Direct and two-step activity-based profiling of proteases and glycosidases

Willems, L.I.

8

Summary and future prospects

The research described in this thesis involves the development of novel chemical tools and methods for the activity-based profiling of proteases and glycosidases. Activity-based protein profiling (ABPP) is a field of research that aims to obtain information on the activity of a protein or protein family within the context of a biological system. In order to enable the monitoring of enzymatic activity rather than protein expression levels, ABPP strategies make use of active-site directed chemical probes, termed activity-based probes (ABPs), that bind in a mechanism-based and irreversible manner to a specific target enzyme or multiple members of an enzyme family. An essential element of an ABP is a means to visualize, isolate, quantify and/or identify the labeled proteins. Depending on the nature of the detectable agent, ABPP experiments can involve either direct or two-step labeling strategies. Direct ABPs are functionalized with a reporter entity such as a fluorescent tag and/or an affinity tag. Alternatively, in two-step ABPs the reporter group is replaced by a small reactive moiety, referred to as 'ligation handle', that provides the option to introduce a tag after binding of the probe to a target enzyme. The attachment of a reporter group in two-step labeling strategies is realized by making use of bioorthogonal ligation reactions, which proceed with high selectivity and efficiency and are non-perturbing to the biological system at hand. In Chapter 1 the principles of ABPP and ABP design are discussed. Chapter 2 presents an overview of the various bioorthogonal ligation reactions that have been developed to date, with an emphasis on their use in two-step profiling of enzymatic activity.

The development of a two-step ABPP strategy based on the Diels-Alder cycloaddition is described in Chapter 3. Previously reported two-step ABPP methods all rely on the use of an alkyne or azide as a ligation handle for labeling of enzymatic activity via Staudinger-Bertozzi ligation or azide-alkyne cycloaddition ('click' reaction), which may either be catalyzed by copper(I) or driven by ring-strain. In contrast, the Diels-Alder approach makes use of ABPs that are functionalized with a conjugated diene to enable reaction with a tagged maleimide reagent as a dienophile. The Diels-Alder strategy was applied to label the activity of endogenously expressed proteasome β-subunits and cysteine proteases of the cathepsin family in cell extracts. Moreover, it was demonstrated that this ligation reaction can be performed in tandem with the Staudinger-Bertozzi ligation for the labeling of two different enzymatic activities in the same sample. Although the Diels-Alder method forms a useful complement to Staudinger-Bertozzi and click ligation strategies, its broad utility is limited by the need to mask cysteine residues prior to the addition of the dienophile, which precludes *in situ* and *in vivo* applications. In addition, a considerable amount of background labeling was observed that might hamper the detection of proteins with low endogenous activity.

To avoid non-specific reaction of the dienophile with cysteine residues, Chapter 4 details the development of a two-step ABPP strategy that is based on the inverse-electron-demand Diels-Alder reaction. By using tetrazine as a diene and norbornene as a dienophile, the selectivity and speed of the ligation reaction is drastically improved. A proteasome ABP functionalized with norbornene as a ligation handle enabled the efficient and selective labeling of endogenous proteasome activity in cell extracts and in cultured cells by reaction with fluorescently labeled or biotin-tagged tetrazine reagents. Furthermore, a triple ligation strategy was developed that combines the tetrazine ligation, Staudinger-Bertozzi ligation and copper(I)-catalyzed click reaction for the simultaneous labeling of three different enzymatic activities with different tags in the same sample. Despite the fact that tetrazines are not directly compatible with copper-catalyzed click chemistry, a simple washing step between these two ligation reactions allows their consecutive use in the same experiment.

The triple labeling procedure developed in Chapter 4 should not only be useful for ABPP applications but also for the simultaneous monitoring of other biomolecules, for instance modified cell surface glycans or post-translationally modified proteins, in complex biological samples. Furthermore, the fact that tetrazine and Staudinger-Bertozzi ligations can both be performed inside living cells[1,2] and in living animals[3,4] provides the possibility to develop tandem labeling procedures *in situ* and *in vivo*. *In situ* labeling via copper(I)-catalyzed click chemistry can be achieved without affecting cell viability by making use of specific ligands that accelerate the cycloaddition and sequester the copper-induced toxicity.[5,6]

Several reports have already described the metabolic incorporation of two differently tagged unnatural sugars for the simultaneous imaging of the corresponding glycan structures on live cell surfaces by strain-promoted click reaction and tetrazine ligation,[7-9] for example by making use of azide-modified *N*-acetylglucosamine derivative **1**[10] and cyclopropene-functionalized mannosamine **2**[7] (Figure 8.1A). The parallel labeling of additional cell surface glycans could be achieved by using alkyne-functionalized sugars, such as *N*-acetylneuraminic acid derivative **3**.[11] Alternatively, unnatural sugars that are tagged with a 3,3-disubstituted cyclopropene moiety, for instance *N*-acetylgalactosamine derivative **4**, may be used for bioorthogonal reaction with nitrile imines, which can be generated from tetrazoles *in situ* by exposure to UV light. In contrast to 1,3-disubstituted cyclopropenes, the 3,3-disubstituted cyclopropene moiety was demonstrated to show virtually no reactivity towards tetrazines, which enables the concurrent use of both ligation handles in a tandem labeling procedure.[12] Together, the ligation handles in compounds **1**-**4** should enable the parallel labeling of up to four different cell surface glycans or, for example, enzymatic activities (Figure 8.1B). Such a tandem ligation strategy would entail a number of steps starting with the inverse-electron-demand Diels-Alder reaction of the 1,3-disubstituted cyclopropene with a tetrazine and the (simultaneous) elaboration of the azide ligation handle via Staudinger-Bertozzi ligation with a phosphine, or alternatively via strain-

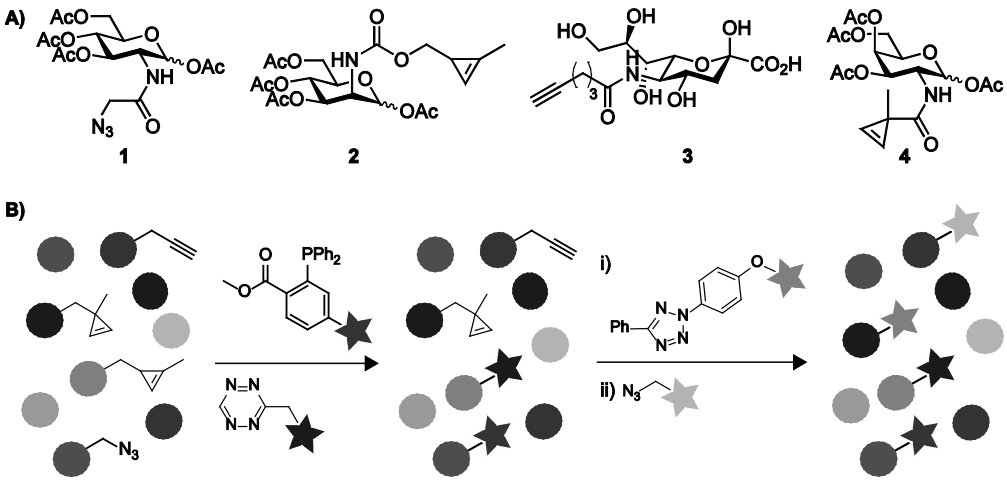


Figure 8.1. A) Peracetylated *N*-azidoacetyl glucosamine (**1**), cyclopropene-functionalized mannosamine derivative (**2**), alkyne-equipped neuraminic acid analogue (**3**) and *N*-acetyl galactosamine derivative with a 3,3-disubstituted cyclopropene (**4**) for bioorthogonal labeling of cell surface glycans. B) Tandem bioorthogonal labeling strategy involving four different ligation reactions.

promoted click reaction with a cyclooctyne. Subsequently, light-induced 1,3-dipolar cycloaddition of the 3,3-disubstituted cyclopropene with a nitrile imine can be performed, followed by copper(I)-catalyzed click reaction of the terminal alkyne with an azide.

The analysis of labeled proteins by mass spectrometry is an important method to identify target proteins and/or active-site peptides and to determine the exact site and mechanism of ABP binding. For this purpose, proteins are usually labeled with a biotin-tagged ABP and subsequently enriched by affinity-purification using streptavidin beads. Since the release of captured proteins from the beads requires harsh conditions and may result in contamination of the purified sample with endogenously biotinylated proteins, these methods benefit greatly from the use of cleavable linker systems. These may be incorporated in a direct ABP or in a bioorthogonal ligation reagent and enable the mild and chemoselective release of target proteins.

With the aim to make the developed tetrazine-based ABPP method suitable for such selective enrichment experiments, a tetrazine reagent was designed in which a hydrazine-cleavable linker separates the biotin tag from the tetrazine moiety (**11**, Figure 8.2).[13] A short polyethylene glycol (PEG) spacer was included to enhance water-solubility of the probe. The synthesis of biotin-cleavable linker-tetrazine **11** commenced with reduction of the azide in PEG spacer **14**[14] to an amine followed by reaction with the acid chloride generated from 6-heptynoic acid to give **15** (Scheme 8.1). After Boc-deprotection and reaction with biotin-OSu, the resulting alkyne-modified compound (**16**) was subjected to copper(I)-catalyzed click reaction with azide-functionalized levulinoyl derivative **18**[13] to give biotinylated cleavable linker **17**. The second building block was synthesized from tetrazine **12** (see Chapter 4), which was extended with an aminohexanoic acid spacer (**13**) to minimize potential steric

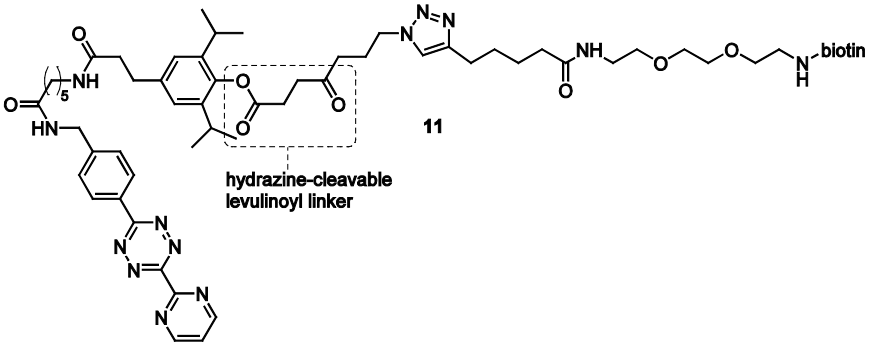
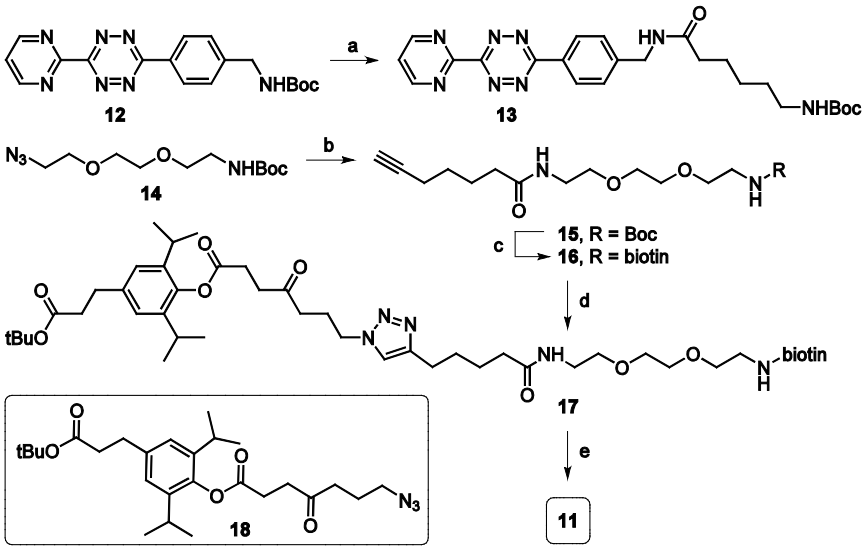


Figure 8.2. Biotinylated hydrazine-cleavable tetrazine reagent **11**.

