD switch also generated a change in the anomeric mixture. However, a concomitant chair conformation occurred over time,\[^{139,140}\] therefore limiting the number of pD switches (Scheme 6). Nevertheless, the reversibility of the process was clearly demonstrated.
2.3.4 **Mechanism of the mutarotation of 1-thioaldoses**

The mechanism of the mutarotation of 1-thioaldoses could hypothetically follow two pathways (Scheme 7). First and similarly to the mutarotation process of natural aldoses, the formation of a thiocarbenium ion intermediate (32) through an exo-ring opening mechanism could occur.\(^{[128, 141]}\) An alternative pathway would involve the formation of an oxocarbenium ion intermediate (33). Unfortunately, no clear experimental evidences could discard one or the other of these mechanisms. However, the formation of trace amount of thiofuranoses during the kinetic experiments indicated that an acyclic intermediate was generated during the mutarotation process. Furthermore, the slow formation of aldoses (35) as well as the characteristic smell of H\(_2\)S confirmed the existence of process other than mutarotation.

2.3.5 **Solvation effects on the mutarotation of 1-thioaldoses**

As mentioned earlier, the factors directing the anomeric mixture composition at equilibrium are likely to involve a combination of steric, electronic and solvation effects. Given the delicate balance between these effects, it proved
difficult to draw any exact conclusions as to what factor plays the major role. For thioaltrose, however, density functional theory calculations (DFT) provided information on these preferences and, \textit{ab initio} calculations with an implicit solvent model (PCM) at the MP2/6-31G(d,p) level were performed (Figure 12). For the protonated and ionic states in solution, the computed free energies of the different 1-thioaltropyranose (19) anomers yielded an $\alpha/\beta$ composition of 9:91 and 89:11, respectively. These values were consistent with the experimental observations. In the ionic state, the formation of a stabilizing internal hydrogen bond in the $\alpha$-anomer justifies the strong $\alpha$-preference in basic conditions. Indeed, such stabilizing effect does not exist for the $\beta$-anomer. At neutral or acidic conditions, the formation of an internal hydrogen bond is not possible and therefore, the solvation effects favored the $\beta$-anomer.

Figure 12. Solution structures for the most stable conformers of the $\alpha$- and $\beta$-anomers of the deprotonated (top) and neutral (bottom) forms of 1-thio-$\alpha$-altrose. Geometries were optimized at the PCM-MP2/6-31+G(d,p) level.

Conclusion

In conclusion, efficient syntheses of thiol carbohydrate analogs have been performed with a particular emphasis on the preparation of 1-thioglycosides. Investigations on the mutarotation process of these 1-thioglycosides suggested a general preference for $\beta$-anomers under acidic conditions. A strong pD-dependence has been demonstrated and thus, under basic conditions, anomerization of the thiol analogs proved restricted except for the derivatives bearing an axial hydroxyl group at the C-2 position of the carbohydrate ring.
Exploration of a new catalytic format for disulfide metathesis: the action of phosphine compounds on disulfides

(Papers III-IV)

3.1 Introduction

Disulfide formation and exchange reactions, widely occurring in nature and extensively explored in DCC protocols, are processes that are intrinsically slow.\cite{142-145} Therefore, when dynamic systems are targeted, extended equilibration times are often needed and the concomitant oxidation of the active species (free thiolate anions) disabling the reversibility of the system generally occurs. It is thus of main interest to provide alternatives to the traditional thiol-disulfide exchange methods. In this context, disulfide metathesis protocols, enhancing the rate of the scrambling process and potentially overcoming the loss of the system reversibility owing to the use of external catalysts and/or initiators, have recently emerged.\cite{94-97} However, metal-free systems for the disulfide metathesis reaction under mild conditions remained to be addressed. In the following studies, we have investigated the use of phosphine-catalyzed and/or -mediated disulfide metathesis under mild conditions for the generation and evaluation of dynamic systems.

3.2 Phosphorous compounds in disulfide chemistry

3.2.1 Reduction of disulfides

Trivalent phosphorous compounds (phosphines, phosphorous triamides and phosphite esters) are well known to encompass strong nucleophilicity and reducing properties.\cite{146} Thus, in the presence of water, the reaction between phosphine derivatives and disulfides most often yields their corresponding phosphine oxide and thiol derivatives (Scheme 8).\cite{147-149} The mechanism involves the formation of a phosphonium salt intermediate and a thiolate anion. Subsequent formation of a strong phosphine-oxygen double bond constitutes the driving force of the disulfide reduction process. The reaction has a broad scope, and both alkyl- and aryl- phosphines generally show high efficiency for the reduction of a large panel of disulfide derivatives. Furthermore, in order to circumvent the generally poor water-solubility of
organophosphines, carboxylate and sulfonate derivatives have been synthesized.\textsuperscript{149-151}

\begin{align*}
\text{Scheme 8. Reduction of disulfides by a phosphine derivative in water.}
\end{align*}

3.2.2 Desulfurization of disulfides

Desulfurization of disulfide derivatives may readily occur in presence of trivalent phosphorus compounds. Indeed, when trialkyl phosphites are reacting with aryl- or alkyl- disulfides, a Michaelis-Arbuzov reaction can take place (Scheme 9a).\textsuperscript{152-154} The displaced thiolate anion reacts through a S\textsubscript{N}2 reaction with the phosphonium salt intermediate and leads to the formation of phosphonate and thioether derivatives.

In addition to phosphite esters, aminophosphines have been successfully reported as potent desulfurizing agents (Scheme 9b).\textsuperscript{155-157} For example, hexaethylphosphorous triamide (HEPA) reacts with organodisulfides to yield the corresponding aminophosphine sulfide and thioethers under mild conditions. The process, developed by Harpp and coworkers, relies on a similar mechanism than the aforementioned Michaelis-Arbuzov reaction.

\begin{align*}
\text{Scheme 9. Desulfurization of disulfides by a) alkyl- and aryl- phosphites and b) hexaethylphosphorous triamide.}
\end{align*}

3.2.3 Disulfide metathesis

As can be observed from the different reaction schemes between a phosphine derivative and a disulfide, the formation of the corresponding phosphonium salt intermediate is a reversible process (Scheme 8 and Scheme 9). Indeed, evidence of a reverse reaction leading to the (re-)formation of a disulfide has been reported previously,\textsuperscript{156} but surprisingly never explored further. Thus, a phosphine-catalyzed or -mediated disulfide metathesis reaction may readily occur under specific conditions; with this in mind, applications in DCC protocols can be envisaged (Scheme 10).
In order to design a suitable system for an exclusive disulfide metathesis process, the factors responsible for the reduction and desulfurization of the disulfides must be identified. In the former case, it is clear that conditions leading to the protonation of the thiolate anion intermediate need to be avoided. In the latter case, the nature of the phosphine displays a key role in the process and thus, aminophosphines as well as phosphite esters may not be appropriate catalysts or initiators of the disulfide scrambling. Therefore, alkyl- and aryl- phosphines were first envisaged for the disulfide metathesis in organic media and, quantum chemical studies were subsequently performed to confirm the reaction pathway (Figure 13). The computed free energies of reaction and activation were obtained for a model system composed of trimethylphosphine and methyl disulfide in acetonitrile.

The formation of the phosphonium salt intermediate was clearly established as the rate-determining step of the reaction. Importantly, the reverse reaction, leading to the recovery of the starting system, was evidenced to have lower activation energy than the hypothetical competitive and irreversible desulfurization process. Therefore, in the presence of organophosphines and under normal conditions, the disulfide metathesis should proceed without concomitant disulfide desulfurization.

**Scheme 10. Phosphine-catalyzed or -mediated disulfide metathesis reaction.**

In order to design a suitable system for an exclusive disulfide metathesis process, the factors responsible for the reduction and desulfurization of the disulfides must be identified. In the former case, it is clear that conditions leading to the protonation of the thiolate anion intermediate need to be avoided. In the latter case, the nature of the phosphine displays a key role in the process and thus, aminophosphines as well as phosphite esters may not be appropriate catalysts or initiators of the disulfide scrambling. Therefore, alkyl- and aryl- phosphines were first envisaged for the disulfide metathesis in organic media and, quantum chemical studies were subsequently performed to confirm the reaction pathway (Figure 13). The computed free energies of reaction and activation were obtained for a model system composed of trimethylphosphine and methyl disulfide in acetonitrile.

**Figure 13. Quantum chemical studies of the phosphine catalyzed reaction in acetonitrile. Energies (kcal mol⁻¹) have been calculated for 298 K and 1 M.**

The formation of the phosphonium salt intermediate was clearly established as the rate-determining step of the reaction. Importantly, the reverse reaction, leading to the recovery of the starting system, was evidenced to have lower activation energy than the hypothetical competitive and irreversible desulfurization process. Therefore, in the presence of organophosphines and under normal conditions, the disulfide metathesis should proceed without concomitant disulfide desulfurization.
3.3 Experimental evaluation and scope of the phosphine-catalyzed disulfide metathesis

3.3.1 Early systems

Our efforts towards phosphine-catalyzed disulfide metathesis were initiated with the evaluation of systems composed of two symmetrical disulfides in the presence of triphenylphosphine (PPh₃, very stable to oxidation) in chloroform-d and at ambient temperature (Scheme 11). The systems were originally constituted by methyl disulfide (36) and a short alkyl chain (ethyl 37a or n-propyl 37b) or an aromatic (phenyl 37c) disulfide derivative. ¹H-NMR spectroscopy was conveniently used to follow the formation of the unsymmetrical disulfide derivatives (38a, 38b or 38c respectively).

