Chapter 6

Summary and future perspectives
6.1 Summary

Techniques used in the field of chemical biology allow the understanding of biological processes at the molecular level. Among these techniques, one is fundamentally based on chemistry and examines biology by means of chemical probes. Many chemical probes are inspired by nature and secondary metabolites, since such compounds are forged to interact with and bind to proteins. Brabantamides, a set of compounds isolated from *Pseudomonas fluorescens* triggered our interest. Brabantamide A, deglycosylated brabantamide A and simplified scaffolds synthesized by Pinto *et al.*,1 potently inhibit lipoprotein-associated phospholipase A₂ (Lp-PLA₂), a mammalian group VII phospholipase A₂ that belongs to the serine-hydrolase superfamily. Brabantamide A also affects bacterial, fungal and oomycete growth and increases phospholipase D activity in oomycetes.1-7 These interesting properties and the fact that the scaffold contains both an enol-carbamate and a Michael acceptor prompted us to study these compounds with the aim to understand their interactions with proteins. Convinced that these compounds covalently modify proteins, we designed and synthesized activity-based probes to allow us the visualization and study of their protein targets.

In Chapter 2, the synthesis of a set of enol-cyclocarbamate derivatives that vary in the substitution pattern is described. The effect on the antibacterial activity towards *Bacillus subtilis* and *Streptococcus pneumoniae* was studied. It was discovered that the bicyclic scaffold and the dodecyl alkyl chain are indispensable for antibacterial activity, that these compounds are bacteriolytic and that they block peptidoglycan synthesis. Competitive activity-based protein profiling experiments with Bocillin-FL identified penicillin-binding protein 4 as one of the targets of our derivatives in *Bacillus subtilis*. The increased understanding of the mechanism of action of these antibacterial compounds may be used for the design of more potent scaffolds.

Chapter 3 describes the synthesis of the first set of chemical probes based on the brabantamide scaffold. With these probes, the targets of enol carbamates were characterized in A549 cells. It was demonstrate that the probes **PJD2d** and **PJD224** bind to aldehyde dehydrogenase 1A1 (ALDH1A1 or RALDH1) and 3A1 (ALDH1A3), which belong to the aldehyde dehydrogenase family. Competition experiments with known inhibitors (disulfiram and
iodoacetamide) revealed that the probes modify a hyper-reactive cysteine of the enzymes. Both in the A549 cancer cell line and in HEK cells transfected with RALDH1, RALDH2 and RALDH3, the probes showed selectivity towards RALDH1. The expression of RALDH1 is generally related with worsened prognosis in cancer and plays a role in cancer drug resistance via inactivation of cyclophosphamide and oxaphosphorines and the chemical probes described in this chapter may therefore serve as tools to study these enzymes. These tools may be further exploited to develop drugs or as a diagnostic tool for cancer.

The reactivity of enol cyclocarbamates towards serine hydrolases is studied in Chapter 4. Competitive activity-based protein profiling on recombinant mammalian Lp-PLA₂, recombinant esterase from Bacillus subtilis and on lysates from Bacillus subtilis and mammalian A549 cells revealed that the chemical probes PJD120 and PJD224 label recombinant Lp-PLA₂ and esterase from Bacillus subtilis. This study characterizes the first enol carbamate-based probes that target recombinant mammalian Lp-PLA₂. PJD224 proved to be a suitable tool to study the inhibitory potency of carbamate inhibitors on Lp-PLA₂ in a competitive activity-based protein profiling assay. The results obtained showed that a hydrophobic linear alkyl chain is necessary to potently inhibit this protein. The broad-spectrum activity-based serine hydrolases probes Fp-rhodamine and Fp-biotin were then used to visualize and identify the targets of the compounds described in Chapter 2 on lysates from Bacillus subtilis and mammalian A549 cell line.