Scheme 8.1 Synthesis of biotin-cleavable linker-tetrazine **11**



Reagents and conditions: a) *i*) TFA/DCM 1/1 (v/v), rt, 15 min, *ii*) Boc-Ahx-OSu, DiPEA, DCE/DMF, rt, overnight, 88%; b) *i*) PPh_3, THF/H_2O, rt, overnight, *ii*) [6-heptynoic acid preactivated with $(COCl)_2$, DMF, toluene, rt, 3 hrs], DiPEA, DCE, rt, overnight, 54%; c) *i*) TFA/DCM 1/1 (v/v), rt, 20 min, *ii*) biotin-OSu, DiPEA, DCE/DMF, rt, overnight, 87%; d) **18**, $CuSO_4$, sodium ascorbate, *t*BuOH/toluene/H_2O/DMF 1/1/1/1 (v/v/v/v), 80 °C, overnight, 60%; e) *i*) TFA/DCM 1/1 (v/v), rt, 30 min, *ii*) [**13** deprotected with TFA/DCM 1/1 (v/v), rt, 20 min], HCTU, DiPEA, DCM/DMF, rt, overnight, 70%.

hindrance of the linker system during the ligation reaction. Removal of the *t*Bu and Boc protecting groups in **17** and **13**, respectively, enabled HCTU-mediated coupling to afford the biotinylated hydrazine-cleavable tetrazine reagent **11**.

Two-step labeling experiments with norbornene-functionalized proteasome ABP **19** (see Chapter 4) and tetrazine reagent **11** demonstrated the selective labeling of the catalytically active proteasome β-subunits (β1, β2, β5) in cell extracts (Figure 8.3). Subsequent treatment with an excess of hydrazine resulted in disappearance of the specifically labeled protein bands on Western blot, indicating that the biotin tag was separated from the probe and thus that cleavage of the levulinoyl linker had occurred. Furthermore, preliminary enrichment experiments have revealed that the catalytically active proteasome β-subunits labeled by ABP **19** and biotin-cleavable linker-tetrazine **11** could be isolated from cell extracts using streptavidin beads and subsequently selectively released by treatment with hydrazine.

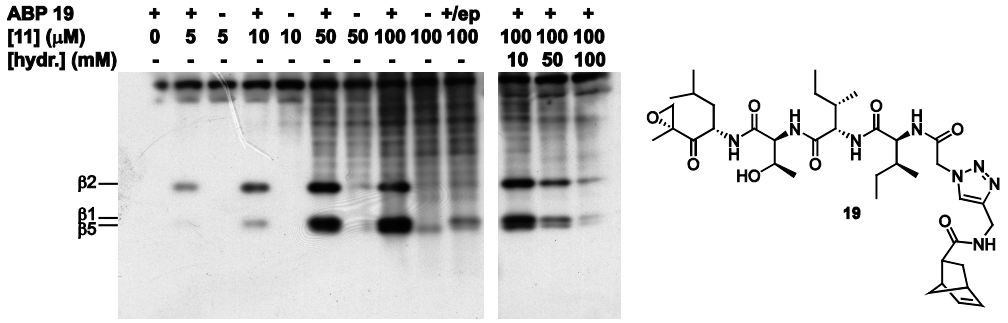


Figure 8.3. Labeling of proteasome activity in HEK cell extracts by 1 μM of ABP **19** for 1 hr followed by 5 - 100 μM of tetrazine **11** for 1 hr. Right panel: after the ligation reaction, samples were treated with 10, 50 or 100 mM hydrazine ('hydr.') overnight to cleave the levulinoyl linker. Proteins were resolved by 12.5% SDS-PAGE and detected by streptavidin Western blotting. 'ep': 100 μM epoxomicin added to compete for proteasome labeling by **19**.

The second part of this thesis describes the synthesis and biological evaluation of novel glycosidase ABPs. Glycosidases are hydrolytic enzymes responsible for the hydrolysis of glycosidic bonds in (oligo)saccharides and glycoconjugates such as glycosphingolipids in lysosomes. A deficiency in one of the lysosomal glycosidases can cause accumulation of the corresponding substrates in lysosomes, which may consequently lead to a lysosomal storage disorder. Despite the similarities in their primary defects, these disorders generally show a completely different disease progress, phenotype and clinical manifestation.[15] ABPP forms an attractive approach to study glycosidases and their involvement in disease.[16,17] At present a limited number of glycosidase ABPs has been developed, which is likely due to the tight substrate preference and the lack of covalent interactions with the substrate in the catalytic mechanism of many of these enzymes. Most of the retaining type of glycosidases, however form a covalent enzyme-substrate intermediate and are therefore amenable to targeting by ABPs that react covalently with the catalytic nucleophile in the active site.

Chapter 5 describes the design and synthesis of two series of irreversible inhibitors and ABPs for retaining α- and β-galactosidases. These probes are equipped with an electrophilic epoxide or aziridine moiety as the warhead in either an α- or a β-configuration to allow attack by the respective active site nucleophiles. The α- and β-galactopyranose-configured epoxide-based compounds include a non-tagged inhibitor as well as three ABPs in which the hydroxyl group at C6 (carbohydrate numbering) is substituted with an azide, a Bodipy fluorophore or a biotin tag. In addition, two aziridine-based α-galactosidase ABPs were synthesized in which an azide or Bodipy tag is installed via acylation of the aziridine nitrogen. Unfortunately, efforts to isolate the β-galactopyranose-configured isomers of these aziridine probes have so far proven unsuccessful, since even the use of a completely acid-free

procedure for HPLC purification and ensuing lyophilization resulted in opening of the acylated aziridine.

The inhibitory potency of the α-configured probes as well as their application for the labeling of human retaining α-galactosidases is described in Chapter 6. The aziridine-based ABPs proved to be very potent inhibitors of the human enzyme α-galactosidase A (αGal A), the deficiency of which is at the basis of the lysosomal storage disorder Fabry disease. The non-tagged epoxide inhibitor displayed a more than 10,000 fold lower potency towards αGal A than the aziridine probes, whereas none of the C6-modified epoxide ABPs showed any inhibition at all, revealing that the primary hydroxyl group is essential for binding of the probes to their target enzyme. Interestingly, the non-tagged epoxide proved to be an equally potent inhibitor of galactocerebrosidase, a human retaining β-galactosidase, while the aziridine ABPs did not inhibit this enzyme. The exact binding mechanism of the α-galactosidase probes to αGal A has yet to be confirmed by mass spectrometry analysis of the labeled active site peptides and/or crystallization of the recombinant enzyme with the probes covalently bound in the active site. The Bodipy-tagged aziridine ABP was demonstrated to enable the fluorescent labeling of endogenous αGal A activity in wild-type fibroblast cell extracts. Besides αGal A, the probe also labeled *N*-acetyl-galactosaminidase (αGal B), a related human enzyme displaying retaining α-galactosidase activity. In addition, the fluorescently labeled ABP was used to directly compare α-galactosidase activity in extracts from wild-type cells with those obtained from classic Fabry patients, which lack αGal A activity and accordingly showed only labeling of αGal B. An interesting application of the ABP would be to profile retaining α-galactosidase activity in samples from Fabry patients that have different mutations in αGal A and/or different phenotypic variants of the disease.

Preliminary *in situ* labeling experiments with the Bodipy-tagged aziridine ABP in fibroblast cell cultures revealed that no specific fluorescent signals could be detected on gel, despite the fact that inhibition of αGal A activity could clearly be demonstrated in extracts of probe-treated cells by measuring residual enzyme activity with the fluorogenic substrate 4-methylumbelliferyl α-D-galactoside (Figure 8.4). A possible explanation for the lack of fluorescent labeling is that the levels of enzymatic activity might be very low and thereby make it difficult to detect the labeled proteins, and/or that the ester bond formed between the enzyme and the probe is labile under the conditions used for SDS-PAGE analysis. The *in situ* inhibition of αGal A and αGal B activity by either tagged or non-tagged aziridine probes (**20** and **21**), regardless of their ability to enable visualization on gel, can be used to provide insight into the nature and extent of glyco(sphingo)lipid accumulation that results from reduced α-galactosidase activity by using mass spectrometry analysis.[18,19]

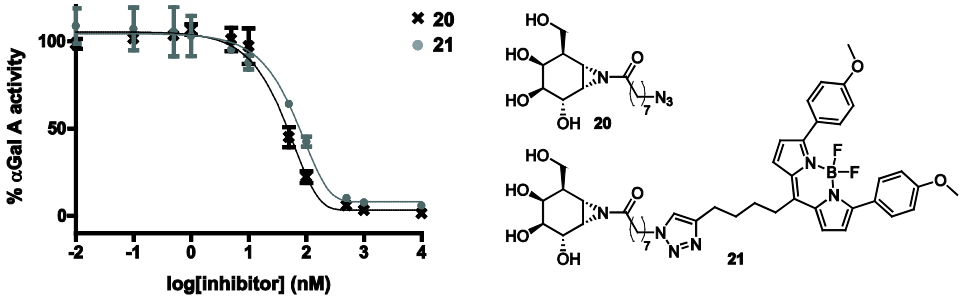


Figure 8.4. Inhibition of αGal A activity in wild-type fibroblast cells by treatment with 0.01 nM - 10 µM of ABPs **20** and **21** for 2 hrs. After cell lysis residual activity was determined from hydrolysis of 4-methylumbelliferyl α-D-galactoside.

In order to enable the visualization of enzymatic activity *in situ* and *in vivo*, it might be useful to improve the intensity and/or signal-to-background ratio of the fluorescent labeling. More sensitive detection of the labeled proteins could for example be realized by using an aziridine that is functionalized with a different (near-infrared) fluorophore such as Cy5 (**22**, Figure 8.5). Alternatively, the background labeling may be strongly reduced by performing two-step labeling with a cyclopropene-modified ABP (**25**) in combination with a quenched tetrazine reagent which only becomes fluorescent after reaction with the dienophile (e.g. **26**[20]). Biotin-tagged aziridines (**23** and **24**) would also form a useful complement to the set of available α-galactosidase ABPs by providing the option to enrich the labeled proteins in order to facilitate subsequent detection on Western blot and/or identification by mass spectrometry. Efforts have been undertaken to synthesize biotin-aziridine **24**, however the

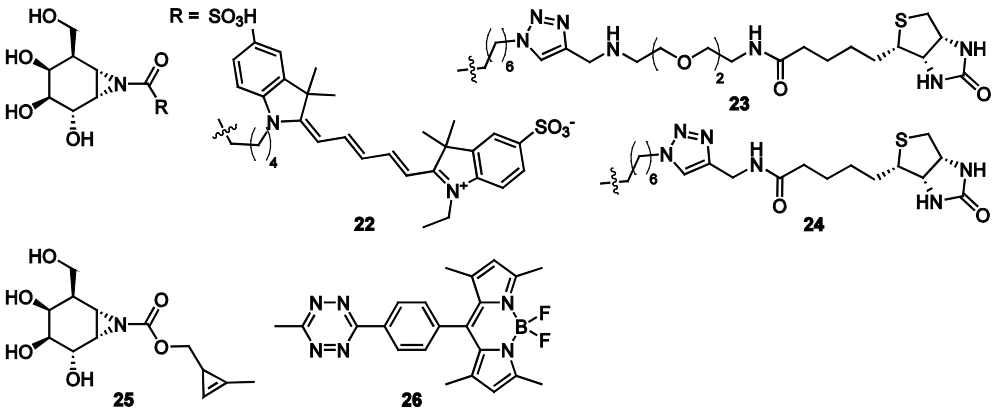


Figure 8.5. Putative aziridine-based retaining α-galactosidase ABPs functionalized with a Cy5 fluorophore (**22**), a biotin tag (**23** and **24**) or a cyclopropene ligation handle (**25**), and quenched Bodipy-tagged tetrazine **26**.

isolation of this compound proved difficult due to solubility issues and opening of the aziridine. It appears that these problems are at least partly due to the low solubility of the biotin spacer, and therefore the introduction of a PEG-spacer such as in probe **23** might offer a more practical alternative.