![Scheme 11. Phosphine-catalyzed disulfide metathesis (system composed of two initial disulfides).](image)

The first set of experiments consisted of the reactions between the individual disulfides (36 or 37a-c) and PPh₃ under the experimental conditions (350 mM per disulfide, chloroform-d, r.t.). As predicted from the quantum chemical studies, no changes were observed even when a PPh₃ loading higher than 50 mol% was used. These observations were in agreement with a disfavored reduction and/or desulfurization processes under these conditions. Subsequently, the disulfide metathesis of the systems displayed in Scheme 11 was evaluated with PPh₃ loading of 5 mol%. The mixture of two symmetrical starting disulfides led to the slow formation of the metathesis product, in low to good yields (Table 1, entries 1-3). The results, confirmed by GC-MS analysis, clearly underlined a trend in which alkyl-alkyl disulfides (38a and 38b) significantly formed at higher rates than alkyl-aryl disulfides (38c).

3.3.2 Screening of the phosphine catalysts

The efficiency of the disulfide metathesis reaction using PPh₃ as phosphine catalyst proved very low. Therefore, a series of phosphine derivatives was screened in order to probe the influence of the catalyst nature on the reaction equilibration rate (Figure 14).
Figure 14. Phosphine derivatives screened for disulfide metathesis.

With aliphatic phosphines being extremely sensitive to oxidation, only cyclohexylphosphine derivatives were selected for investigation (Table 1, entries 4-15). PCy$_3$, the most oxidation-prone phosphine in the series, was however constituted by 46% of its corresponding phosphine oxide at normal conditions ($^{31}$P-NMR analysis). In addition to organophosphines, triethyl phosphite and HEPA were used to complete the series (Table 1, entries 16 and 17, respectively). The results were highly conspicuous and, PCy$_3$ as well as HEPA proved to be the most efficient catalysts. Thus, formation and equilibration of unsymmetrical alkyl-alkyl disulfides could be reached within 1 hour (Table 1, entries 4, 5 and 17). However, for HEPA systems, irreversible desulfurization was, as expected, competing with the exchange reaction, resulting in the loss of the catalyst and the formation of side products with time. Consequently, PCy$_3$ was concluded to be the most suitable catalyst for the generation of dynamic disulfide systems and, control experiments confirmed that it indeed was the active catalyst (Table 1, entries 18 and 19).
Table 1. Screening of the phosphine catalysts for the disulfide metathesis process.

<table>
<thead>
<tr>
<th>Entry</th>
<th>RSSR</th>
<th>Catalyst</th>
<th>36b (%)</th>
<th>37b (%)</th>
<th>38b (%)</th>
<th>t (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37a</td>
<td>HPh3</td>
<td>23.5</td>
<td>23.5</td>
<td>53</td>
<td>44</td>
</tr>
<tr>
<td>2</td>
<td>37b</td>
<td>PPh3</td>
<td>22.5</td>
<td>22.5</td>
<td>55</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>37c</td>
<td>PPh3</td>
<td>36</td>
<td>36</td>
<td>28b</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>37a</td>
<td>PCy3</td>
<td>24.5</td>
<td>24.5</td>
<td>51</td>
<td>0.28</td>
</tr>
<tr>
<td>5</td>
<td>37b</td>
<td>PCy3</td>
<td>24</td>
<td>24</td>
<td>52</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>37c</td>
<td>PCy3</td>
<td>26</td>
<td>26</td>
<td>48</td>
<td>68</td>
</tr>
<tr>
<td>7</td>
<td>37a</td>
<td>PCy2Ph</td>
<td>27</td>
<td>27</td>
<td>46c</td>
<td>68</td>
</tr>
<tr>
<td>8</td>
<td>37b</td>
<td>PCy2Ph</td>
<td>40</td>
<td>40</td>
<td>20c</td>
<td>68</td>
</tr>
<tr>
<td>9</td>
<td>37c</td>
<td>PCy2Ph</td>
<td>34</td>
<td>34</td>
<td>32c</td>
<td>68</td>
</tr>
<tr>
<td>10</td>
<td>37a</td>
<td>PCy2Ph</td>
<td>27</td>
<td>27</td>
<td>27c</td>
<td>68</td>
</tr>
<tr>
<td>11</td>
<td>37h</td>
<td>PCy2Ph</td>
<td>77.5</td>
<td>77.5</td>
<td>45c</td>
<td>68</td>
</tr>
<tr>
<td>12</td>
<td>37c</td>
<td>PCy2Ph</td>
<td>37.5</td>
<td>37.5</td>
<td>25c</td>
<td>68</td>
</tr>
<tr>
<td>13</td>
<td>37a</td>
<td>P(BiPh)Cy2</td>
<td>45</td>
<td>45</td>
<td>10c</td>
<td>68</td>
</tr>
<tr>
<td>14</td>
<td>37b</td>
<td>P(BiPh)Cy2</td>
<td>45</td>
<td>45</td>
<td>10c</td>
<td>68</td>
</tr>
<tr>
<td>15</td>
<td>37c</td>
<td>P(BiPh)Cy2</td>
<td>41</td>
<td>41</td>
<td>18c</td>
<td>68</td>
</tr>
<tr>
<td>16</td>
<td>37a</td>
<td>P(OEt)3Cy2</td>
<td>47.5</td>
<td>47.5</td>
<td>5c</td>
<td>68</td>
</tr>
<tr>
<td>17d</td>
<td>37a</td>
<td>P(NEt2)Cy2 / HEPA</td>
<td>22.5</td>
<td>22.5</td>
<td>50</td>
<td>0.13</td>
</tr>
<tr>
<td>18b</td>
<td>37a</td>
<td>OPCy3</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>19b</td>
<td>37a</td>
<td>-</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>68</td>
</tr>
</tbody>
</table>

* All experiments were performed with 5 mol% of catalyst, 350 mM of each disulfide at room temperature and in chloroform-d. b Conversions are based on the methyl group. c The exchange reaction did not proceed to completion. d 5% of the final mixture corresponds to products from the desulfurization process. e No exchange was observed.

3.3.3 Solvent effect on the disulfide metathesis rate

Further investigations consisted in evaluating the solvent effect on the equilibration time. The ion-pair nature of the reaction intermediate suggests that polar solvents would facilitate the disulfide metathesis process. Thus, the disulfide metathesis was explored in a wide range of solvent polarities (Table 2). The recorded equilibration times were fully consistent with the predictions and DMSO-d6, similarly to acetonitrile-d3, provided very fast equilibration times.
Table 2. Equilibration times in different solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\varepsilon_r$</th>
<th>$E_T$</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene-$d_6$</td>
<td>2.3</td>
<td>0.111</td>
<td>11 d</td>
</tr>
<tr>
<td>Chloroform-$d$</td>
<td>4.9</td>
<td>0.259</td>
<td>17 min</td>
</tr>
<tr>
<td>Acetonitrile-$d_3$</td>
<td>35.9</td>
<td>0.460</td>
<td>&lt; 5 min</td>
</tr>
<tr>
<td>DMSO-$d_6$</td>
<td>46.4</td>
<td>0.404</td>
<td>&lt; 5 min</td>
</tr>
</tbody>
</table>

* Methyl disulfide 36 (350 mM), ethyl disulfide 37a (350 mM), PCy$_3$ (5 mol%), r.t.; $\varepsilon_r$: dielectric constant; $E_T$: normalized empirical solvent polarity scale.  
  b Values from reference 158, in non-deuterated solvents.

Subsequent experiments were aimed to analyzing the influence of the disulfide structure on the equilibration time in DMSO-$d_6$. In contrast with the trend previously observed in chloroform-$d$, both alkyl-alkyl and aryl-alkyl systems equilibrated within the same time frame (< 5 minutes). Additional investigations on the catalyst efficiency indicated the limitations of the system. Therefore, when PCy$_3$ loading was decreased from 5 mol% to 1 mol% in DMSO-$d_6$, the disulfide metathesis was still equilibrated within 5 minutes. However, lower concentrations resulted in a significant effect on the equilibration rate. Thus, 45 minutes were required for the equilibration of alkyl-alkyl disulfide system (disulfides 36 and 37a, 350 mM each) when a loading of 0.5 mol% of PCy$_3$ was used.

3.4 Exploration of phosphine-catalyzed disulfide metathesis in DCC protocols

3.4.1 System reversibility

The great efficiency of the catalytic system for a metathesis system composed of two initial disulfides was very promising for use in DCC protocols. However, the reversibility of the system remained to be demonstrated. A common strategy for the determination of the reversibility of a dynamic system is based on the addition of a new starting component to an equilibrated system. Thus, the addition of $n$-propyl disulfide (37b) in a system composed of methyl and ethyl disulfide species (36, 37a and 38a) resulted in the formation of all the possible disulfide combinations in almost statistical distributions (Figure 15). Therefore, the phosphine-catalyzed disulfide metathesis was proven reversible.
3.4.2 First dynamic systems

The generation of larger dynamic systems was performed with PCy$_3$ (5 mol%) as phosphine source and DMSO-$d_6$ as solvent; conditions that proved very efficient for the disulfide metathesis at ambient temperature. The dynamic system was initially constituted of three alkyl-chain disulfides (36, 37a and 37b) and two aromatic disulfides (37c and 37d), rapidly forming ten new disulfides (38a-d, 39a-c, 40a-b and 41, Figure 16). Surprisingly, the statistical product distribution of the system could not be reached even after extended reaction times. Further investigations evidenced that, under these conditions, a desulfurization process occurred concomitantly with the disulfide metathesis when aromatic disulfides were present in the library pool. However, this phenomenon was not observed at higher concentrations in the systems composed of two initial disulfides (e.g. formation of 38c), likely owing to a higher disulfide metathesis rate. In order to determine if the desulfurization process was exclusively caused by aromatic disulfides or if it was due to steric factors, a new system, composed of disulfides 36, 37a-b and i-propyl disulfide, was generated under the same experimental conditions. Again, the resulting reaction mixture contained desulfurization products in addition to the metathesis products. Therefore, the phosphine-catalyzed disulfide metathesis proved sensitive to disulfides possessing steric constraints at the carbon adjacent to the sulfur atom.