Chapter 5 describes an NMR study towards the reactivity of enol carbamates. This study showed the versatility of this intriguing scaffold containing two putative electrophilic traps, a carbamate and a Michael acceptor. The NMR experiments with the model substrate confirmed that thiols, amines and alcohols react differently with this scaffold leading to different adducts. Thiols react with the Michael acceptor. Initial 1,4 addition of thiophenol followed by retro Michael reaction with CO₂ extrusion led to a decarboxylated thiophenol adduct. Besides addition, isomerization of the original double bond was observed, presumably via thiophenol expulsion. Alcohols and amines react with the carbamate. Treating the carbamate with benzylamine and methanol gives the benzyl urea and the methyl carbamate adduct respectively. The benzylamine adduct can react further to the bicyclic ring closed urea adduct. The outcome of the study allowed us to understand the reactivity of the enol carbamate scaffold. This information
was used to design new scaffolds with tuned reactivity such as the vinyl sulfone based probes VDG30 and VDG33, the ester probes PJD243 and PJD252 and the monocyclic derivatives PJD251 and PJD253. VDG30 and VDG33 showed impressive selectivity towards RALDH1. Interestingly ester-based probe PJD253 did not label RALDH1. It rather selectively targeted two other proteins, presumably serine hydrolases, and identification of these targets may open new applications for this probe.

In conclusion, the research in this thesis shows that enol carbamates react with hyper-reactive cysteine and serine residues within proteins. By equipping the scaffold with a reporter group, chemical probes can be obtained that label enzymes containing these residues. The reactivity of the scaffold can be tuned to increase the potency and to generate selectivity for proteins of interest.

6.2 Future perspectives

The work reported in this thesis has merely focused at gaining insight in the reactivity of the enol carbamate scaffold, determining the bacterial targets responsible for antibiotic activity and establishing the suitability of this electrophilic scaffold in the development of activity-based probes. The prepared panel of inhibitors and probes based on the Brabantamide scaffold and profiling studies increased the understanding of the properties of the scaffold and as such the research in this thesis forms a solid foundation for the further development of novel inhibitors for serine hydrolases, aldehyde dehydrogenases and antibacterial agents based on this scaffold.

However, a variety of questions and challenges remain to be addressed before the potential of the enol carbamate scaffold can be fully exploited. First of all, despite the fact that several serine hydrolases and aldehyde dehydrogenases were identified as targets, the remaining targets of the probes and inhibitors in various cell types have to be determined. Abundant proteins may obscure the detection of other relevant targets. The observation that PJD2d strongly labeled RALDH1 in the A549 cell line and that other enzymes react with enol carbamate probes in HEK cells indicates that the high expression level of RALDH1 in A549 cells may outcompete the labeling of other enzymes such as Lp-PLA₂ by the probe. In HEK cells a 40-45 kDa protein is strongly labeled by the probes PJD2d and PJD224 and several other proteins are labeled less intensely. Identification of these
proteins may provide further insight in the reactivity profile of the enol carbamate family. In order to obtain insightful data about the target engagement of the synthesized compounds and reliably identify their targets, the quantitative mass-spectrometry methods that are nowadays available should be employed both for the enrichment of the probe adducts and in reactive cysteine, serine and lysine profiling experiments. This approach should enable determining the enrichment ratio rather than solely identification.

A limitation specific to unraveling the mechanism of action of this compound class in bacteria is that the available chemical probes do not display antibacterial activity. Therefore ABPs based on the scaffold of active compound 2a (Figure 1A) should be prepared. Incorporating a terminal alkyne or azide into a dodecyl chain of 2a will provide ABPs that likely display antibacterial activity and this will open the possibility to further study the protein targets in a direct manner.

![Figure 1. New scaffolds: containing an azide as click handle inspired by compound 2a (A); a sulfone closely related to VDG30 (B) or a compound bearing biotin that can be prepared from VDG30 (C).](image)

Finally, several probes show interesting labeling profiles in different lysates, but these probes lack an affinity-handle that can be used for the enrichment. For example, we observed in chapter 5 that VDG33 targets selectively a protein in B. subtilis. This protein could not be identified because VDG33 lacks a purification handle and VDG30 does not target this protein. Although competitive protein profiling with broad-spectrum probes may enable the identification of the protein of interest, preparing a derivative
with a long alkyl chain to mimic the hydrophobicity of VDG33 (Figure 1B) or a derivative of the sulfone probe VDG30 containing an affinity handle may allow direct identification (Figure 1C). As a general solution for this problem an azido-BODIPY® could be implemented into the scaffold leading to a fluorescent probe that can be modified using the azide handle (Figure 2A, 2B) for affinity purification purposes.

**Figure 2.** Probes containing an azido-bodipy at two different positions (A, B) or a norbornene and an alkyne (B) to be used to both visualize and identify targeted proteins.