Chapter 7 describes the evaluation of the β-galactopyranose-configured epoxide probes for their ability to inhibit and label recombinant galactocerebrosidase, a human retaining β-galactosidase. The non-tagged epoxide proved to be the most potent inhibitor of this enzyme. While the inhibitory potency was almost 2,000-fold decreased for the ABP with an azide at C6, the installment of a Bodipy tag at the same position partially restored the inhibitory potency. A similar trend has been reported for the inhibition of retaining β-glucosidases by C6-modified β-glucopyranose-configured epoxide probes, although the beneficial effect of the Bodipy dye was much larger in that case.[16] In contrast, neither of the C6-functionalized α-galactopyranose-configured epoxide isomers (Chapter 6) appeared to inhibit retaining α-galactosidases, indicating that the substrate tolerance and restrictions in the active site of each individual glycosidase play an important role for the successful targeting with epoxide-based ABPs. The Bodipy- and biotin-functionalized β-galactosidase ABPs were shown to enable the visualization of catalytically active recombinant galactocerebrosidase on gel. It remains to be determined whether these probes also target other human retaining β-galactosidases, for instance lysosomal β-galactosidase, and whether they can be used to label endogenously expressed β-galactosidases in cell extracts and living cells. In addition, the exact binding mechanism of the ABPs to the active site of their target enzyme(s) has yet to be confirmed. Notably, the epoxide probes selectively labeled galactocerebrosidase without affecting the related enzymes α-galactosidase A and glucocerebrosidase. The fact that this latter enzyme can be targeted selectively by ABPs that differ only in the configuration of a single hydroxyl substituent from the β-galactosidase probes described here[16] demonstrates the potential to target different classes of retaining (exo)glycosidases by using similar ABPs that are modified in such a way that they mimic the natural substrate of the target enzyme.

Considering the results obtained with the α-galactopyranose- and β-glucopyranose-configured aziridine-based ABPs (see above), the synthesis of aziridine-based probes for β-galactosidases will likely provide ABPs with higher reactivity and/or altered selectivity than the epoxide-based probes. The availability of broad-spectrum as well as specific fluorescently labeled β-galactosidase ABPs would be useful to analyze differences in substrate preference of the different retaining β-galactosidases and to monitor enzymatic activity in the various lysosomal storage disorders that are associated with β-galactosidase

deficiency. A deficiency in galactocerebrosidase is at the basis of Krabbe disease while a deficiency in lysosomal β-galactosidase can lead to either Morquio B syndrome or GM1 gangliosidosis. The potential overlap in substrate preferences and the interplay between these enzymes have not yet been fully elucidated, and in this respect a set of β-galactosidase probes with different selectivity profiles might provide useful research tools. In addition, the (non-tagged) epoxide inhibitor may also be a valuable tool to study the accumulation of glyco(sphingo)lipids that occurs after inhibition of retaining β-galactosidase activity.

The instability of acylated aziridines is a recurring problem during the synthesis of aziridine-based ABPs and is mainly due to the electron-withdrawing nature of the carbonyl group, which activates the aziridine towards nucleophilic ring-opening. At the same time this property is probably also responsible for the superior reactivity of aziridine probes as compared to their epoxide analogues (Chapter 6).[16,21] An optimal balance between synthetic access and reactivity of an aziridine-based ABP may be realized by changing the nature of the functional group added to the aziridine nitrogen, for example by alkylation or sulfonylation as in probes **27** and **28** (Figure 8.6). Moreover, since the high reactivity of acylated aziridine probes was demonstrated to be accompanied by a loss in selectivity,[21] the synthesis of other substituted aziridines with varying reactivity may provide the option to tune their selectivity and thereby develop both highly specific and broad-spectrum ABPs. A non-substituted aziridine has a ring strain similar to an epoxide but is in principle less electrophilic due to its lower electronegativity,[22] so that the intrinsic reactivity of a non-activated aziridine (**27**) is likely lower than that of an epoxide. However, the aziridine-based ABPs have the advantage that the hydroxyl substituents are not used for the attachment of a tag and are therefore all available for making interactions with the active site of their target enzymes, potentially resulting in more tight binding and an increase in potency. In addition, it seems likely that the aziridine nitrogen will be protonated by the general acid/base residue in the active site, thereby strongly activating the aziridine towards nucleophilic ring-opening. Alkylated β-aziridine **27** can be derived from α-epoxide **29** (see Chapter 5) by opening of the epoxide with azido-PEG-amine **30**, mesylation of the resulting hydroxyl group and subsequent treatment with base to achieve aziridine formation.[23] A fluorophore or an affinity tag can then be introduced by copper(I)-catalyzed click reaction of the azide in **27** with an alkyne-functionalized tag. Sulfonylated aziridine **28** may be synthesized from deprotected aziridine **31** in a manner similar to the acylated aziridines described in Chapter 5 by selective reaction of the aziridine nitrogen with an activated ester of sulfonic acid **32** (e.g. an *N*-hydroxybenzotriazole ester).[24]

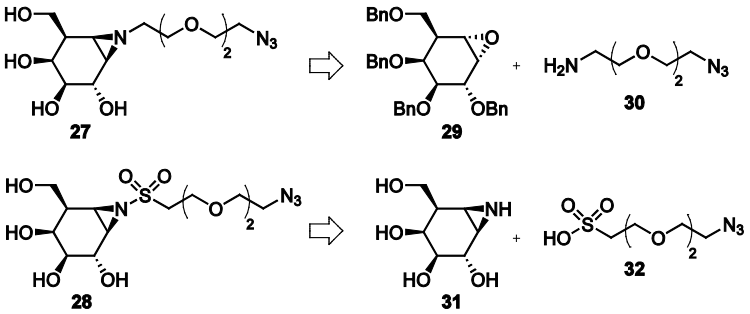
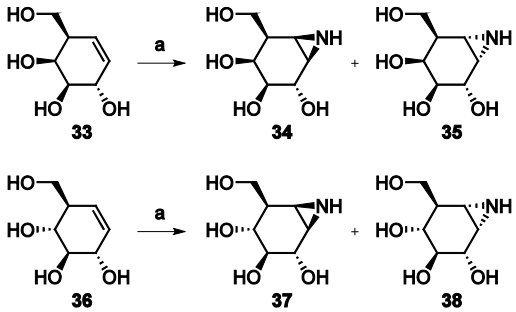


Figure 8.6. Alkylated (**27**) and sulfonylated (**28**) β-galactopyranose-configured aziridines.

Recently, a new method has been reported for the direct and mild conversion of olefins to *N*-H aziridines via a rhodium-catalyzed reaction with *O*-(2,4-dinitrophenyl) hydroxylamine (DPH) in trifluoroethanol.[25] This reaction can be performed in the presence of various functional groups, including unprotected alcohols, and may offer a useful alternative strategy to synthesize aziridine-based glycosidase ABPs. For example, galactopyranose- and glucopyranose-configured aziridines **34**-**35** and **37**-**38** can be obtained in a single step from the appropriately substituted cyclohexene precursors (**33** and **36**, respectively) (Scheme 8.2). This method would eliminate the need to first synthesize an epoxide or to form the aziridine with the aid of the primary hydroxyl group, procedures that were applied in Chapter 5. Although in general both diastereomeric aziridine products are formed, it seems likely that directing effects by the unprotected hydroxyl groups will affect the stereochemical outcome of the reaction. Hence, protection of one or more hydroxyl groups may be necessary to achieve formation of the desired α- and/or β-configured aziridines.

Scheme 8.2 Proposed new strategy for aziridine formation during the synthesis of glycosidase ABPs



Reagents and conditions: a) DPH, $Rh_2(esp)_2$ (Du Bois' catalyst), CF_3CH_2OH.

The synthetic strategy towards the galactosidase probes that was developed in this thesis can also be applied to the synthesis of a number of differently configured probes. For example, α-*N*-acetylgalactopyranose-configured probes (**39** and **40**, Figure 8.7) may enable the selective targeting of α-*N*-acetylgalactosaminidase (αGal B), the deficiency of which is at the basis of the lysosomal storage disorder Schindler disease. Although this enzyme was targeted by the aziridine-based α-galactosidase probes described in Chapter 6, the labeling was not selective. In fact, it was demonstrated that these probes label αGal A more efficiently than αGal B. Since this latter enzyme binds α-linked *N*-acetylgalactosamine substrates with much higher efficiency than α-galactosides, it is likely that substitution of the hydroxyl group at C2 (carbohydrate numbering) in the ABPs with an acetylated amine will increase their selectivity for αGal B and might even completely abolish the labeling of αGal A. A proposed strategy towards the synthesis of these probes is depicted in Scheme 8.3 and involves the same synthetic route as was used for the galactosidase probes but starts from a differently configured and orthogonally protected aldehyde (**43**). This compound may be obtained by procedures similar to those described in literature,[26] using D-ribose as the starting material to achieve the required stereochemistry.[27] Aldol condensation with oxazolidinone **44**, removal of the Evans template and ring-closing metathesis as described in Chapter 5 will lead to compound **45**. Next, several protective group manipulations are necessary to selectively unmask the hydroxyl group at C2 (**46**), which can then be activated with triflic anhydride and substituted with TMS azide to yield compound **47**. Reduction of the azide to an amine and subsequent acetylation may provide compound **48**, from which the epoxide- and aziridine-based probes **39** and **40** can be synthesized.

Other compounds that can be synthesized via a similar synthetic strategy are α-L-fucopyranose-configured probes (**41**, Figure 8.7), which might enable the labeling of human retaining α-L-fucosidases. Epoxide-based inhibitors are also an option (**42**), but since the natural substrates of these enzymes lack a substituent at C6 the attachment of a tag at this position will likely not be tolerated, so that the tag would need to be introduced at a less straightforward position. The synthesis of probes **41** and **42** requires the use of the enantiomers of the starting materials that were used in Chapter 5, i.e. oxazolidinone **50**

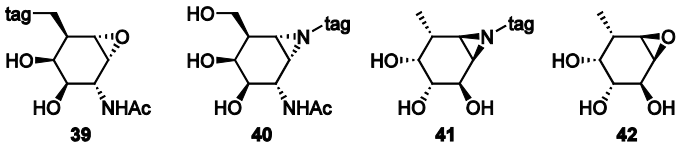
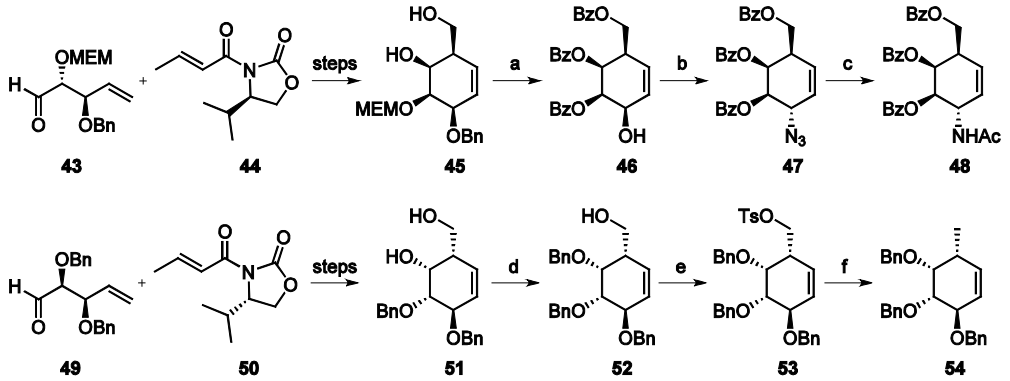


Figure 8.7. Putative inhibitors and ABPs to target α-*N*-acetylgalactosaminidase (**39**, **40**) and α-L-fucosidase (**41**, **42**).