Figure 15. Reversibility of the reaction between methyl disulfide (36) and ethyl disulfide (37a) in the presence of PCy$_3$ (5 mol%) in chloroform-$d$: a) after equilibration between 36 and 37a and b) new equilibrium 50 min after addition of n-propyl disulfide (37b).
Figure 16. Disulfide metathesis in dynamic system generation. Aromatic-containing system formation and $^1$H-NMR spectrum of the reaction mixture. Experimental conditions: Disulfides 36, 37a-d (10 mM each), PCy$_3$ (5 mol%), r.t., DMSO-$_d_6$.

3.4.3 Optimized dynamic disulfide system

Aromatic disulfides 37c and 37d from the previous dynamic system were subsequently replaced by allyl disulfide (37e) and benzyl disulfide (37f), so that the desulfurization process could be avoided during the metathesis process (Figure 17). After optimization of the disulfide concentration (5 mM each) and catalyst loading (10 mol%), the system equilibrated within 55 minutes. In this case, concomitant thioether formation could not be detected during the reaction, thus demonstrating the convenient and efficient use of the phosphine-catalyzed disulfide metathesis for the generation of dynamic systems.
3.5 Towards phosphine-catalyzed disulfide metathesis in aqueous systems

3.5.1 Low water-content systems

The success of the use of phosphine derivatives for the disulfide metathesis in organic media and the observation of the rapid formation of metathesis products relative to the desulfurization process led us to envisage the exploration of similar systems in aqueous media. Indeed, in the cases where concomitant thioether formation was evidenced, it could be noted that the disulfide metathesis performed at higher rates than the desulfurization process. Thus, in aqueous systems, it could be hypothesized that the predicted disulfide reduction would occur at lower rate than the disulfide scrambling, therefore enabling the generation of a dynamic system. Investigations on mixed systems (D$_2$O/DMSO-d$_6$, 5:95 to 25:75) were initiated for the disulfide metathesis reaction between disulfides 36 and 37a in the presence of PCy$_3$ (5 mol%).
results were highly conspicuous and, despite a general $^1$H-NMR signal broadening due to the poor solubility of the initial disulfides in water-containing systems, solely the disulfide metathesis product 38a was formed during the reaction. Therefore, phosphine derivatives proved suitable for the disulfide metathesis in aqueous media.

### 3.5.2 Biphasic systems

In addition to being prone to oxidation, PCy$_3$ has a very poor solubility in water. Therefore, biphasic systems were investigated: a chloroform phase contained methyl disulfide (36) and the phosphine derivative whereas 1-thio-β-D-galactoside dimer (27) was dissolved in water. The mixture was vigorously stirred at ambient temperature and the reaction was followed by $^1$H-NMR for both phases. Unfortunately, even when the loading of catalyst was increased up to 200 mol%, disulfide metathesis did not occur. The initial disulfides were thus fully recovered in their corresponding preferential solvents.

### 3.6 Exploration of phosphine-mediated disulfide metathesis in water

#### 3.6.1 System optimization

The achievements of low water-content dynamic systems were contrasting with the unsuccessful attempts in biphasic media. However, these differences could probably be explained by a greater difficulty for the phosphine derivative to perform the metathesis reaction at the interface of the two phases. Therefore, we initiated the exploration of dynamic disulfide systems in aqueous media. Three glycosyl disulfides were conveniently selected for investigation and optimization of the disulfide metathesis reaction. Thus, 1-thio-β-D-galactoside dimer (27), 1-thio-β-D-glucoside dimer (28) and 1-thio-α-D-mannoside dimer (29) constituted the starting disulfides. PCy$_3$, although poorly soluble in water, was initially used as catalyst. Furthermore, in order to control the pH after addition of the phosphine which, besides being a good nucleophile, also can act as a base, the reactions were performed in buffer solutions (pD 8). The pD-value was strategically set higher than the pKa-value (7.7)$^{[58]}$ of the different thiolate anion intermediates from the carbohydrate system, so that protonation would be avoided. However, equilibration of the system did not occur under these conditions (50 mM for each disulfide, 50 mol% of PCy$_3$) and the disulfide metathesis products only amounted to a few percents. Consequent increase of the phosphine loading, up to several equivalents, did not provide any improvements. Interestingly, thiol formation and other hypothetical side products (except of tricyclohexylphosphine oxide,
OPCy₃) were not observed. Therefore, it was concluded that PCy₃ was not suitable for the catalysis of the disulfide metathesis in water.

Alternatives involved, for example, the use of different phosphine derivatives. After analysis of the results obtained in organic media, HEPA naturally appeared as a potential candidate to overcome the problematic issues involved with PCy₃. However, this system would be expected to generate some aminophosphine sulfide and thioether derivatives disabling the system reversibility. Surprisingly, the addition of HEPA (100 mol%) to the glycosyl disulfide mixture (27, 28 and 29, 50 mM each) did not yield any thioether formation but, instead, solely the metathesis reaction occurred. The equilibration proved very slow (32 h) and further optimization was needed. After a series of experiments, it was concluded that the most efficient alternative way to increase the equilibration rate was through the addition of acetonitrile or DMSO. Thus, in the presence of acetonitrile-d₃ (10%, v/v) and HEPA (100 mol%), glycosyl disulfides 27, 28 and 29 (15 mM each) generated three new disulfides (43, 44 and 45) and equilibration of the system occurred within 3 hours (Figure 18a-c). Subsequent addition of a fourth disulfide (46) yielded the formation of three new carbohydrate-containing species (47, 48 and 49); thus, demonstrating the reversibility of the reaction (Figure 18d). Furthermore, the product redistribution, within the system components, generated by the presence of thiocholine disulfide (46, thiol pKa of 7.7)[58] proved that the scope of such protocols is not restricted to carbohydrate derivatives.

The structure of the exchanging disulfides was however demonstrated to affect the final product distribution and/or equilibration rate. For example, in two starting disulfide systems, the non-sterically hindered disulfide 46 resulted in rapid displacement of the equilibrium towards the formation of the unsymmetrical disulfides at the exception of the mannose-containing derivative 49 (Figure 19). Furthermore, the mannose-species required longer equilibration times than the other species. The reason could be attributed to steric effects and a more difficult access to the disulfide bridge for an α-carbohydrate conformer. The different systems proved stable for a period of over a month under normal conditions and therefore, disulfide metathesis still readily occurred in the reaction mixture.
Figure 18. Disulfide metathesis in dynamic carbohydrate system generation: a) $^1$H-NMR spectra of the reaction mixture after equilibration of the library; enlarged area displaying the anomeric protons of the carbohydrate species at b) $t = t_0$, c) $t = 175$ min and d) reversibility of the system after the addition of disulfide 46.
3.6.2 Phosphine concentration effect on the disulfide metathesis

The study of the disulfide metathesis reaction at different phosphine concentrations provided further insights into the limitations and efficiency of the system. The formation and equilibration of the dynamic carbohydrate system (starting disulfides 27, 28 and 29, 15 mM each) was investigated with phosphine concentrations varying from 25 mol% to 200 mol% (Figure 20). The results were highly conspicuous and a significant concentration effect was undoubtedly displayed. Thus, higher phosphine concentrations yielded faster equilibration of the dynamic carbohydrate system.

Unexpectedly, when phosphine concentrations lower than 75 mol% were used, the system did not produce the same final composition. However, the
hypothetical loss of the phosphine efficiency (for 50 and 25 mol% loadings), which could have explained the distribution differences, was clearly discarded during a reversibility control experiment. The addition of thiocholine dimer (46, 15 mM) led to a consequent but slow readjustment of the system composition. From these series of experiments, it was therefore concluded that a phosphine loading corresponding to a minimum of 75 mol% of the disulfide concentration was necessary for a nearly statistical product distribution.

3.7 Exploration of the system biocompatibility

3.7.1 Target protein: Concanavalin A (Con A)

Concanavalin A (Con A) is a well-studied lectin (carbohydrate binding protein) isolated from a legume plant: *Canavalia ensiformis*, commonly known as Jack bean (Figure 21). At neutral or basic pH, it forms a tetrameric structure in which each subunit, composed of 237 amino acid residues, is identical.[159] Two antiparallel β-sheets of six and seven strands, respectively, constitute the subunit structure. Con A is a lectin which possesses specificity primarily for α-D-mannose and α-D-glucose structures. The shallow carbohydrate recognition domain (CRD), present in each monomer, allows the binding of mono-, oligo- and polysaccharides. In addition, it has been demonstrated that two metal cations (Mn²⁺ and Ca⁺) at the CRD are essential for the carbohydrate-lectin interactions.[112]

![Figure 21. a Monomeric subunit of Concanavalin A. Two metal ions (manganese (pink) and calcium (grey)) are located at the carbohydrate recognition domain (CRD). a Figure made with PyMOL[160] software (reference 1I3N[161] from the Protein Data Bank[162]).](image-url)
3.7.2 Direct resolution of a dynamic carbohydrate system for the binding of Concanavalin A

Investigations on the biocompatibility of the phosphine-mediated disulfide metathesis were thus conducted with the model receptor, Con A. $^1$H Saturation transfer difference-NMR ($^1$H STD-NMR, see Chapter 4.5.1 for description of the technique) was conveniently adopted for the direct identification of the best binders from the carbohydrate system (starting disulfides: 27, 28 and 29) to Con A (0.1 mM). The on-resonance and off-resonance frequencies were strategically set at 8 and 100 ppm, corresponding to spectral regions where no carbohydrate signals and no system signals were present, respectively. The Mannose species were naturally expected to be identified in the STD spectra while the other carbohydrate derivatives possessing no or very weak affinity with the lectin should not be displayed. First, in order to probe the binding experiment conditions, a system constituted of the starting glycosyl disulfides (10 mM each) in the absence of phosphine was investigated. Mannosyl disulfide 29 was the only carbohydrate derivative observed in the STD spectra, thereby validating the method. A second experiment consisted of the binding analysis resulting from the dynamic carbohydrate system (20 mM of each starting disulfide; 75 mol% of PCy$_3$) after equilibration with Con A (Figure 22). Thus, mannose derivatives 29, 43 and 44 were clearly identified as the best binding species respectively. Therefore, the use of trivalent phosphine derivatives, and more particularly HEPA, did not influence the biological activity of the target protein.