It also has been demonstrated that the tetrazine ligation, the Staudinger-Bertozzi ligation and the copper(I)-catalyzed click reactions can be used simultaneously⁹ so we believe that a scaffold containing a norbornene and an alkyne (Figure 2B) could be used as a platform for both visualization and affinity purification for target identification. Using one handle for visualization (by “pre-click”, prior to incubation with lysates or proteins) and the other for affinity purification in one experiment and using them in a reverse manner in another experiment is likely to give different results based on the importance of the modification on these positions observed in Chapter 2 (compound 4b is inactive whereas compound 2a is active against B. subtilis).

As described for VDG33 in bacterial cell lysates, the targets of ester derivative PJD252 remain to be identified but based on the composite image
of PJD252 and Fp-rhodamine found in Chapter 5 it seems that the two main targets of PJD252 are serine hydrolases that therefore can be identified using Fp-biotin.

The results described in chapter 3, 4 and 5 suggest that both the electrophilic sites within the enol carbamate scaffold display biological activity, and that selectivity may be obtained for one or another residue by tuning the reactivity and the substitution pattern on the scaffold. To further tune the reactivity and the selectivity, a next library should implement combinations of various linear chain length (or bulkiness), Michael acceptor (based on sulfone, ester or amide) and ring(s) (monocyclic or 5,5 fused membered ring). The position of the click handle should also be adequate as it should be accessible for the reaction to occur and can also influence greatly the activity of a compound (Figure 3A and 3B). By adjusting the substitution pattern on the scaffold, selectivity may be generated for different enzymes.

**Figure 3.** Probes bearing a long alkyl chain, an ester and a click handle at different positions (A); monocyclic sulfone probe (B) and probe appending a potent probe using the enol carbamate as a trap to modify a residue in proximity (C).

Furthermore, the enol carbamate scaffold may find use as chemical crosslinker in proximity-based protein labeling reagents. Appending the enol carbamate to a ligand should target the electrophilic trap to a protein of interest, where it will react with a nucleophile in the proximity. Due to the high local concentration, the reagent will likely react with the target protein, while leaving other proteins untouched. An added advantage of the enol carbamate is that it can react with various nucleophiles, which increases the chances on adduct formation with the protein of interest. To test the suitability of this approach, biotin may be functionalized with an alkyn containing enol carbamate trap. The resulting ligand-directed enol
carbamate will be used in protein labeling experiments (Figure 3C).

Figure 4. Probe containing a phosphocholine moiety (A) and reaction catalyzed by Lp-PLA₂ cleaving platelet activating factor (PAF) to form acetate and lyso-PAF (B).

To further investigate the interaction between Lp-PLA₂ and the enol carbamate scaffold, a probe containing a phosphocholine moiety (Figure 4A) could be prepared to better mimic PAF (platelet activating factor), one of the endogenous substrate of Lp-PLA₂ (Figure 4B). PAF is involved in the inflammatory (especially in allergic reactions) and thrombotic cascades and a potent and selective probe targeting Lp-PLA₂ could help understanding better these mechanisms.

In future, I am convinced that enol cyclocarbamate probes can be used to screen and develop covalent inhibitors towards RALDH1 using competitive activity-based protein profiling assays. These probes can also be used to assess RALDH1 expression and might be considered as diagnostic tools for cancer. Indeed, by comparing a healthy tissue and a cancerous tissue at the protein level, the probes developed in this thesis should allow to determine protein expression differences that might have biological relevance. To conclude we believe that the tools and inhibitors developed in this thesis can be used as a platform to study both antibacterial and anticancer mechanisms at the protein level. This statement is empowered by the work presented in chapter 5; probes VDG30 and VDG33 showed higher potency towards RALDH1 and can be used to screen specific inhibitors towards this enzyme.

As a complement, not only will these optimized procedures and compounds
find use in mammalian and bacterial cells, screening the compounds against the plant pathogens *Phytophthora capsici*, *Pythium ultimum*, and *Phytophthora infestans* (which causes the serious potato and tomato disease known as late blight or potato blight at the origin of the Great Irish Famine 1845-1852) will aid to the understanding of the anti-oomycete activity of brabantamide A. This will help to unravel how phospholipase D activity is stimulated by these compounds.\(^{10}\)

Based on the work reported in this thesis, further investigation is needed to allow exploiting the full potential of this intriguing and versatile scaffold.

### 6.3 References