Scheme 8.3 Proposed route of synthesis towards building blocks **48** and **54**



Reagents and conditions: a) *i*) $ZnBr_2$, DCM, *ii*) BzCl, pyridine, *iii*) BCl_3, DCM; b) *i*) Tf_2O, DMAP, pyridine, DCM, *ii*) $TMSN_3$, TBAF, THF; c) *i*) Ph_3P, H_2O, THF, *ii*) Ac_2O, pyridine; d) *i*) Ph_3CCl, Et_3N, DMAP, DMF, *ii*) BnBr, NaH, TBAI, DMF, *iii*) $BF_3.OEt_2$, MeOH/toluene; e) *p*TsCl, pyridine; f) $LiAlH_4$, THF.

derived from D-valine instead of L-valine and aldehyde **49**, which can be obtained from L-xylose instead of D-xylose (Scheme 8.3). Aldol condensation, removal of the Evans template and ring-closing metathesis should lead to compound **51**, after which several protective group manipulations may afford tribenzylated compound **52**. The free hydroxyl group can then be selectively activated by tosylation (**53**) and subsequently substituted with a hydride donor to provide **54**, which serves as a basis for the synthesis of the α-L-fucopyranose-configured inhibitors (**41**, **42**).

The absence of covalent interactions with a substrate in the catalytic mechanism of an enzyme of interest hampers the development of 'classic' ABPs that act via direct alkylation of the catalytically active amino acid residue. A possible solution is the use of suicide substrates, which are cleaved by the enzyme in the same manner as a natural substrate and consequently release a highly reactive species that can react with a nearby nucleophilic amino acid residue. This type of ABPs has for instance been applied to the activity-based profiling of a number of inverting glycosidases.[28-31] Another possibility to label enzymes that do not form a covalent enzyme-substrate intermediate is the use of affinity-based probes (A_fBPs), which also carry a (latent) reactive group but do not require activation by the catalytic machinery of the enzyme. Instead, these probes bind with high affinity to a specific site on the enzyme of interest and achieve labeling by non-specific covalent reaction of an amino acid residue with an electrophile or a photoreactive group in the A_fBP.[32,33] Frequently used photoaffinity labels include aryl azides, benzophenones and diazirines, which are all transformed into a highly reactive species upon activation by light of the appropriate

wavelength and can subsequently crosslink to proximal amino acid residues. A limitation of the use of photoaffinity probes is the propensity of the activated molecules to react with water, so that the success of labeling strongly depends on the speed of crosslinking as well as on the ability of the probes to tightly bind their target enzymes. Since these probes do not bind in a mechanism-based manner, they are strictly no 'true' ABPs. Nonetheless, A_fBPs can be valuable tools to examine the structural integrity of a target enzyme, for example if binding only occurs to an intact active site. Moreover, these probes can be useful to image the cellular localization and/or degradation of proteins and to identify the targets of drugs and inhibitors.[33]

A class of enzymes that does not employ a catalytic nucleophile in the active site is that of the α-ketoglutarate dependent oxygenases, also named 2-oxoglutarate (2-OG) oxygenases.[34-35] These enzymes catalyze the oxidation of C-H bonds in proteins, nucleotides, lipids and small molecules. This process results in hydroxylation of the substrates and can also lead to lysine demethylation. More than 60 different 2-OG oxygenases are known in humans and these are involved in a wide variety of cellular processes, including for example gene control, fatty acid metabolism and the response to hypoxia. The catalytic mechanism of 2-OG oxygenases involves an Fe(II) cofactor that is bound in the active site and in turn coordinates to the cosubstrate 2-OG (Figure 8.8A).[35] Binding of a substrate and O_2 results in oxidative decarboxylation of 2-OG with concomitant formation of CO_2 and oxidation of the substrate. Since the cofactor and cosubstrate binding sites are highly conserved, these sites

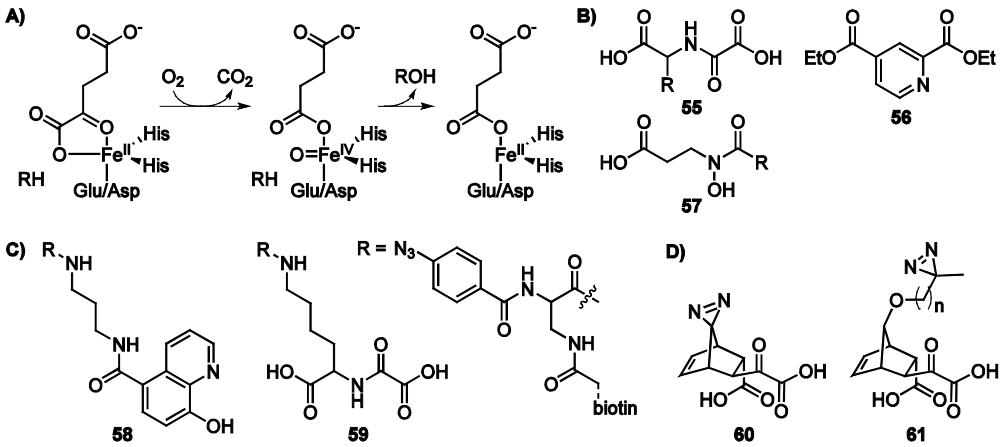


Figure 8.8. A) Catalytic mechanism of 2-OG oxygenases. B, C) Known 2-OG oxygenase inhibitors (**55**-**57**) and probes (**58**-**59**). D) Putative two-step photoaffinity probes for 2-OG oxygenases (**60**, **61**).

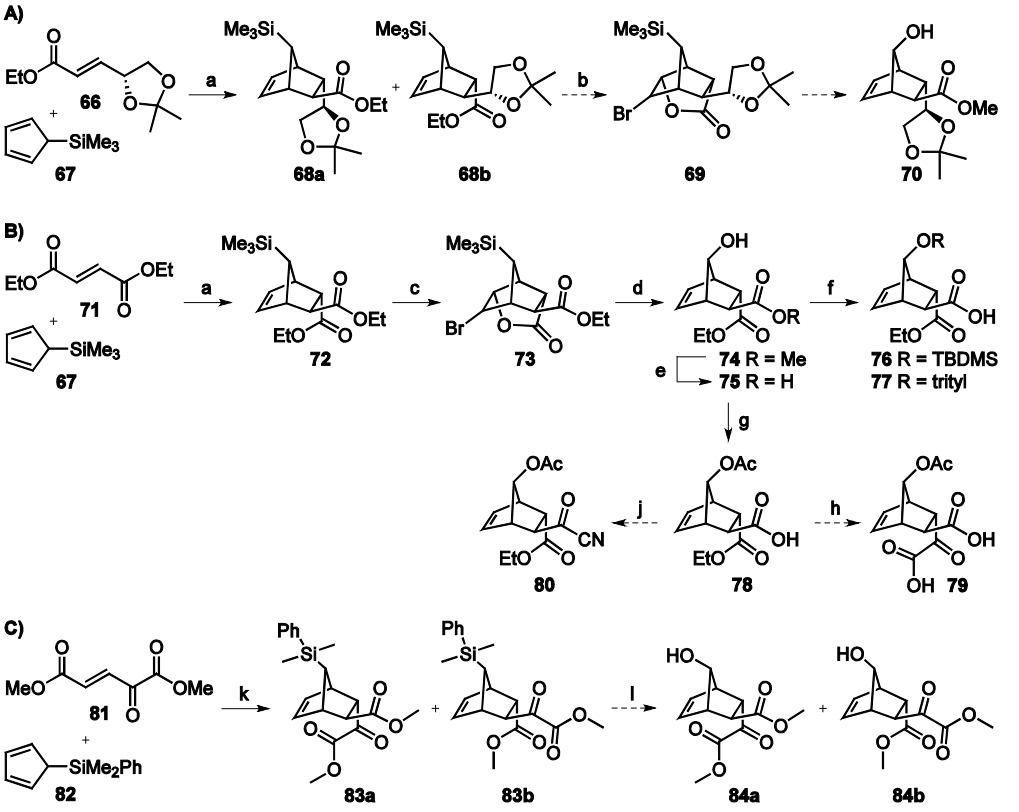
form attractive targets for the development of broad-spectrum inhibitors or A$_f$BPs. A number of reversible 2-OG oxygenase inhibitors has been reported in literature, including metal ions, substrate derivatives and analogues of 2-OG such as compounds **55**-**57** (Figure 8.8B), which act via coordination to Fe(II) and competition with binding of 2-OG in the active site.[36-40] The only probe that has been described so far for the labeling of this class of enzymes in complex biological samples is the hydroxyquinoline-based probe **58**, which is derivatized with a biotin tag and an aryl azide as a photoaffinity label (Figure 8.8C).[41] In the same study, the oxalylglycine derivative **59** proved to be only a very weak inhibitor of a small set of 2-OG oxygenases. Hence it appears that in this case the attachment of the relatively large photoreactive group and the biotin tag is not tolerated by the majority of 2-OG oxygenases, indicating that the modifications made to the 2-OG core should be sterically limited. The development of a two-step labeling probe may provide a means to enhance affinity by minimizing the steric bulk that is added to the probe scaffold and may also increase cell permeability. Therefore, two photoreactive 2-OG oxygenase probes were designed that are based on the structure of 2-OG integrated into a norbornene moiety for two-step labeling via tetrazine ligation (**60** and **61**, Figure 8.8D). A diazirine photoaffinity tag is included for crosslinking of the probe to target proteins and is added either directly onto the norbornene core (**60**) or via a short spacer (**61**).

Several strategies have been explored to synthesize A$_f$BPs **60** and **61**. It was reasoned that **60** can be derived from compound **62** (Figure 8.9) by conversion of the ketone into a diazirine moiety via a previously reported procedure[42] followed by deprotection and oxidation of the hydroxyl groups. Compound **61** can be obtained from **63** by functionalization of the hydroxyl group with a diazirine-containing spacer. These key building blocks may in turn be derived from for instance compound **64**, with the hydroxyl groups orthogonally protected to enable separate oxidation steps, or from compound **65** in which an α-keto acid or ester would need to be installed prior to introduction of the diazirine moiety. Initial efforts to synthesize compounds **62** and **63** started with the Et_2AlCl-catalyzed Diels-Alder reaction of silylated diene **67** and dienophile **66** (Scheme 8.4A), which gave a mixture of various diastereomers assumed to be the regio-isomers with the silyl group away

Figure 8.9. Key building blocks for the synthesis of A$_f$BPs **60** and **61**.

from the other substituents (**68a** and **68b** plus the corresponding endo/exo isomers). Next, cyclization of **68b** with bromine was attempted in order to enable silver nitrate-induced desilylation of **69** and intramolecular rearrangement as described in literature (see below).[43] However, the acetonide was not stable towards treatment with bromine. Other conditions for bromocyclization of **68b**, including hydrolysis of the ester with aqueous NaOH followed by reaction with *N*-bromosuccinimide, also failed to give the expected product (**69**).