Conclusion

During these studies, we have successfully demonstrated the use of phosphine-catalyzed and phosphine-mediated disulfide metathesis for the efficient generation of dynamic systems in organic and aqueous media, respectively. The use of phosphine derivatives also proved to have no effect on the biological evaluation of a disulfide system with a target protein. Therefore, the use of phosphine for the disulfide metathesis provides a potent alternative to traditional thiol-disulfide exchange methods.
Figure 22. $^1$H STD-NMR binding studies of the dynamic carbohydrate system with Concanavalin A: a) full system spectra; b) enlarged area of the carbohydrate region. * Signals from the remaining solvent and phosphine derivatives.
4

Direct resolution of a dynamic hemithioacetal system using $^1$H STD NMR: identification of $\beta$-galactosidase inhibitors

(Paper V)

4.1 Introduction

In order to further expand the scope of DCC and ensure its increasing popularity and development, the implementation of new tools for the generation of dynamic systems and for the identification of their best component associations remains essential. Thus, in the set of reversible reactions available for DCC, but yet unexplored, hemithioacetal (HTA) formation was selected for investigation. This choice was mainly governed by the biocompatibility properties offered by the HTA chemistry and, therefore, proteins ($\beta$-galactosidase) could be targeted. Subsequent competitive inhibition studies on the dynamic hemithioacetal system were performed to determine the best component associations against $\beta$-galactosidase activity. In addition, the following study challenged the use of $^1$H saturation transfer difference (STD) NMR spectroscopy in DCC, a technique enabling the direct identification of the best component associations (Figure 23).

Figure 23. Concept of direct STD NMR identification of enzyme inhibitors from a virtual dynamic hemithioacetal system.
4.2 Target protein: E. coli β-galactosidase (EC 3.2.1.23)

4.2.1 Biological function and mechanism of β-galactosidase

β-Galactosidase from Escherichia coli (E. coli) is a large tetrameric enzyme produced upon activation of the lac operon. Each subunit, composed of 1023 amino acids, comprises five distinct domains organized around a central α/β barrel (Figure 24). β-Galactosidase belongs to the hydrolase class of enzymes. The enzyme active site, located at the third domain, catalyzes the hydrolysis of β-galactosides into their corresponding free β-galactose and alcohols. In addition, disaccharides can undergo a transglycosylation reaction at the enzyme active site. Thus, lactose, the natural substrate, produces allolactose, an inducer of the lac operon.

Figure 24. Subunit of E. coli β-galactosidase tetramer. Representation of the five domains and location of the active site in which a lactose molecule (red) is in complex. Figure made with PyMOL software (reference 1JYN from the Protein Data Bank).

β-Galactosidase is a retaining enzyme, meaning that the initial anomeric conformation of the substrate is maintained in the product. The proposed catalytic mechanism involves a double displacement reaction in which galactosylation and degalactosylation steps successively take place (Figure 25). The glycosidic bond cleavage and the formation of the galactosyl-enzyme intermediate constitute the first step in the overall hydrolysis reaction. The second step corresponds to the attack of a water molecule on the galactosyl-enzyme intermediate, leading to the formation of free β-galactose. It has been demonstrated that the presence of metal ions such as magnesium (Mg²⁺) or sodium (Na⁺) at the active site enhances the catalytic activity of β-galactosidase. However, the exact role of some important enzyme
residues for the catalytic reaction still remains somewhat controversial. For many years, residue Glu461 was believed to be the active nucleophile during the substitution reaction. More recently, however, Glu461 has been identified as the proton donor/acceptor initiating the acid/base catalyzed process, Glu537 being the active nucleophile. \[171, 175, 176\] In addition, the role of Tyr503, evidenced as important during the catalytic mechanism, was for a long time not clearly defined.\[177\] Recent computational studies have however proposed that Tyr503 stabilizes Glu537 and the oxocarbenium ion of the galactose structure by hydrogen bonding during the degalactosylation of the galactosyl-enzyme intermediate.\[170\]

![Proposed catalytic mechanism of E. coli β-galactosidase for the hydrolysis of lactose.](image)

**Figure 25.** Proposed catalytic mechanism of E. coli β-galactosidase for the hydrolysis of lactose.

### 4.2.2 Inhibition of β-galactosidase

To date, the inhibition of β-galactosidase has been explored using a fair range of competitive inhibitors; i.e., molecules that closely resemble the structure and geometry of the substrate. The binding site structure and binding mode between the enzyme and the substrate are essential elements in the design of potent inhibitors. At the entrance of the active site, galactoside derivatives first bind in a “shallow” mode. Various aglycon fragments can therefore be accommodated, justifying that a wide range of galactosides are good β-galactosidase substrates. In addition to this “shallow” binding mode, a “deep” binding mode occurs inside the active site, where the catalytic mechanism is performed. The presence of a tryptophan residue (Trp568) provides a hydrophobic platform for the galactose ring and promotes its stacking in the
deep binding mode. The binding mode of the aglycon part would offer important information for the design of inhibitors. Unfortunately, the aglycon binding to the enzyme, except for a stacking interaction with Trp999, has yet to be clearly defined.

The design of a good β-galactosidase competitive inhibitor should encompass several structural properties, the most important being naturally the presence of a galactose fragment in the molecule. In addition, the glycosidic linkage should possess improved resistance to hydrolysis. In this context, thiogalactosides have demonstrated high efficiency and, isopropyl-β-D-1-thiogalactopyranoside (IPTG) is widely used in molecular biology as a ligand and inhibitor of β-galactosidase. Similarly, C-glycosides and amino carbohydrate derivatives have also shown good inhibition properties. Furthermore, the importance of the structure of the aglycon fragment has been underlined in several studies. Thus, a significant increase of the inhibitory potency occurs when aromatic substituents or, to a lower extent, long alkyl-chain derivatives are used. This specificity is consistent with the presence of a hydrophobic region (Trp999) in the aglycon subunit site.

4.2.3 The selection of β-galactosidase as target protein

The selection of β-galactosidase as the target protein was the result of a strategic analysis of the requirements needed for the study of dynamic HTA systems. Thus, the receptor should not interfere with the formation of the component associations and any side reaction between the target protein and the system components should be avoided. In this context, enzymes containing cysteine residues at the active site (including the active site vicinity) needed to be excluded. Indeed, the reaction between a cysteine residue and an aldehyde from the library pool is likely to form an enzyme-system component HTA product competing with the dynamic system at the active site. β-Galactosidase, a cysteine-free enzyme, revealed to be an excellent candidate. In addition, other criteria such as the use of thiol carbohydrate analogs previously synthesized (see Chapter 2.2.1) and the easy follow up of the inhibition reactions using UV-Vis spectrometry and/or 1H-NMR spectroscopy also oriented our choice towards β-galactosidase.

4.3 Dynamic hemithioacetal system in aqueous media

4.3.1 Starting component design

The design of the starting components for the dynamic HTA system comprises two key features. First, the single constituents and component associations should be compatible with aqueous systems and with the target protein. In
addition, the final system should bring diversity, so that unexpected \( \beta \)-galactosidase inhibitors can be identified. The system also needs to provide specificity, so that the chances of successful hits are increased. Thus, a series of five thiols and six aldehydes were initially chosen for investigation of HTA formation (Figure 26). The thiol series comprised three 1-thioglycosides and two substituted alkyl derivatives; all having a thiol pKa of 7.7\(^{[58]}\) thereby offering a similar reactivity. The galactose fragment \( \mathbf{1} \) was expected to form the best dynamic enzyme inhibitors. In the aldehyde series, a set of aromatic and branched alkyl derivatives was selected. Their structures somewhat resemble the aglycon fragment of known substrate \( o \)-nitrophenyl-\( \beta \)-galactoside (ONPG) and ligand IPTG of \( \beta \)-galactosidase. The aromatic fragments (\( \mathbf{A} \), \( \mathbf{B} \) and \( \mathbf{C} \)) would be expected to provide the best inhibitors while the strength of the enzyme inhibition would decrease in respect to the length of the alkyl aglycon derivatives.

**Figure 26. Selection of the starting components for the intended dynamic system formation.**

### 4.3.2 Hemithioacetal formation in water: the unexpected virtuality

As previously reported (see Chapter 1.3), the addition of a thiol derivative to an aldehyde reversibly produces a HTA (Scheme 12). However, in presence of water, aldehydes are well known to undergo reversible hydrate formation. Therefore, the intended dynamic system will be constituted of hemiacetal products, the desired component associations, as well as the starting thiols and aldehydes, all in equilibrium.

**Scheme 12. Hemithioacetal and hydrate formation in water.**

The selection of HTA chemistry, a reaction known to be acid and base catalyzed, is compatible with the enzymatic conditions set for this study (pD 7.2, 25 °C).\(^{[105-107, 184, 185]}\) However, the equilibration time of the reaction,
presumed to be fast, remained to be determined. Thus, investigations on HTA formation, at pD 7.2, were performed and $^1$H-NMR spectroscopy was conveniently used to follow the reaction. Somewhat surprisingly, the addition of compound 1 to aldehyde A did not result in any observation of the characteristic signals of product 1-A (Figure 27b). Indeed, the proton of the new chiral center in the HTA product, expected to have a similar chemical shift than the corresponding hydrate (A') proton, could not be observed by $^1$H-NMR under these conditions. However, the general signal broadening of the thiol and aldehyde protons suggested a rapid exchange reaction (Figure 27b). In order to verify this hypothesis and aiming to decrease the dissociation rate, the same reaction was performed under acidic conditions (Figure 27c). This control experiment proved successful and the characteristic HTA signal could immediately be identified at lower pH.