Scheme 8.4 Synthetic routes towards building blocks **62** and **63**



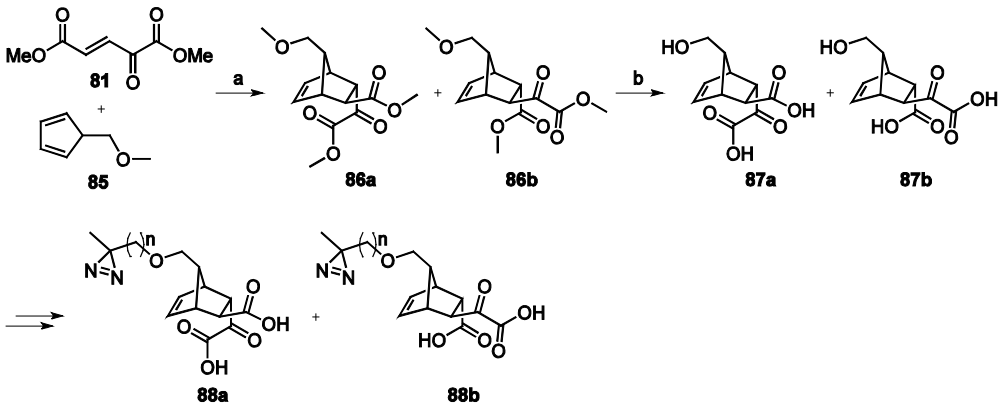
Reagents and conditions: a) Et_2AlCl, toluene, -78 °C → rt, overnight, **68** 86%, **72** quant.; b) Br_2, pyridine, DCM, 0 °C → rt, 6 hrs; c) Br_2, DCM, 0 °C → rt, 4.5 hrs, 83%; d) $AgNO_3$, MeOH, 70 °C, 3.5 hrs, 68%; e) Me_3SnOH, toluene, 100 °C, 5 hrs, 83%; f) TBDMS-Cl, imidazole, DMF, rt, overnight, **76** 28% or TrCl, DMAP, pyridine, 60 °C, 6 hrs, **77** <30%; g) Ac_2O, DMAP, pyridine, 40 °C, 1.5 hrs, 65%; h) *i*) LDA, diethyl oxalate, THF/TMEDA, rt, overnight, *ii*) HCl, 100 °C, 5 hrs; j) *i*) $(COCl)_2$, DMF, DCM, 0 °C → rt, 5 hrs, *ii*) CuCN, ACN, 90 °C, overnight; k) toluene, rt, 1 hr, 80%; l) KBr, NaOAc, AcOOH, AcOH, rt, 1.5 hrs or $Hg(OTFA)_2$, AcOK, AcOH, AcOOH, 0 °C → rt, 4.5 hrs.

Taking into account the difficulties that were associated with the use of **66** as a starting material, resulting in the formation of complicated mixtures of Diels-Alder products including undesired compound **68a**, a second strategy was designed starting from diethyl fumarate (**71**) (Scheme 8.4B). Diels-Alder reaction with silylated diene **67** gave Diels-Alder adduct **72** (plus its enantiomer) in quantitative yield. Treatment with bromine afforded compound **73**, which was then reacted with silver nitrate in methanol to induce desilylation with concomitant opening of the lactone and intramolecular rearrangement,[43] giving compound **74**. Attempts to increase the yield of this reaction by using a longer reaction time resulted in transesterification of the ethyl ester to a methyl ester. Selective hydrolysis of the methyl ester in **74** with Me_3SnOH gave **75**, after which efforts were undertaken to protect the hydroxyl group by silylation or tritylation. Both procedures, however, proceeded slowly and gave low yields of products **76** and **77**, respectively. Moreover, the main product isolated after reaction with TBDMS-Cl appeared to be disilylated (at the hydroxyl group and the carboxylic acid). Acetylation of **75**, on the other hand, successfully afforded compound **78**, which corresponds to one of the potential building blocks shown in Figure 8.9 (**65**). The introduction of an α-keto acid was then attempted by Claisen condensation with diethyl oxalate followed by decarboxylation under acidic conditions (**79**). An alternative procedure involved the formation of an acid chloride and reaction with copper cyanide to give acyl cyanide **80**, which can be hydrolyzed to an α-keto acid. Unfortunately, neither of these procedures proved to yield the desired products.

Therefore, a third route of synthesis was designed in which an α-keto ester is already present in the starting material (**81**) (Scheme 8.4C). Since this procedure does not involve the bromocyclization and rearrangement steps as in routes A and B, a cyclopentadienyl-silane reagent was synthesized in which one of the methyl substituents is replaced with a phenyl substituent (**82**) so that the silyl group can be oxidized to a hydroxyl group. Diels-Alder reaction of **81** and **82** gave a mixture of products (presumably **83a** and **83b** and their enantiomers), which were subjected to several Fleming oxidation procedures. However, compounds **84a** and **84b** could not be isolated. The use of differently substituted silane reagents and/or the use of different oxidation conditions may provide a means to obtain the hydroxylated products **84a** and **84b** (which correspond to building block **63**, Figure 8.9). Alternatively, synthetic route B also holds potential to synthesize the target compounds via selective reduction of the ethyl ester in **78** to an aldehyde, e.g. with DIBAL-H (which will likely also require the use of another hydroxyl protecting group). Stille reaction or Grignard reaction with a hydroxymethylated reagent may then provide an α,β-diol as a precursor of the α-ketoacid.

Another promising option to synthesize putative A_fBPs for 2-OG oxygenases is by making use of 5-methoxymethyl-cyclopenta-1,3-diene (**85**)[44] as a starting material (Scheme 8.5). This compound contains a methoxymethyl substituent that acts as a latent attachment site for the photoreactive group, since it can be mildly converted into a hydroxyl group during a later stage of synthesis. As depicted in Scheme 8.5, the use of cyclopentadiene **85** would lead to probes **88a** and **88b**, the latter of which is structurally similar to compound **61** (Figure 8.8D) except for the placement of the diazirine-containing linker: in probes **88a** and **88b** an extra carbon atom is added between the norbornene core and the alkoxy substituent. The proposed synthetic strategy commences with Diels-Alder reaction of cyclopentadiene **85** with compound **81** to provide adducts **86a** and **86b**, with the hydroxymethyl group away from the (α-keto) ester substituents.[45] In order to prevent isomerization of **85** to 1-methoxymethyl-1,3-cyclopentadiene, Et_2AlCl can be used as a catalyst to accelerate the Diels-Alder reaction and to allow the reaction to proceed at low temperatures (see also Schemes 8.4A and 8.4B). Subsequently, demethylation of the hydroxymethyl group may be achieved by reaction with BBr_3 in DCM and the methyl esters can be hydrolyzed under acidic conditions, procedures that have both been reported to proceed well in the presence of an α-keto moiety.[46-47] The resulting compounds **87a** and **87b** contain the 2-OG motif as well as a primary hydroxyl group for further functionalization with a photoaffinity label.

Scheme 8.5 Proposed strategy to synthesize A_fBPs **88a** and **88b**



Reagents and conditions: a) Et_2AlCl, toluene, -78 °C → 0 °C; b) *i*) BBr_3, DCM, *ii*) HCl/H_2O.

Conclusion

The work described in this thesis provides novel approaches for the two-step profiling of enzymatic activity and for the simultaneous bioorthogonal labeling of multiple cellular targets, and presents novel probes for the activity-based profiling of two specific classes of enzymes, the retaining α- and β-galactosidases. Two-step labeling strategies are fundamental for enzymes that do not tolerate the attachment of a bulky reporter group onto an inhibitor scaffold. In addition, the application of ABPs for the imaging of enzymatic activity *in vivo* poses additional demands on the cell-permeability of the probes and may therefore necessitate the use of two-step ABPP strategies. At the same time there is an increasing interest in the use of tandem bioorthogonal ligation strategies that allow the monitoring of multiple targets simultaneously. Tandem labeling strategies such as those described in this thesis may provide valuable approaches to study multiple components involved in a particular biological processes or disease state at the same time.

Originally, the development of ABPs was mainly directed towards a few classes of hydrolytic enzymes with well-defined catalytic mechanisms, including serine hydrolases and cysteine/threonine proteases.[48] Nowadays, specific ABPs have also been developed for a variety of other enzyme classes including protein tyrosine phosphatases,[49-51] monooxygenases,[52] monoamine oxidases,[53] and carbohydrate processing enzymes.[54] For those enzymes that do not make use of an active site nucleophile in their catalytic mechanism or that are not susceptible to targeting by ABPs for another reason, the use of A_fBPs can provide a valuable alternative. Such probes have for instance proven successful for the labeling of metalloproteases, phosphatases, histone deacetylases, 2OG oxygenases and kinases.[32] Although A_fBPs do not label their target proteins in an activity-based manner, the careful design of a probe such that binding is dependent on the integrity of the active site may provide a means to monitor the functional state of an enzyme. As the field of ABPP is gradually moving towards the targeting of enzyme classes with different and sometimes poorly understood catalytic mechanisms, tight substrate specificity and/or low expression levels, continuing efforts are being made to generate novel strategies for the activity-based profiling of such difficult to target enzymes. This thesis presents a number of strategies that open up new opportunities for future research directions, both for the activity-based profiling of new enzyme classes as well as for the bioorthogonal labeling of enzymatic activity and other biomolecules.

Experimental procedures

A. Synthesis

General

All reagents were commercial grade and were used as received unless stated otherwise. MeOH was obtained from Biosolve. Toluene, EtOAc and PetEt (Riedel-de Haën) used for column chromatography were of technical grade and distilled before use. DCE, DCM, DMF, and THF (Biosolve) were of analytical grade and when used under anhydrous conditions stored over flame-dried 4 Å molecular sieves. Reactions were monitored by TLC-analysis using DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254/366 nm), spraying with a solution of 20% H_2SO_4 in EtOH or a solution of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4{\cdot}2H_2O$ (10 g/L) in 10% aqueous sulfuric acid followed by charring at ~150 °C or by spraying with an aqueous solution of $KMnO_4$ (7%) and K_2CO_3 (2%). Column chromatography was performed on silica gel (Screening Devices BV, 0.040 - 0.063 mm, 60 Å). LC/MS analysis was performed on an LCQ Adventage Max (Thermo Finnigan) ion-trap spectrometer (ESI+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 5µm particle size, Phenomenex). The applied buffers were A: H_2O, B: ACN and C: 1 % aqueous TFA. Reported gradients represent the percentage of buffer B in buffer A with 10% buffer C. HRMS analysis was performed on an LTQ Orbitrap (Thermo Finnigan) mass spectrometer equipped with an electronspray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL min−1, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150 - 2000) and dioctylphtalate (m/z = 391.28428) as a "lock mass". The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). ^{1}H- and ^{13}C-APT-NMR spectra were recorded on a Brüker AV-400 (400/100MHz) instrument. Chemical shifts are given in ppm (δ) relative to the solvent peak or to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Peak assignments are based on 2D ^{1}H-COSY and ^{13}C-HSQC NMR experiments.

***N*-(Boc-6-aminohexanoyl)-4-(6-(2-pyrimidinyl)-1,2,4,5-tetrazin-3-yl)benzylamine (13)**

Boc-protected tetrazine **12** (0.50 mmol, 0.18 g) was treated with TFA/DCM (1/1, v/v) for 15 min and then concentrated *in vacuo* in the presence of toluene. The residue was dissolved in DCE/DMF under argon atmosphere and DiPEA (1.0 mmol, 0.17 mL, 2.0 eq.) and Boc-6-Ahx-OSu (0.50 mmol, 0.16 g, 1.0 eq.) were added. The reaction mixture was stirred at room temperature overnight before being concentrated *in vacuo*. The crude product was purified by column chromatography (DCM → 2% MeOH in DCM), followed by recrystallization from DCM/n-hexane to yield Boc-Ahx-tetrazine **13** as purple crystals (0.21 g, 0.44 mmol, 88%).^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.15 (d, *J* = 4.9 Hz, 2H), 8.70 (dd, *J* = 8.3, 1.3 Hz, 2H), 7.61 (t, *J* = 4.9 Hz, 1H), 7.55 (d, *J* = 8.1 Hz, 2H), 6.19 (d, *J* = 7.4 Hz, 1H), 4.61-4.59 (m, 3H), 3.15-3.11 (m, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.79-1.71 (m, 2H), 1.55-1.48 (m, 2H), 1.44 (s, 9H), 1.43-1.35 (m, 2H). ^{13}C NMR: (100 MHz, $CDCl_3$): δ (ppm) 172.95, 164.28, 163.08, 159.57, 158.41, 156.04, 144.43, 130.38, 129.15, 128.57, 122.50, 79.11, 43.23, 40.30, 36.49, 29.80, 28.43, 26.40, 25.25. LC/MS analysis: R_t 6.9 min (linear gradient 10 → 90% B in 15 min), m/z 479.0 $[M+H]^+$, 379.2 $[M\text{-}Boc+H]^+$, 957.2 $[2M+H]^+$. HRMS: calcd. for $[C_{24}H_{31}N_8O_3]^+$ 479.25136, found 479.25184; calcd. for $[C_{24}H_{30}N_8O_3Na]^+$ 501.23331, found 501.23367.