Figure 27. $^1$H-NMR spectra of: a) aldehyde A at pD 7.2, b) system 1-A at pD 7.2 and c) system 1-A at pD 4. The characteristic HTA signal is only observed under acidic conditions.
Each thiol/aldehyde combination from the intended dynamic system was subsequently prepared at pH 4 and the presence of the characteristic HTA proton signal, always adjacent to the corresponding hydrate signal, was confirmed by $^1$H-NMR. The HTA signal for each component association was recorded in very similar proportions, indicating that the intended dynamic system would most likely have a similar product distribution. In conclusion, HTA formation occurs readily and spontaneously in water. However, the rapid dissociation rate under neutral conditions precludes the observation of the HTA characteristic $^1$H-NMR signals. Thus, the HTA products can be considered as virtual under the enzymatic conditions. Here, the terminology “virtual” underlines the invisible but existing HTA formation during the $^1$H-NMR experiments.[30, 51, 186]

4.3.3 Dynamic hemiacetal system formation

The reaction between the selection of thiol derivatives and aldehydes theoretically led to the spontaneous formation of 30 discrete HTA products (60 if considering diastereoisomers) as depicted in Scheme 13. Owing to the “virtual” character of the system, the different stereoisomers could not be observed and therefore identified under the experimental conditions. Thus, the dynamic HTA system was totally composed of at least 47 discrete members (5 thiols, 6 aldehydes, 6 hydrates and 30 HTA adducts).

Scheme 13. Virtual dynamic hemithioacetal system formation in aqueous media.
4.4 Determination of the inhibitory potency of the component associations

4.4.1 $^1$H-NMR competitive inhibition evaluation and screening

β-Galactosidase activity and inhibition are usually determined using UV-Vis spectrometry. Thus, ONPG, a well-known β-galactosidase substrate and chromogenic compound, has been commonly used in such studies. Indeed, the hydrolyzed ONPG product, o-nitrophenol (ONP), in the deprotonated state can be easily monitored by UV-Vis spectrometry owing to its intense yellow color. However, in this study, $^1$H-NMR was instead adopted to follow the substrate hydrolysis rates. The subsequent binding studies involving STD-NMR spectroscopy, the use of $^1$H-NMR for the inhibition experiments appeared as the most appropriate to maintain consistency in the analysis method.

First, a blank reaction, following the hydrolysis rate of ONPG (0.83 mM) in a $d$-buffer solution (pD 7.2) of β-galactosidase and in the absence of any inhibitors, was optimized. The β-galactosidase concentration was calibrated so that ONPG hydrolysis would be complete in a reasonable time frame (less than 60 min). In the literature, the presence of magnesium ions (Mg$^{2+}$) is described to enhance the enzyme catalytic activity. Thus, a control experiment was performed using the same buffer solution in the presence of MgCl$_2$ (1 mM). Surprisingly, ONPG hydrolysis rate was not affected by the addition of Mg$^{2+}$ in the reaction. Therefore, the subsequent kinetic experiments were carried out without any addition of metal ions, although sodium ions were naturally present in the phosphate buffer solution.

After optimization of the blank reaction ($t_{50} = 23$ min, time required for 50% of ONPG hydrolysis), competitive inhibition experiments were performed for each component association with an initial substrate (ONPG)/inhibitor ratio of 1:1. However, at these conditions, no significant inhibition was recorded for any of the individual HTA adducts. Three hypotheses were therefore envisaged: firstly, the system was not adapted to β-galactosidase inhibition; secondly, an incubation time was needed for the inhibitor to penetrate the deep active site of the enzyme; finally, the active HTA concentration was not high enough to affect the substrate hydrolysis. The first hypothesis was not likely to be the cause of the lack of inhibition. Indeed, the dynamic system was especially designed to include some transient HTA products that closely resemble known enzyme inhibitors. The second hypothesis was dismissed after a control experiment, in which the potential system inhibitors were incubated for 30 min with β-galactosidase prior to the addition of the substrate. No effect on the ONPG hydrolysis was recorded, thus precluding any incubation effect. The third hypothesis could however be supported and subsequent optimization of the constituent concentration was needed. After further investigation, it was found that the use of five equivalents of dynamic starting components (4.15
mM of thiol and aldehyde derivatives) provided, for some component associations, a severe decrease in the ONP formation rate (Figure 28).

**Figure 28.** Inhibition studies of the system components and constituents. Inhibition $= \frac{t_{50(\text{inh})}}{t_{50(\text{blank})}}$, for which $t_{50}$ = time for 50% ONPG hydrolysis. Darker data represents the constituents and component associations of the dynamic HTA system for the binding studies.

The results were highly conspicuous and only the galactose-containing entities showed significant inhibitory effects. A transient association between the galactose fragment and any aldehyde from the system was needed in order to observe an effect on ONPG hydrolysis. Indeed, none of the other single constituents and their transient component associations demonstrated any inhibitory potency against $\beta$-galactosidase activity. When looking closer to the galactose-containing systems, one can observe a clear trend in the inhibitory potency of the different component associations. Aromatic-containing systems [1-A], [1-B], and [1-C] were the most efficient dynamic inhibitors and decreased the ONPG hydrolysis 11.8, 6.1 and 5.6 times respectively. These coefficients were calculated as the ratio of the $t_{50}$ value of the inhibition reaction over the $t_{50}$ value of the blank reaction. The results of the aromatic series combined with the inhibition observed with the branched alkyl aldehydes D, E and F (inhibition value of 3.7, 2.8 and 2.6 respectively) were fully consistent with a deep binding mode for the transient component associations (See Chapter 4.2.2).
4.4.2 Dynamic hemithioacetal system for the in situ binding studies

The use of $^1$H STD-NMR for the subsequent binding studies in a proof-of-principle investigation led us to envisage a smaller system (Scheme 14). Indeed, the previously described system results in a very complex 1D $^1$H-NMR spectrum. The analysis of the inhibition studies directed us to a simplified system in which the best aromatic and branched alkyl chain fragments (constituents A and D respectively) were equilibrated with a series of five thiols, leading to a system of 19 members (10 HTA products, Figure 28 darker data).

Scheme 14. Reduced-size virtual dynamic hemithioacetal system formation.

4.5 Binding studies: direct identification of the best β-galactosidase binders

4.5.1 Saturation transfer difference (STD) NMR

STD-NMR spectroscopy has become established as a powerful method for the determination of binding events of ligands to target proteins.$^{[187-191]}$ The absolute advantage of STD-NMR over other methods such as, transferred nuclear overhauser enhancement spectroscopy (trNOE), structure activity relationship by NMR (SAR) and competitive binding spectroscopy, relies on the observation of such events in their natural environment. Indeed, STD-NMR does not require any labeling or immobilization of the ligands or the target protein. The technique, necessitating only a small amount of protein (ca 100 μM), is based on the selective saturation of part of the receptor’s protons
which, by spin diffusion, propagates across the entire receptor and any bound molecules (Figure 29). Thus, difference spectra between an “on resonance” spectrum in which the protein is selectively saturated and an “off resonance” spectrum without saturation of the receptor allows the direct observation of the bound ligands. In addition to the ligand identification, STD-NMR offers relevant information on the binding mode to the protein. An epitope mapping of the ligands, correlating the proximity of the ligand substituents with the surface of the target protein, is often possible.

**Figure 29.** $^1$H STD-NMR principle: a) for small molecules or library. Off resonance: the protein is not saturated and the bound ligands are not affected. On resonance pulse: the protein is selectively saturated and the saturation is transferred to the bound ligands; b) for big molecules. Off resonance: the protein is not saturated and the bound large molecule is not affected. On resonance: the protein is selectively saturated and the saturation is transferred to the closest protons of the bound ligand.

### 4.5.2 $^1$H STD-NMR resolution of the dynamic hemithioacetal system

STD-NMR, despite a high potency to resolve binding events to a receptor within a pool of ligands, has never been applied to the direct resolution of a dynamic system. Therefore, we decided to investigate this new approach for the direct identification of the best β-galactosidase ligands from the dynamic HTA system. The on resonance frequency was strategically chosen at -2 ppm
where there were no system component signals and the off resonance frequency was settled at 100 ppm, a spectral region free of any signal. During the binding experiments, the β-galactosidase concentration was set at 20 μM in a 100:1 ligand/receptor ratio.

The results on the full dynamic HTA system were highly conspicuous despite the virtual character of the transient component associations (Figure 30). Thus, the complex ¹H-NMR spectra (off resonance) became much better resolved in the STD spectra and the enzyme specificity for thiogalactose (1*), the only thiol fragment significantly identified in the STD spectra, was undoubtedly demonstrated during the experiments (Figure 30c). Binding analysis of the aldehyde fragments suggested a stronger effect for the aromatic-containing components (A*) than for the branched alkyl chain aldehyde derivatives (D*).

**Figure 30.** ¹H STD-NMR binding studies: a) full system spectrum; enlarged areas of b) the aromatic region and c) the aliphaic region. * Signals from the corresponding constituents.
A preliminary conclusion of the full system study would hypothesize that the component associations of systems [1-A] and, to a lower extent, [1-D] provided the best β-galactosidase inhibitors. The virtual character of the HTA products hindered the determination of the exact constitution of the best ligands and, therefore, more investigations needed to be performed in order to identify the effective binders.