***N*-Boc-2-(2-(2-amino-*N*-(1-heptyn-7-oyl)ethoxy)ethoxy)ethylamine (15)**

Boc-protected compound **14**[14] (1.9 mmol, 0.53 g) was dissolved in THF and triphenylphosphine (2.3 mmol, 0.60 g, 1.2 eq.) was added. The reaction mixture was stirred overnight at room temperature, before a few drops of H_2O were added. After stirring for an additional 2 hrs, toluene was added and the mixture was extracted with H_2O. The aqueous layer was washed with toluene and the combined organic layers were again extracted with H_2O until no more product was detected in the organic phase. The combined aqueous layers were concentrated *in vacuo* to give

the monoprotected PEG-diamine. A solution of 6-heptynoic acid (1.9 mmol, 0.24 mL, 1.0 eq.) in toluene was put under argon atmosphere, after which oxalyl chloride (2.9 mmol, 0.25 mL, 1.5 eq.) and one drop of DMF were added. After stirring for 3 hours at room temperature, the mixture was concentrated *in vacuo* and coevaporated with toluene (3x). The acyl chloride was then redissolved in DCE under argon atmosphere, neutralized with DiPEA (2.9 mmol, 0.50 mL, 1.5 eq.) and added to the crude PEG-amine. The mixture was stirred overnight at room temperature, quenched with MeOH and concentrated *in vacuo*. Purification by column chromatography (30% EtOAc in PetEt → EtOAc) yielded alkyne-functionalized PEG spacer **15**, contaminated with some triphenylphosphine oxide (0.37 g, ~1.0 mmol, 54% over two steps). This product was used without further purification for the next step. LC/MS analysis: R_t 6.7 min (linear gradient 10 → 90% B in 15 min), m/z 356.9 $[M+H]^+$, 257.2 $[M\text{-}Boc+H]^+$, 379.1 $[M+Na]^+$. HRMS: calcd. for $[C_{18}H_{33}N_2O_5]^+$ 357.23840, found357.23855; calcd. for $[C_{18}H_{32}N_2O_5Na]^+$ 379.22034, found 379.22042.

***N*-biotin-2-(2-(2-amino-*N*-(1-heptyn-7-oyl)ethoxy)ethoxy)ethylamine (16)**

Boc-protected compound **15** (0.60 mmol, 0.21 g) was treated with TFA/DCM (1/1, v/v) for 20 min and then concentrated *in vacuo* in the presence of toluene. The residue was dissolved in DCE/DMF (1/1, v/v) under argon atmosphere and DiPEA (1.2 mmol, 0.21 mL, 2.0 eq.) and biotin-OSu (0.60 mmol, 0.20 g, 1.0 eq.) were added. The reaction mixture was stirred at room temperature overnight before being concentrated *in vacuo*. The crude product was purified by column chromatography (DCM → 10% MeOH in DCM). To remove final impurities, the product was redissolved in DCM and washed with H_2O (2x), after which the combined aqueous layers were extracted with DCM/MeOH (9/1, v/v) until no more product was detected in the aqueous phase. The combined organic layers were dried over $MgSO_4$, filtered and evaporated *in vacuo* to give alkyne-functionalized and biotin-tagged PEG spacer **16** (0.25 g, 0.52 mmol, 87%). ^{1}H NMR (400 MHz, MeOD): δ (ppm) 4.52 (ddd, *J* = 7.9, 5.0, 1.0 Hz, 1H), 4.33 (dd, *J* = 7.9, 4.5 Hz, 1H), 3.65 (s, 4H), 3.57 (dt, *J* = 5.6, 1.1 Hz, 4H), 3.39 (t, *J* = 5.5 Hz, 4H), 3.23 (ddd, *J* = 8.8, 5.9, 4.5 Hz, 1H), 2.95 (dd, *J* = 12.8, 5.0 Hz, 1H), 2.73 (d, *J* = 12.7 Hz, 1H), 2.27-2.18 (m, 6H), 1.84-1.37 (m, 11H). ^{13}C NMR (100 MHz, MeOD): δ (ppm) 174.75, 174.61, 164.70, 83.28, 69.90, 69.22, 68.40, 61.97, 60.23, 55.60, 39.65, 38.89, 35.34, 35.07, 28.37, 28.10, 27.80, 25.44, 24.70, 17.40. LC/MS analysis: R_t 5.0 min (linear gradient 10 → 90% B in 15 min), m/z 483.3 $[M+H]^+$, 965.1 $[2M+H]^+$. HRMS: calcd. for $[C_{23}H_{39}N_4O_5S]^+$ 483.26357, found 483.26329; calcd. for $[C_{23}H_{38}N_4O_5SNa]^+$ 505.24551, found 505.24477.

Biotinylated levulinoyl linker 17

Azide-modified levulinoyl derivative **18**[13] (0.50 mmol, 0.24 g) and biotin-tagged alkyne **16** (0.50 mmol, 0.24 g, 1.0 eq.) were dissolved in *t*BuOH/toluene/H_2O (2/2/1, v/v/v) (10 mL). Copper(II)sulphate pentahydrate (50 µmol, 1 mL 50 mM in H_2O, 0.1 eq.) and sodium ascorbate (75 µmol, 1 mL 75 mM in H_2O, 0.15 eq.) were added, followed by a few drops of DMF until the mixture became clear. The reaction mixture was heated to 80 °C and stirred overnight, before being concentrated *in vacuo*. The product was purified by column chromatography (DCM/acetone/MeOH, 8/2/0 (v/v/v) → 6.5/2/1.5 (v/v/v)), redissolved in DCM and washed with H_2O, upon which the biotinylated levulinoyl linker **17** precipitated from the organic phase as white crystals (0.29 g, 0.30 mmol, 60%). ^{1}H NMR (400 MHz,$CDCl_3$): δ (ppm) 7.34 (s, 1H), 6.96 (s, 2H), 6.89 (bs, 1H), 6.71 (bs, 1H), 6.63 (bs, 1H), 5.86 (bs, 1H), 4.49 (d, *J* = 7.0 Hz, 1H), 4.33-4.31 (m, 3H), 3.64-3.53 (m, 8H), 3.44-3.42 (m, 4H), 3.14-3.13 (m, 1H), 2.97-2.62 (m, 12H), 2.55-2.50 (m, 4H), 2.24-2.15 (m, 6H), 1.81-1.60 (m, 8H), 1.43 (s, 11H), 1.17 (d, *J* = 6.9 Hz, 12H). ^{13}C NMR (100 MHz,$CDCl_3$): δ (ppm) 207.22, 173.46, 173.28, 172.34, 171.69, 164.18, 143.74, 140.12, 138.76, 123.81, 121.11, 80.31, 70.07, 70.01, 69.94, 69.85, 63.85, 61.80, 60.27, 55.63, 48.98, 40.45, 39.16, 39.11, 38.81, 37.06, 37.00, 36.06, 35.91, 31.00, 29.68, 28.81, 28.20, 28.08, 27.72, 27.43, 25.57, 25.22, 25.12, 24.24. LC/MS analysis: R_t 8.8 min (linear gradient 10 → 90% B in 15 min), m/z 956.5 $[M+H]^+$. HRMS: calcd. for $[C_{49}H_{78}N_7O_{10}S]^+$ 956.55254, found 956.55331; calcd. for $[C_{49}H_{77}N_7O_{10}SNa]^+$ 978.53448, found 978.53480.

Biotin-levulinoyl-tetrazine 11

*t*Bu-protected compound **17** (50 µmol, 48 mg) was treated with TFA/DCM (1/1, v/v) for 30 min and then concentrated *in vacuo* in the presence of toluene. Boc-protected tetrazine **13** (0.10 mmol, 48 mg, 2.0 eq.) was also treated with TFA/DCM (1/1, v/v) for 20 min and then concentrated *in vacuo* in the presence of toluene. The residue was dissolved in DCE/DMF under argon atmosphere, added to the deprotected levulinoyl acid and the mixture was neutralized with DiPEA (0.2 mmol,33 µL, 4.0 eq.). After addition of HCTU (0.15 mmol, 62 mg, 3.0 eq.), the reaction mixture was stirred at room temperature overnight. The mixture was then washed with H_2O, the aqueous layers was extracted with DCM (2x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (DCM → 10% MeOH in DCM) followed by recrystallization from DCM/PetEt yielded biotin-levulinoyl-tetrazine **11** as a purple powder (44 mg,35 µmol, 70%).^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 9.12 (d, *J* = 4.9 Hz, 2H), 8.64 (d, *J* = 8.0 Hz, 2H), 7.60 (t, *J* = 4.9 Hz, 1H), 7.52 (d, *J* = 8.1 Hz, 2H), 7.32 (s, 1H), 7.04-6.92 (m, 4H), 6.89 (t, *J* = 5.6 Hz, 1H), 6.45 (s, 1H), 6.20 (t, *J* = 5.8 Hz, 1H), 5.77 (s, 1H), 4.55 (d, *J* = 5.9 Hz, 2H), 4.46 (dd, *J* = 7.9, 4.8 Hz, 1H), 4.32-4.26 (m, 3H), 3.57-3.52 (m, 8H), 3.41-3.40 (m, 4H), 3.20 (q, *J* = 6.7 Hz, 2H), 3.11 (d, *J* = 4.7 Hz, 1H), 2.98-2.56 (m, 12H), 2.52-2.44 (m, 4H), 2.28 (t, *J* = 7.5 Hz, 2H), 2.21-2.13 (m, 6H), 1.70-1.65 (m, 10H), 1.52-1.28 (m, 6H), 1.14 (d, *J* = 6.9 Hz, 12H). ^{13}C NMR (100 MHz,$CDCl_3$): δ (ppm) 207.35, 173.52, 173.39, 173.35, 172.43, 171.84, 164.29, 164.01, 163.02, 159.47, 158.40, 147.81, 144.82, 143.77, 140.23, 139.06, 130.15, 129.01, 128.49, 123.80, 122.57, 121.23, 70.06, 70.01, 69.91, 69.84, 61.75, 60.20, 55.58, 48.92, 43.09, 40.45, 39.17, 39.15, 39.10, 38.77, 38.47, 36.92, 36.20, 35.98, 35.84, 31.75, 29.68, 29.14, 28.74, 28.14, 28.03, 27.77, 27.42, 26.39, 25.55, 25.11, 25.09, 24.22. LC/MS analysis: R_t 6.9 min (linear gradient 10 → 90% B in 15 min), m/z 1260.8 $[M+H]^+$, 631.1 $[M+H]^{2+}$. HRMS: calcd. for $[C_{64}H_{90}N_{15}O_{10}S]^+$ 1260.67103, found 1260.67164; calcd. for $[C_{64}H_{89}N_{15}O_{10}SNa]^+$ 1282.65298, found 1282.65323.