4.5.3 **Determination of the binding nature of the aldehyde derivatives**

The nature of binding events from a ligand to a receptor can be divided into two categories: specific binding and non-specific binding. In the case of specific binding, the ligand is accommodated in the active site of the target protein whereas a non-specific ligand binds, for example, to another site of the receptor. In the latter case, the enzyme activity would not be affected. Thus, subsequent investigations on the binding nature of the best molecular fragments were performed. Naturally, component 1 maintains a specific binding mode to the enzyme, and was consequently observed in the difference spectra of its single component analysis. It was somehow more unexpected to observe binding evidence for aldehydes A and D. However, with hydrophobic regions present in the structure of β-galactosidase, non-specific binding of the single aldehydes can be hypothesized. Therefore, competitive inhibition studies were performed for aldehydes A (Figure 31) and D in the presence of different concentrations of IPTG, a known competitive inhibitor. In both aldehyde cases, the presence of a large excess of IPTG did not produce any decrease in aldehyde binding. Considering the fact that IPTG is in complex with β-galactosidase in the active site, these results undoubtedly demonstrated the non-specificity of the binding of single aldehydes A and D respectively.

**Figure 31.** STD spectra of the $^1$H STD-NMR competition experiments between A (2 mM) and IPTG with β-galactosidase (20 μM): a) with 350 eq. of IPTG, b) with 100 eq. of IPTG, c) with 50 eq. of IPTG and d) without IPTG.
4.5.4 Binding evidences for the determination of the best ligands

The idea behind the following experiments was to provide evidence on the nature of the ligand constitution during the binding events to β-galactosidase. Indeed, the full dynamic system $^1$H STD-NMR experiment displayed information on the different fragment molecules ($1^*$, $A^*$ and $D^*$) involved in the binding to the enzyme. It was also of importance to determine the exact composition of these ligand mixtures: single constituents or their corresponding component associations. Therefore, $^1$H STD-NMR and 1D $^1$H-NMR experiments involving constituents 1, A and D as well as their transient component associations (1-A and 1-D) in the presence or absence of enzyme, were performed and analyzed.

1D $^1$H-NMR spectra comparison

The analysis of the different galactose-containing systems displayed a change in the chemical shift displacements as well as a significant signal broadening upon binding to β-galactosidase (Figure 32). In the presence of enzyme, the signals from the carbohydrate protons shifted downfield compared to the chemical shifts recorded for the same system in the absence of the target protein. Importantly, all the protons from the galactose fragment were significantly and differently affected by the magnetic field in the single constituent (1) and component associations (1-A and 1-D) experiments (Figure 32a, c and e). This observation indicated that the binding of the galactose fragment to the enzyme active site in the dynamic HTA system was not originated by the single constituent (1) but rather by the component associations 1-A and 1-D. Therefore, 1D $^1$H-NMR spectra analysis of the galactose-containing systems confirmed the original idea that the best binders to β-galactosidase from the dynamic system would be the transient HTA products. In addition, the chemical shift displacements are consistent with a deep binding mode of the component associations and the active site. Indeed, the hydroxyl groups at positions 2, 3 and 6 of the carbohydrate ring of the galactose fragment have been reported to establish important hydrogen bonding for β-galactosidase catalytic activity.\cite{170, 192} A stronger chemical shift displacement could probably be associated with a stronger binding to the enzyme. Therefore, since system [1-A] signals are the most affected by the introduction of enzyme, it could be deduced that the component association adduct or HTA 1-A presents the greatest interaction within the dynamic system.
Figure 32. Chemical shift displacements and signal broadening upon binding (1D $^1\text{H}$-NMR spectra): a) $1\text{-A} + \beta$-galactosidase, b) $1\text{-A}$, c) $1\text{-D} + \beta$-galactosidase, d) $1\text{-D}$, e) $1 + \beta$-galactosidase and f) $1$. * From corresponding constituents.

$^1\text{H}$ STD-NMR spectra comparison

Other evidence on the binding events of component associations ($1\text{-A}$ and $1\text{-D}$) to the enzyme was obtained by analysis of the different STD spectra. Indeed, the epitope mapping of the galactose fragment in the different experiments provided information on the binding modes of the system components and the active site. More importantly, the comparison of the epitope ratio between the single constituent experiment and the different component association experiments indicated a different 3D orientation of the ligands at the $\beta$-galactosidase active site (Figure 33). Thus, the binding of component
associations 1-A and 1-D in the dynamic HTA system, rather than the binding of the thiogalactose component 1, was demonstrated.

Figure 33. Epitope mapping and \(^1\)H STD NMR spectra of: a) constituent 1 and b) component association 1-A. * From corresponding constituent.

4.6 Confirmation of the structure of the best inhibitors

The results from the binding studies were in total agreement with the competitive inhibition studies. Therefore, the association of constituent 1 with aldehydes A and D, respectively, provided the best \(\beta\)-galactosidase inhibitors. However, one important question remained to be clarified: are the dynamic enzyme inhibitors HTA products, or adducts of the constituents? The virtual character of HTA formation can be confusing when the different results have to be analyzed. However, a simple control experiment could answer this question and was therefore subsequently performed. In order to demonstrate the nature of the best component associations, two additional inhibition studies involving galactopyranose (52) and reduced aldehyde A (constituent G) were carried out (Figure 34). In a first analysis, constituent 52 and G were tested individually against ONPG hydrolysis. The results confirmed a lack of inhibitory effect of these constituents. A second experiment, more informative on the constitution of the best dynamic inhibitors, consisted in the evaluation of two systems composed of constituents 1 and G and compounds 52 and A, respectively. The observation of a significant \(\beta\)-galactosidase inhibitory effect in one or both of these systems, both incapable of forming HTA products, would reject the hypothesis that HTA products are responsible for the enzyme inhibition.
However, no inhibition was recorded during these experiments, therefore supporting the action of HTA products 1-A and 1-D as the true β-galactosidase inhibitors.

**Figure 34.** Control inhibition experiments with non-HTA systems. a Inhibition = \( \frac{t_{50}(\text{Inh})}{t_{50}(\text{blank})} \), for which \( t_{50} \) = time for 50% ONPG hydrolysis; b The inhibition of constituent 1 alone is 2 times.

**Conclusion**

In conclusion, we have implemented HTA chemistry in DCC for the generation of dynamic systems. HTA products were also demonstrated to form spontaneously in aqueous conditions. Despite the virtuality of the component associations, inhibition and binding studies successively allowed the determination of the best inhibitors and ligands to β-galactosidase. HTA 1-A and 1-D exhibited the greatest potency against the substrate hydrolysis. Importantly, we first demonstrated the use of STD-NMR for the resolution of a virtual dynamic system. This technique proved rapid and successfully allowed the direct determination of the best receptor-binding ligands in a dynamic system.
5
Dynamic hemithioacetal system-based drug design of β-galactosidase inhibitors
(Paper VI)

5.1 Introduction

Since the first DCL examples on ligand templating by a biological receptor in the mid-1990s, DCC has been demonstrated as a promising alternative to conventional combinatorial methods in the drug discovery area. Thus, numerous dynamic systems have proven highly efficient for the identification of potent protein ligands and enzyme inhibitors as well as for the preparation of dynamic receptors targeting the binding of natural ligands.\[31-33, 36-38\] To date, however, the DCC approach still remains at the proof-of-principle stage in the drug discovery process and therefore, drug candidates have only rarely originated and been optimized from a dynamic system-based strategy.\[197-200\] When looking at the different steps leading to a drug candidate, one can easily observe the long way to go before the systematic use of the DCC approach in drug discovery (Figure 35). In the following study, we challenged the lead optimization of the best β-galactosidase inhibitors identified from a dynamic hemithioacetal system (see Chapter 4). Therefore, by demonstrating that the synthesis of non-dynamic (or “static”) inhibitors, exclusively designed from the best component associations of a dynamic system, yields greatly improved inhibitory potencies, the DCC approach could be promoted a step further towards the discovery of drug candidates. This new step can be defined as a dynamic drug discovery (DDD) strategy for the optimization of potent leads.

![Figure 35. Dynamic combinatorial chemistry (DCC) and dynamic drug discovery (DDD) approaches in drug discovery.](image-url)
5.2 The fragment-based strategy in drug discovery

5.2.1 Introduction to the fragment-based approach

Fragment-based drug discovery (FBDD) relies on the screening, identification and chemical modification of small molecules possessing a weak affinity for a target protein.\cite{201-207} Subsequent expansion, merging and/or linkage of the most promising molecular fragments constitute the final leads (Figure 36a). The conceptual idea of a fragment-based approach for the discovery of potent ligands surfaced in the early-1980s.\cite{208} However FBDD has only emerged over the last 15 years benefiting from the considerable advances made in some analytical techniques for molecular recognition (e.g. NMR, X-Ray, SPR).\cite{205, 209} The pressure from the pharmaceutical industry to increase the efficiency of the whole drug discovery process has also contributed to its recent popularity. FBDD, by an \textit{in situ} screening and identification of the best molecular fragments in a step prior to the lead synthesis, requires a significantly smaller synthetic effort than conventional combinatorial and high-throughput screening methods. In addition, the use of small molecular entities allows a more effective screening of the “druggable” molecular and chemical spaces.\cite{205, 210, 211} However, the efficient linkage of the molecular fragments remains the major shortcoming of FBDD and alternatives are needed in order to continue the rapid development of this method.

![Figure 36. Concepts of a) fragment-based drug discovery (linkage of the best molecular fragments) and b) dynamic combinatorial chemistry (target-driven dynamic lead formation) in drug discovery processes.](image)

5.2.2 Dynamic combinatorial chemistry: a fragment-based approach

DCC, by exploring molecular recognition through the reversible association of small molecules, can be considered as a fragment-based strategy. Therefore, the labile character of the component associations in a dynamic system provides a good option considering the issues of the fragment linkage encountered in FBDD. However, these two approaches rely on different strategies, notably for the hit/lead identification, demonstrating a higher potential for the DCC approach. Indeed, in contrast to FBDD which uses
information from the binding events of molecular fragments to synthesize a lead, DCC allows the target-driven selection of the best component associations and the dynamic-lead formation in a single process (Figure 36). Thus, the possible over-expression of the best ligands from a dynamic system in the presence of a target protein can greatly simplify the hit identification. The recent reports on the implementation of the DCC strategy in a FBDD process have demonstrated high efficiency for the rapid mapping of potent molecular fragments through site recognition and therefore, new perspectives for FBDD can be envisaged.[206, 212-214]

5.3 Design and synthesis of β-galactosidase inhibitors

5.3.1 The design strategy

The synthesis of “static” molecular mimics of “dynamic” enzyme inhibitors requires some careful design. First and foremost, the static ligands must be destitute of any reversible property and should structurally and geometrically resemble the dynamic analogs. Further, the introduction of a non-reversible scaffold connecting the different molecular fragments should not decrease the binding affinity of the lead to the target protein. In order to maintain the molecular recognition, the non-dynamic connecting fragments should incorporate similar functional groups and/or group of atoms. Based on these criteria, the design and synthesis of two series of β-galactosidase inhibitors, synthetic equivalents of the best HTA component associations from a dynamic system (see Chapter 2), were addressed (Figure 37).

![Dynamic drug design of hemithioacetal-like β-galactosidase inhibitors.](image)
The design of HTA mimics deprived of labile linkages relied on the conservation of either the sulfur- or the hydroxyl group in the final core structure. Indeed, the reversible formation/dissociation of the HTA scaffold is well known to originate from the vicinity of these two groups of atoms. Therefore, thioglycoside and C-glycoside structures provided a good static alternative to the HTA derivatives (Figure 38). The glycosides constituting both inhibitor series were based on component associations 1-A and 1-D and, in addition to the galactoside derivatives (expected inhibitors), the corresponding glucose analogs were also investigated for the inhibition of β-galactosidase (see Chapter 4.3.1 for the dynamic inhibitors design). Thus, the specificity of the enzyme would be demonstrated during the competitive inhibition studies.

**Figure 38.** Design of static β-galactosidase inhibitors mimic of the HTA component associations 1-A and 1-D: a) thioglycoside series and b) C-glycoside series.

Similar to a FBDD approach, small modifications on the structure of the original fragment A and the hydroxyl-group of the HTA scaffold were performed. These structural changes, respectively intended to demonstrate the importance of the aglycon aromatic ring constitution, and the influence of the ligand nature and geometry. Indeed, keto-C-glycosides (compounds 57 and 62) have a more flat structure and possess a weaker hydrogen bond donor character than their corresponding diastereoisomeric alcohols (compounds 56 and 61). The set of designed inhibitors should provide a good range of β-galactosidase inhibitors (see Chapter 4.2.2). Furthermore, compound 55 has already been reported to be a potent β-galactosidase inhibitor, and could serve as a reference compound for the validation of our results.\[^{[179]}\]
5.3.2 Synthesis of the thioglycoside series

Thioglycosides are increasingly popular in glycochemistry and therefore, several efficient methods have been developed for their synthesis. Two strategies, corresponding to the reaction between a glycosyl donor and a halogen derivative as well as to the nucleophilic attack of a thiol derivative on a glycosyl acceptor, have subsequently been adopted (Scheme 15).

Thus, inhibitors 53 and 58 were conveniently obtained by reaction of thioglycosides 1 or 18 with the commercially available bromo-pyridyl derivative, in the presence of base. The synthesis of the rest of the thioglycoside series (compounds 54, 55, 59 and 60) was efficiently performed using the so-called “BF\(_3\)·O(Et)\(_2\)” method in chloroform. The nucleophilic attack of a thiol derivative on a Lewis-Acid activated glycoside provided the acetylated thioglycoside in almost quantitative yields. Subsequent deacetylation (Zemplén conditions) afforded the desired thioglycosides.

Scheme 15. Synthetic routes to the thioglycoside series, (a) KOH, MeOH, r.t., 6h, 69-77%; (b) BF\(_3\)·O(Et)\(_2\), CHCl\(_3\), r.t., 15 min, 90-98%; (c) NaOMe, MeOH, r.t., 3h, quant.

5.3.3 Synthesis of the β-C-Glycoside series

C-Glycosides have demonstrated high resistance to hydrolytic enzymes and other chemical reactions. Thus, during the last 20 years, glycochemists have shown an increasing interest for their preparation. However, only a handful of methods have proven general for the synthesis of various C-glycoside structures. In addition, the route to β-C-glycoside anomers is often only possible when 1,2-cis-glycosides are targeted. However, a synthetic strategy leading to β-C-glycosides, independent of the initial carbohydrate configuration, has been recently reported and was therefore used for the access of compounds 56, 57, 61 and 62 (Scheme 16). The reaction between a free glycopyranose (52 and 63) and a β-diketone derivative (64) yielded the desired β-C-glycosides 57 and 62 under basic conditions and reflux temperature, in a one-pot process. Subsequent reduction of the keto derivatives 57 and 62 by a hydride source yielded C-glycosides 56 and 61 in good yields. The separation of the resulting diastereoisomeric mixtures (3:1 and 1:1
respectively) proved impossible. However, the addition of a catalytic amount of acetic acid during the reduction of the ketone 57, yielded the opposite diastereoisomeric composition (1:2). The evaluation of the stereochemistry during the inhibition experiments could therefore be addressed.

**Scheme 16.** Synthetic route and mechanism of the β-C-glycoside series: (a) NaHCO₃, EtOH/H₂O (4:1), reflux, overnight, 38-64%; (b) NaBH₄, MeOH, r.t., 1h, 78-86%.

5.4 Evaluation of the inhibitory potency of the static library components to β-galactosidase

5.4.1 ¹H-NMR competitive inhibition studies

Investigations on the ONPG hydrolysis by β-galactosidase in the presence or absence of inhibitor were performed following the same method as the one described in Chapter 4.4.1. Therefore, the inhibitions recorded for the dynamic HTA system and the set of static ligands would be possible to compare. The substrate/inhibitor ratio was however conveniently set at 1:1 during the whole competitive inhibition studies. Indeed, the use of the same substrate/inhibitor ratio (1:5) as during the dynamic system evaluations would lead to extremely slow substrate hydrolysis.

The results were highly conspicuous and the enzyme specificity for the galactose derivatives was demonstrated (Figure 39). Traces or very weak inhibition were recorded for the set of glucose-containing derivatives (compounds 58-62). However, more important information about the affinity strength of the ligands to β-galactosidase could be depicted by analyzing the galactoside series. First, one can notice the influence of the aglycon fragment on the substrate hydrolysis rate. In the thiogalactoside series, aromatic-containing ligands 53 and 55 were, as expected from our previous dynamic studies and the enzyme aglycon subsite structure, the best inhibitors from the
designed pool. In contrast, the branched alkyl chain derivative 54 exhibited the weakest effect on the substrate hydrolysis rate. However, the higher affinity for a benzyl group compared to a pyridyl derivative was somewhat unexpected. A single substitution of a carbon atom by nitrogen on the benzyl ring, offering a better hydrogen bond acceptor character, reduced the inhibitory effect of the ligand (32.9 and 12.4 respectively). This difference could however be explained by the presence of hydrophobic platforms at the enzyme active site. The analysis of the C-galactoside series also provided precious information on the binding affinity of the ligand to β-galactosidase. Thus, the combination of a glycosidic carbon atom and a keto- or hydroxyl- benzylic aglycon fragment dramatically affected the inhibitory effect. Indeed, the keto derivative 57 demonstrated only a trace effect on the substrate hydrolysis rate (inhibitory value of 1.5). However, the corresponding diastereoisomeric alcohol 56 exhibited a better affinity with the enzyme and, interestingly, β-galactosidase showed higher affinity for one of the diastereoisomers (Figure 39, compounds 56a and 56b).

<table>
<thead>
<tr>
<th>Thigalactoside series</th>
<th>Thiglucoside series</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="53" alt="Chemical Structure" /></td>
<td><img src="58" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><img src="54" alt="Chemical Structure" /></td>
<td><img src="59" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><img src="55" alt="Chemical Structure" /></td>
<td><img src="60" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C-Galactoside series</th>
<th>C-Glucoside series</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="56" alt="Chemical Structure" /></td>
<td><img src="61" alt="Chemical Structure" /></td>
</tr>
<tr>
<td><img src="57" alt="Chemical Structure" /></td>
<td><img src="62" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>

Figure 39. Evaluation of the inhibitory effect of the static ligands. Diastereoisomeric ratio of \( \text{a} \): 3:1 and \( \text{b} \): 1:2 (Inhibition = \( t_{50(\text{inhib})}/t_{50(\text{blank})} \), for which \( t_{50} = \) time for 50% ONPG hydrolysis).
5.4.2 UV-Vis competitive inhibition studies

The presence of potent β-galactosidase inhibitors in the set of static molecules led us to envisage the determination of their IC\textsubscript{50} values (half maximal inhibitory concentrations) and K\textsubscript{i} values (inhibition constants). In order to obtain these values, subsequent UV-Vis studies were performed with the galactoside series of inhibitors. A typical experiment consisted of recording the kinetics of the ONP formation ($\lambda_{\text{max}} = 420$ nm) for different concentrations of inhibitors over a period of 15 minutes. Then, the determination of the rate of ONPG hydrolysis for each inhibitor concentration allowed the construction of a dose-response plot in which the IC\textsubscript{50} values for the different inhibitors could be easily estimated (Figure 40a and b). The Michaelis constant (K\textsubscript{M}), defined as the concentration of substrate providing half maximal activity of the enzyme, is an essential constant for the determination of K\textsubscript{i}. Therefore, the kinetics of the ONP formation for different concentrations of substrate (in absence of any inhibitor) was also performed. Subsequent Michaelis-Menten analysis in which the rate of ONPG hydrolysis is expressed as a function of the substrate concentration provided an estimation of the K\textsubscript{M} value (14.6 μM).\[229\] Then, the K\textsubscript{i} values for the different galactoside inhibitors were calculated using the Cheng-Prusoff equation (Figure 40c).\[230\]