Ethyl 3-(2,2-dimethyl-1,3-dioxolan-4-yl)-7-(trimethylsilyl)bicyclo[2.2.1]hept-5-ene-2-carboxylate (68)

Compound **66** (5.0 mmol, 0.98 mL) was dissolved in toluene and cooled to -78 °C under argon atmosphere, before Et_2AlCl (5.0 mmol, 2.8 mL 1.8 M in toluene, 1.0 eq.) was added. After stirring for 15 min at -78 °C, cyclopenta-2,4-dien-1-yltrimethylsilane (**67**) (10 mmol, 1.7 mL, 2.0 eq.) was added dropwise and the reaction mixture was stirred overnight while allowing the temperature to slowly rise to room temperature. The reaction was then quenched with saturated aqueous $NaHCO_3$, the aqueous layer was extracted with Et_2O (2x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 1.5% acetone in PetEt) gave Diels-Alder adduct **68** in two separate fractions with mixtures of 3 and 2 diastereomers, respectively (1st fraction: 1.4 g, 4.1 mmol, 81%; 2nd fraction: 78 mg, 0.23 mmol, 5%). ^{1}H NMR (400 MHz, $CDCl_3$) fraction 1 (3 diastereomers): δ (ppm) 6.25-6.16 (m, 1Ha+1Hb), 6.16-6.08 (m, 2Hc), 5.93 (dd, *J* = 5.6, 2.7 Hz, 1Ha), 5.91-5.87 (m, 1Hb), 4.20-3.87 (m, 4Ha+4Hb+3Hc), 3.79-3.60 (m, 1Ha+1Hb+1Hc), 3.47 (dtd, *J* = 10.6, 5.6, 2.5 Hz, 1Hc), 3.24 (bs, 1Hb), 3.22-3.17 (m, 1Ha), 3.18-3.10 (m, 1Hc), 3.08 (bs, 1Hc), 3.06-2.99 (m, 1Ha), 2.88 (q, *J* = 3.3, 2.5 Hz, 1Hb), 2.56 (d, *J* = 3.0 Hz, 1Hb), 2.45-2.35 (m, 1Ha+1Hc), 2.02 (ddd, *J* = 7.1, 4.5, 2.1 Hz, 1Hb), 1.97-1.88 (m, 1Ha), 1.68 (dd, *J* = 5.0, 2.0 Hz, 1Hc), 1.44 (s, 3Ha+3Hb+3Hc), 1.40 (s, 3Ha+3Hb+3Hc), 1.36 (m, 1Ha+1Hb+1Hc), 1.35-1.19 (m, 3Ha+3Hb+3Hc), -0.02--0.16 (m, 9Ha+9Hb+ 9Hc). ^{1}H NMR (400 MHz, $CDCl_3$) fraction 2 (2 diastereomers): δ (ppm) 6.20 (ddd, *J* = 8.1, 5.5, 3.2 Hz, 1Ha+1Hb), 5.97-5.92 (m, 1Ha), 5.90 (dd, *J* = 5.6, 2.8 Hz, 1Hb), 4.14-4.04 (m, 3Ha+3Hb), 4.04-3.93 (m, 1Ha+1Hb), 3.80-3.68 (m, 1Ha+1Hb), 3.28-3.23 (m, 1Hb), 3.21 (tdq, *J* = 2.7, 1.8, 1.0 Hz, 1Ha), 3.02 (t, *J* = 2.1 Hz, 1Ha), 2.89 (t, *J* = 4.0 Hz, 1Hb), 2.60-2.55 (m, 1Hb), 2.41 (dd, *J* = 4.7, 3.5 Hz, 1Ha), 2.03 (dd, *J* = 7.9, 4.4 Hz, 1Hb), 1.91 (dd, *J* = 8.9, 4.8 Hz, 1Ha), 1.44 (s, 3Ha+3Hb), 1.39 (bs, 3Ha+4Hb), 1.36-1.33 (m, 1Ha), 1.24 (t, *J* = 7.1 Hz, 3Ha+3Hb), -0.04--0.09 (m, 9Ha+9Hb). ^{13}C NMR (100 MHz,$CDCl_3$) fraction 1: δ (ppm) 175.40, 175.06, 173.58, 137.92, 137.64, 136.01, 135.35, 133.44, 133.41, 109.05, 108.94, 108.81, 79.64, 79.44, 79.21, 69.03, 68.70, 68.63, 60.55, 60.35, 60.14, 50.72, 50.46, 49.82, 49.73, 49.20, 48.99, 48.95, 48.72, 48.66, 48.60, 48.45, 48.22, 48.09, 47.53, 47.50,

47.19, 26.96, 26.79, 26.72, 25.94, 25.88, 25.72, 14.27, 14.23, 14.20, -0.18, -0.23. ^{13}C NMR (100 MHz,$CDCl_3$) fraction 2: δ (ppm) 173.96, 173.63, 144.61, 137.94, 133.45, 122.42, 109.09, 108.97, 79.66, 79.46, 69.04, 68.64, 60.39, 60.17, 49.84, 49.23, 49.01, 48.73, 48.67, 48.62, 48.47, 48.12, 47.55, 47.52, 26.80, 26.73, 25.95, 25.73, 14.28, 14.24, -0.18, -0.23. LC/MS analysis: fraction 1: R_t 7.7, 7.8 and 8.3 min (linear gradient 10 → 90% B in 15 min), m/z 338.9 $[M+H]^+$, 299.0 [M-acetonide+H]$^+$; fraction 2: R_t 7.8 and 8.0 min (linear gradient 10 → 90% B in 15 min), m/z 338.8 $[M+H]^+$, 299.0 [M-acetonide+H]$^+$.

(1*R*,2*S*,3*S*,4*S*,7*R*)-diethyl 7-(trimethylsilyl)bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylate and (1*R*,2*R*,3*R*,4*S*,7*R*)-diethyl 7-(trimethylsilyl)bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylate (72)

Diethyl fumarate (**71**) (10 mmol, 1.7 mL) was dissolved in toluene (10 mL) and cooled to -78 °C under argon atmosphere, before Et_2AlCl (10 mmol, 5.6 mL 1.8 M in toluene, 1.0 eq.) was added. After stirring for 15 min at -78 °C, cyclopenta-2,4-dien-1-yltrimethylsilane (**67**) (20 mmol, 3.3 mL, 2.0 eq.) was added dropwise and the reaction mixture was stirred overnight while allowing the temperature to slowly rise to room temperature. The reaction was then quenched with saturated aqueous $NaHCO_3$, the aqueous layer was extracted with Et_2O (3x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 2% EtOAc in PetEt) gave Diels-Alder adduct **72** (3.1 g, 10 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 6.16 (dd, *J* = 5.7, 3.1 Hz, 1H), 5.94 (dd, *J* = 5.6, 2.8 Hz, 1H), 4.23-4.09 (m, 2H), 4.09-4.03 (m, 2H), 3.34 (t, *J* = 3.9 Hz, 1H), 3.31-3.26 (m, 1H), 3.13 (d, *J* = 3.1 Hz, 1H), 2.71-2.66 (m, 1H), 1.32-1.14 (m, 7H), -0.07--0.17 (m, 9H). ^{13}C NMR (100 MHz,$CDCl_3$): δ (ppm) 174.32, 172.93, 136.95, 134.32, 60.54, 60.26, 50.61, 49.95, 49.17, 48.89, 48.45, 14.10, -0.50.

(1*S*,2*S*,3*S*,7*S*,8*S*,9*S*)-ethyl 2-bromo-5-oxo-8-(trimethylsilyl)-4-oxatricyclo[4.2.1.03,7]nonane-9-carboxylate and (1*R*,2*R*,3*R*,7*R*,8*R*,9*R*)-ethyl 2-bromo-5-oxo-8-(trimethylsilyl)-4-oxatricyclo[4.2.1.03,7]nonane-9-carboxylate (73)

Compound **72** (25 mmol, 7.8 g) was dissolved in DCM under argon atmosphere and cooled to 0 °C. Bromine (50 mmol, 2.6 mL, 2.0 eq.) was added and the reaction mixture was stirred at 0 °C for 40 min and then at room temperature for 4 hrs, before being quenched with saturated aqueous $NaHCO_3$. The aqueous layer was extracted with DCM (3x) and the combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 8% EtOAc in PetEt) gave bromo-functionalized compound **73** (7.5 g, 21 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 4.92 (d, *J* = 4.7 Hz, 1H), 4.26-4.11 (m, 2H), 3.78 (d, *J* = 2.1 Hz, 1H), 3.27 (t, *J* = 4.6 Hz, 1H), 3.11-3.01 (m, 2H), 2.79 (d, *J* = 2.1 Hz, 1H), 1.35-1.30 (m, 1H), 1.26 (t, *J* = 7.2 Hz, 3H), 0.19 (s, 9H). ^{13}C NMR (100 MHz,$CDCl_3$): δ (ppm) 177.34, 170.38, 88.02, 61.86, 53.12, 52.50, 52.32, 49.04, 42.45, 36.98, 14.17, 0.32.

(1*R*,2*S*,3*S*,4*S*,7*R*)-2-ethyl 3-methyl 7-(hydroxy)bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylate and (1*R*,2*R*,3*R*,4*S*,7*S*)-2-methyl 3-ethyl 7-(hydroxy)bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylate (74)

To a solution of bromo-functionalized compound **73** (1.0 mmol, 0.36 g) in MeOH (10 mL) under argon atmosphere was added $AgNO_3$ (5.0 mmol, 0.84 g, 5.0 eq.) and the reaction mixture was stirred at 70 °C for 3.5 hrs. The mixture was then filtered, concentrated *in vacuo* and redissolved in EtOAc. After addition of H_2O, the layers were separated and the aqueous layer was extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (10% EtOAc in PetEt → 50% EtOAc in PetEt) to yield alcohol **74** (0.16 g, 0.68 mmol, 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 6.23 (dt, *J* = 4.5, 2.3 Hz, 1H), 5.95 (dd, *J* = 6.3, 3.2 Hz, 1H), 4.15 (qd, *J* = 7.1, 2.4 Hz, 2H), 4.03 (bs, 1H), 3.81-3.72 (m, 4H), 3.58 (t, *J* = 4.5 Hz, 1H), 3.13 (td, *J* = 3.4, 1.6 Hz, 1H), 3.11-3.03 (m, 1H), 2.82 (d, *J* = 5.0 Hz, 1H), 1.27 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz,$CDCl_3$): δ (ppm) 175.87, 173.81, 135.63, 132.73, 83.80, 60.82, 52.27, 50.38, 49.79, 45.89, 45.51, 14.08. LC/MS analysis: R_t 5.7 min (linear gradient 10 → 90% B in 15 min), m/z 240.9 $[M+H]^+$.

(1*R*,2*S*,3*S*,4*S*,7*R*)-2-ethyl 7-(hydroxy)bicyclo[2.2.1]hept-5-ene-2-carboxylate-3-carboxylic acid and (1*R*,2*R*,3*R*,4*S*, 7*S*)-3-ethyl 7-(hydroxy)bicyclo[2.2.1]hept-5-ene-3-carboxylate-2-carboxylic acid (75)

Diester **74** (1.2 mmol, 0.29 g) and Me_3SnOH (1.4 mmol, 0.46 g, 1.2 eq.) were dissolved in toluene and stirred at 100 °C for 5 hrs. The mixture was then cooled to room temperature, washed with 1 M HCl and the aqueous layer was extracted with EtOAc (6x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by column chromatography (PetEt → 50% EtOAc in PetEt) to yield monoester **75** (0.23 g, 1.0 mmol, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.44 (bs, 1H), 6.21-6.10 (m, 1H), 5.94-5.86 (m, 1H), 4.09 (q, *J* = 7.2 Hz, 2H), 3.74 (t, *J* = 1.9 Hz, 1H), 3.59 (t, *J* = 4.3 Hz, 1H), 3.10 (dd, *J* = 3.7, 1.8 Hz, 1H), 3.03 (td, *J* = 3.6, 1.8 Hz, 1H), 2.78 (d, *J* = 4.7 Hz, 1H), 1.21 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz,$CDCl_3$): δ (ppm) 179.30, 174.00, 135.63, 132.97, 83.61, 60.96, 50.06, 50.00, 45.98, 45.47, 14.20.