![Figure 40. UV-Vis competitive inhibition studies of the static galactosides: a) IC\textsubscript{50} determination (normalized rate of ONPG hydrolysis); b) IC\textsubscript{50} and K\textsubscript{i} values for the set of inhibitors, K\textsubscript{M} = 14.6 μM; c) Cheng-Prusoff equation for the conversion of IC\textsubscript{50} into K\textsubscript{i} values. Diastereoisomeric ratio of $^a$ 3:1 and $^b$ 1:2. Conditions: 25 °C, pH = 7.4.](image)

<table>
<thead>
<tr>
<th>Galactoside</th>
<th>IC\textsubscript{50} (μM)</th>
<th>K\textsubscript{i} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>70.9</td>
<td>17.6</td>
</tr>
<tr>
<td>54</td>
<td>315.0</td>
<td>78.2</td>
</tr>
<tr>
<td>56</td>
<td>18.4</td>
<td>4.6</td>
</tr>
<tr>
<td>56\textsubscript{a}</td>
<td>253.4</td>
<td>62.9</td>
</tr>
<tr>
<td>56\textsubscript{b}</td>
<td>176.0</td>
<td>43.7</td>
</tr>
<tr>
<td>57</td>
<td>864.8</td>
<td>214.6</td>
</tr>
</tbody>
</table>

The trend of the IC\textsubscript{50} and K\textsubscript{i} values for the different static inhibitors was fully consistent with the results from the \textsuperscript{1}H-NMR competitive inhibition studies. In addition, the previous report of 1/K\textsubscript{i} for the reference compound 55 enabled the validation of our results.\[179\] The benzyl thiogalactoside derivative 55 had previously been estimated with a K\textsubscript{i} value of 4.3 μM (at 30 °C, pH 7.5) and...
therefore, the determination of an inhibition constant of 4.6 μM for compound 55 (at 25 °C, pH 7.4) supported the results of the inhibition studies.

5.5 Important fragments for β-galactosidase inhibition

The inhibition studies clearly demonstrated the importance of specific fragments for the inhibition of β-galactosidase. In contrast, some atoms or group of atoms have demonstrated a very weak or no affinity with the enzyme. Thus, a map depicting the different fragments investigated and their relative importance during the inhibition process can be established and could become a useful tool for future inhibitor design (Figure 41). At the glycosidic position, a sulfur atom undoubtedly provides the best inhibitors from the set of ligands. However, it remains difficult to fairly compare the thiogalactoside series with the C-glycoside series as they have not been studied bearing the same aglycon fragment. A tentative prediction would still favor the sulfur analogs. The reason would involve the interactions (hydrogen bonding) between the glycosidic atom and some enzyme residues (notably Glu461) during the catalytic mechanism. Therefore, a sulfur atom, possessing a more pronounced hydrogen bond acceptor than a carbon atom would probably have a greater affinity with the enzyme.

Figure 41. * Schematic representation of the importance of the substituent for the inhibition of β-galactosidase. Substituent providing (+) a very good inhibition, (≈) good inhibition and (-) a poor inhibition. * Figure made with PyMOL[160] software (reference 1JYN[171] from the Protein Data Bank[162]).

The influence of the aglycon counterpart evidenced a large preference for aromatic fragments. The reason for this tendency is likely the presence of hydrophobic platforms at the aglycon subsite and has already been detailed previously. However, the observation that a pyridyl derivative significantly
decreases the inhibition allowed a trend to be delineated. Thus, a phenyl fragment seems to be the most effective for the enzyme inhibition. Finally, the presence of a keto- and, to lower extent, a hydroxyl group at the benzylic position dramatically decreases the binding affinity of the ligand to the enzyme. At this position, a tetrahedral bi-substituted carbon appears to be the most appropriate structure for optimal inhibition.

Conclusion

In conclusion, the transformation of the best component associations of a dynamic HTA system into their structurally and geometrically synthetic equivalents has been achieved in a dynamic drug-design strategy. The evaluation of a set of static ligands was performed in the presence of β-galactosidase and the compounds in the static series showed a significant increase of the inhibitory potency in comparison to their dynamic homologues. Thus, these results successfully demonstrated the high potency and efficiency of the dynamic drug discovery approach for the fragment-hit identification, design and optimization as well as the synthesis of potent leads.
6

Summary of results and concluding remarks

The development of new protocols for the generation and biological evaluation of dynamic systems by means of sulfur-containing reversible reactions constitutes the major achievements reported in this thesis.

Screening

Disulfide metathesis and HTA formation have been successfully explored in dynamic system generation, in organic and/or aqueous media and under mild conditions. Thus, in a proof-of-principle study, a system composed of 15 disulfides has been efficiently generated using the disulfide metathesis process in organic solvent. Further optimization and expansion of the reaction in aqueous media allowed the preparation of dynamic carbohydrate systems (up to 10 disulfides). In addition, HTA formation has been demonstrated as a convenient and rapid approach for the generation of dynamic systems in neutral aqueous systems, despite a truly virtual and transient existence under these conditions.

Evaluation

Efficient synthesis and evaluation of the intrinsic properties of thioglycoside derivatives – starting components of the different dynamic systems – have been performed. Thus, the pH-profile of the mutarotation process of 1-thioaldoses was established in aqueous media. A strong β-anomer preference was found for all the structures under acidic or neutral conditions. However, this trend was reversed under basic conditions exclusively for derivatives bearing an axial hydroxyl group at the C-2 position of the carbohydrate ring.

Biological evaluations for the different dynamic systems have been performed in the presence of a lectin protein (Concanavalin A, for disulfide system) or a glycosidase enzyme (β-Galactosidase, for HTA system). Thus, 1H STD-NMR has been implemented for the direct, rapid and in situ identification of the best binders from a dynamic system of 6 glycosyl disulfides or 19 discrete components (10 HTA, 5 thiols, 2 aldehydes and 2 hydrate derivatives). In the case of the dynamic HTA system evaluation, inhibition studies have confirmed the binding studies results. Thus, the component associations resulting from the reaction between the thiogalactose analog and an aromatic aldehyde provided the best enzyme inhibitors. Finally, a dynamic drug design approach has been successfully adopted for the synthesis of potent lead mimicking the best dynamic HTA β-galactosidase inhibitors.
Catalysis

Phosphine derivatives such as PCy$_3$ and HEPA have demonstrated a great ability to catalyze the disulfide metathesis reaction under mild conditions and, therefore, provide a very efficient alternative to traditional thiol-disulfide exchange protocols. In polar organic solvents, the reaction rate proved very fast and low catalytic loading of catalyst was sufficient. However, a concomitant desulfurization process could be observed when hindered disulfides and/or HEPA were used. In aqueous media, HEPA was demonstrated as the most adequate phosphine derivative to perform the disulfide metathesis in a reversible manner. This phosphine-mediated disulfide scrambling proved very stable.
I would like to express my deepest gratitude to all people who have helped, supported and directed me in different ways during these years. Naturally, there are some persons that I wish to thank more explicitly.

First and foremost, I would like to thank my supervisor, Professor Olof Ramström, for giving me the opportunity to join his group, for his support, guidance and advices during the last four years. Olof, it has been truly fantastic in many aspects to work under your supervision.

Professor Torbjörn Norin for constructive criticisms and valuable comments on this thesis.

Dr. Eugene Mahon and all the members of the Ramström Research Group for proof-reading and comments on this thesis.

My co-authors, Professor Tore Brinck, Professor Hai Dong, Professor Jesús Jiménez-Barbero, Professor Zhichao Pei, Dr. Luis Amorim, Dr. Pornrapee Vongvilai, Lingquan Deng, Martin Rahm, João Ribeiro and Morakot Sakulsombat for fruitful collaborations and pleasant team-work.

The European Union (Marie-Curie training program), Kungliga Tekniska Högskolan and the Swedish Research Council for financial supports.

The Aulin-Erdtman foundation and the Lars Gunnar Silléns minnesfond for travel grants.

My colleagues and friends in the Ramström Research Group. Marcus, Morakot, Oscar, Vince, Yan, Germain, Chelsea, Yen and Katharina, it has been a great pleasure and a lot of fun to have you as co-workers. I sincerely enjoyed my time in the group and it is of course mainly due to all of you.

All the past members of the group; especially Rikard, Jom, Zhichao and Hai for their friendship and helpful assistance when I just started in the group.

All the members of the DYNAMIC network. It has been an outstanding experience for me, on both professional and personal standpoints. João, Eugene, Maxime, Gunnar, Rikard, Oscar and the other “crazy guys”, from Namur to Stockholm, I only have great memories from our successful team building activities.

I would like to express my sincere gratitude to Professor Mihail Barboiu and Professor Jesús Jiménez-Barbero for hosting me in their respective laboratories and making me feel as a member of their group during those short periods. Eugene and João, it has been a real pleasure, some hard work and a lot of fun to collaborate with you.
Dr. Ulla Jacobsson and Dr. Zoltan Szabo for supporting with the NMR issues.
Henry Challis, Lena Skowron and Ilona Mozsi for all kind of help.
All the past and present coworkers at the organic chemistry department.

I would like to warmly thank my closest relatives for always being there when I need it and for their invaluable support.

Maman et papa, merci de m’avoir toujours soutenu et laissé libre de mes décisions. C’est avec un immense plaisir que je vous dédie cette thèse; sans vous je n’en serai pas arrivé là aujourd’hui.

Alesia, thank you for your patience, understanding and everlasting love.
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 performed part of the experimental work.

Paper II: I contributed to the formulation of the research problems, performed the majority of the experimental work and wrote the manuscript, excluding the DFT calculations part.

Paper III: I contributed to the formulation of the research problems, performed the majority of the experimental work and wrote the manuscript, excluding the quantum chemical studies part.

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

Paper V: I contributed to the formulation of the research problems, performed the majority of the experimental work and wrote the manuscript, excluding the $^1$H STD-NMR part.

Paper VI: I contributed to the formulation of the research problems, performed the majority of the experimental work and wrote the article.
References


[162] The Protein Data Bank. (http://www.rcsb.org/).