(1*R*,2*S*,3*S*,4*S*,7*R*)-2-ethyl 7-((tert-butyl-dimethyl-silyl)hydroxy)bicyclo[2.2.1]hept-5-ene-2-carboxylate-3-carboxylic acid and (1*R*,2*R*,3*R*,4*S*,7*S*)-3-ethyl 7-((tert-butyl-dimethyl-silyl)hydroxy)bicyclo[2.2.1]hept-5-ene-3-carboxylate-2-carboxylic acid (76)

Compound **75** (0.53 mmol, 0.12 g) was dissolved in DMF under argon atmosphere, after which imidazole (0.80 mmol, 54 mg, 1.5 eq.) and TBDMS-Cl (0.66 mmol, 99 mg, 1.3 eq.) were added. The reaction mixture was stirred at room temperature overnight, diluted with H_2O and extracted with EtOAc (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 2% EtOAc in PetEt → 50% EtOAc in PetEt) gave monosilylated compound **76** (50 mg, 0.15 mmol, 28%) as well as disilylated byproduct (0.10 g, 0.22 mmol, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) **76**: δ (ppm) 6.18 (ddd, *J* = 6.3, 3.6, 1.1 Hz, 1H), 5.92 (ddd, *J* = 6.3, 3.2, 1.0 Hz, 1H), 4.18-4.03 (m, 2H), 3.73-3.64 (m, 2H), 3.12 (dd, *J* = 3.6, 1.8 Hz, 1H), 2.93 (td, *J* = 3.5, 1.6 Hz, 1H), 2.80 (d, *J* = 4.4 Hz, 1H), 1.24 (t, *J* = 7.1 Hz, 3H), 0.83 (s, 9H), 0.04 (s, 3H), 0.01 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) **76**: δ (ppm) 179.54, 174.04, 135.29, 132.87, 84.30, 60.62, 50.72, 50.55, 46.31, 44.89, 25.50, 14.26, -5.11, -5.30. ^{1}H NMR (400 MHz, $CDCl_3$) disilylated product: δ (ppm) 6.23-6.15 (m, 1H), 5.88-5.83 (m, 1H), 4.09 (dd, *J* = 7.1, 2.7 Hz, 2H), 3.67-3.57 (m, 2H), 3.03 (dd, *J* = 3.7, 1.8 Hz, 1H), 2.88 (dt, *J* = 3.8, 1.8 Hz, 1H), 2.73 (d, *J* = 5.1 Hz, 1H), 1.21 (t, *J* = 7.1 Hz, 3H), 0.89 (s, 9H), 0.84 (s, 9H), 0.24 (s, 3H), 0.23 (s, 3H), 0.03 (s, 3H), 0.01 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) disilylated product: δ (ppm) 174.33, 172.90, 135.85, 132.21, 84.52, 60.42, 50.99, 50.11, 48.10, 44.72, 25.80, 25.44, 18.08, 17.53, 14.26, -4.69, -4.80, -5.00, -5.02. LC/MS analysis: **76**: R_t 10 min (linear gradient 10 → 90% B in 15 min), m/z 340.9 $[M+H]^+$; disilylated product: R_t 14 min (linear gradient 10 → 90% B in 15 min), m/z 455.0 $[M+H]^+$.

(1*R*,2*S*,3*S*,4*S*,7*R*)-2-ethyl 7-((trityl)hydroxy)bicyclo[2.2.1]hept-5-ene-2-carboxylate-3-carboxylic acid and (1*R*,2*R*, 3*R*,4*S*,7*S*)-3-ethyl 7-((trityl)hydroxy)bicyclo[2.2.1]hept-5-ene-3-carboxylate-2-carboxylic acid (77)

Compound **75** (0.44 mmol, 0.10 g) was dissolved in pyridine under argon atmosphere, after which TrCl (0.88 mmol, 0.24 g, 2.0 eq.) and DMAP (88 µmol, 11 mg, 0.2 eq.) were added. The reaction mixture was stirred at 60 °C for 6 hrs, allowed to cool temperature and quenched with MeOH. Next, H_2O was added and the mixture was extracted with EtOAc (5x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. After purification by column chromatography (PetEt/EtOAc 1/0 → 6/4) a mixture of product **77** and starting material **75** (ratio 3:7) was obtained that was not further purified. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.45-7.39 (m, 6H **77**), 7.32-7.20 (m, 9H **77**), 6.21 (dd, *J* = 6.2, 3.5 Hz, 1H **75**), 6.05 (dd, *J* = 6.2, 3.6 Hz, 1H **77**), 5.95 (dd, *J* = 6.1, 3.2 Hz, 1H **75**), 5.73 (dt, *J* = 6.2, 3.0 Hz, 1H **77**), 4.18-4.09 (m, 2H **75** + 2H **77**), 3.83-3.72 (m, 1H **75** + 1H **77**), 3.71-3.66 (m, 1H **77**), 3.65-3.61 (m, 1H **75**), 3.57-3.52 (m, 1H **77**), 3.15-3.05 (m, 2H **75**), 2.84 (d, *J* = 4.8 Hz, 1H **75**), 2.77 (d, *J* = 4.9 Hz, 1H **77**), 2.69 (d, J = 3.5 Hz, 1H **77**), 1.30-1.21 (m, 3H **75** + 3H **77**).

(1*R*,2*S*,3*S*,4*S*,7*R*)-2-ethyl 7-((acetyl)hydroxy)bicyclo[2.2.1]hept-5-ene-2-carboxylate-3-carboxylic acid and (1*R*,2*R*, 3*R*,4*S*,7*S*)-3-ethyl 7-((acetyl)hydroxy)bicyclo[2.2.1]hept-5-ene-3-carboxylate-2-carboxylic acid (78)

Compound **75** (0.20 mmol, 45 mg) was dissolved in pyridine under argon atmosphere, after which Ac_2O (0.40 mmol, 40 µL, 2.0 eq.) and DMAP (20 µmol, 2.4 mg, 0.1 eq.) were added. The reaction mixture was stirred at 40 °C for 1.5 hrs and then allowed to cool temperature. Next, H_2O was added and the mixture was extracted with DCM (3x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (PetEt → 25% EtOAc in PetEt) gave acetylated product **78** (35 mg, 0.13 mmol, 65%).^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 6.28 (ddd, *J* = 6.2, 3.6, 1.1 Hz, 1H), 6.02 (ddd, *J* = 6.2, 3.3, 0.9 Hz, 1H), 4.54 (t, *J* = 2.0 Hz, 1H), 4.16 (q, *J* = 7.2 Hz, 2H), 3.63 (t, *J* = 4.3 Hz, 1H), 3.40 (dt, *J* = 4.5, 1.5 Hz, 1H), 3.27 (dq, *J* = 4.5, 1.6 Hz, 1H), 2.94 (d, *J* = 4.6 Hz, 1H), 1.96 (s, 3H), 1.27 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 178.53, 172.89, 169.91, 135.21, 132.76, 83.20, 60.99, 47.63, 47.45, 46.01, 45.03, 20.70, 14.19.

Cyclopenta-2,4-dien-1-yl-dimethylphenylsilane (82)

Freshly distilled cyclopentadiene (20 mmol, 1.7 mL) was dissolved in THF under argon atmosphere and cooled to -78 °C, before *n*-BuLi (20 mmol, 13 mL 1.6 M in hexanes, 1.0 eq.) was added. The reaction mixture was stirred at -78 °C for 20 min, then at 0 °C for 40 min and then cooled back to -78 °C. $Me_2PhSiCl$ (20 mmol, 3.5 mL, 1.0 eq.) was added and the reaction mixture was stirred overnight while the temperature was allowed to rise to room temperature. After concentrating the solvents *in vacuo*, H_2O was added and the mixture was extracted with Et_2O (5x). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Half of the crude product (**82**) was then purified by column chromatography (pentane) and used directly for the next step (0.2 g, 1.0 mmol, 10%).

Dimethyl 7-(dimethylphenylsilyl)bicyclo[2.2.1]hept-5-ene-3-(2-oxoacetylate)-2-carboxylate and dimethyl 7-(dimethylphenylsilyl)bicyclo[2.2.1]hept-5-ene-2-(2-oxoacetylate)-3-carboxylate (83)

Diester **81**[55] (0.5 mmol, 86 mg) and silane **82** (1.0 mmol, 0.2 g, 2.0 eq.) were dissolved in toluene and stirred at room temperature for 1 hr, before being concentrated *in vacuo*. Purification by column chromatography (pentane → 10% EtOAc in pentane) gave Diels-Alder adduct **83** (0.15 g, 0.40 mmol, 80%) as a mixture of 2 isomers (ratio 3:2). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.60-7.23 (m, 5Ha + 5Hb), 6.32-6.24 (m, 1Hb), 6.18 (t, *J* = 4.2 Hz, 1Ha), 6.11-6.03 (m, 1Hb), 5.93-5.79 (m, 1Ha), 4.05 (t, *J* = 4.2 Hz, 1Ha), 3.96-3.80 (m, 3Ha + 3Hb), 3.73 (s, 3Ha), 3.66 (s, 3Hb), 3.58-3.45 (m, 1Ha + 2Hb), 3.44-3.30 (m, 1Hb), 3.32-3.22 (m, 1Ha + 1Hb), 2.83 (d, *J* = 4.5 Hz, 1Ha), 1.77-1.67 (m, 1Ha), 1.44-1.34 (m, 1Hb), 0.32-0.19 (m, 6Ha + 6Hb). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 193.48, 192.40, 174.43, 173.07, 161.67, 160.58, 138.85, 138.70, 137.60, 136.85, 135.53, 133.98, 133.63, 133.62, 128.93, 127.77, 55.44, 53.66, 53.12, 53.01, 52.19, 51.89, 51.02, 50.63, 49.55, 49.46, 48.87, 48.25, 47.75, 47.12, -1.36, -1.47.

B. Biochemistry

General

HEK-293T cells were cultured in DMEM supplemented with 10% (v/v) FCS, 10 units/mL penicillin and 10 µg/mL streptomycin in a 5% CO_2 humidified incubator at 37 °C. Cells were harvested, washed with PBS (2x) and lysed in digitonin lysis buffer (50 mM Tris pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, 0.025% digitonin) for 30 min on ice. After centrifugation of the cells at 16,100 g for 20 min at 4 °C, the supernatants were collected and the protein concentration was determined by Bradford assay. Fibroblasts were obtained with consent from control donors and cultured in DMEM/F-12 medium (Invitrogen) supplied with 10% (v/v) FBS in an incubator at 5% CO_2 and 37 °C. Precipitation of proteins was done using a chloroform/methanol (c/m) precipitation protocol.[56] Western blotting: the proteins were transferred from an SDS-PAGE gel onto a PVDF membrane using a Bio-Rad

Trans-Blot cell system. Membranes were blocked with 1% BSA in TBS-t(+) (0.1% Tween 20) overnight at room temperature, hybridized with Streptavidin-HRP for 1 hr at room temperature (1:10,000 in blocking buffer) (Molecular Probes, Life Technologies), washed with TBS-t(+) and TBS and then visualized using an ECL+ Western blotting detection kit (Amersham Biosciences).

***In vitro* two-step labeling with biotin-cleavable linker-tetrazine 11**

HEK cell lysates (20 µg total protein per experiment) in digitonin lysis buffer (9 µL) were exposed to 1 µM of ABP **19** (1 µL 10 µM in DMSO) for 1 hr at 37 °C in the presence or absence of 100 µM epoxomicin (1 µL 1 mM in DMSO). Next, the indicated concentrations of tetrazine **11**(1.1 µL 10x solution in DMSO) were added and the mixtures were again incubated for 1 hr at 37 °C. After quenching by c/m precipitation, proteins were taken up in 15 µL Laemmli's sample buffer containing 2-mercaptoethanol, boiled for 5 min at 100 °C and resolved on 12.5% SDS-PAGE. Biotinylated proteins were detected by streptavidin Western blotting.

Hydrazine cleavage. After boiling in sample buffer, the samples were treated with 10, 50 or 100 mM hydrazine (5 µL 4x solution in H_2O) and incubated at room temperature overnight. The samples were then again boiled for 5 min at 100 °C and resolved on 12.5% SDS-PAGE. Biotinylated proteins were detected by streptavidin Western blotting.

***In situ* inhibition of α-galactosidase A activity**

Cultured WT fibroblasts were grown to confluency in 12-wells plates and incubated with 0.01 nM - 10 µM aziridine **20** or **21** in medium for 2 hrs at 37 °C. The cells were washed with PBS and lysed by scraping in 25 mM potassium phosphate buffer (pH 6.5, 0.1% (v/v) Triton X-100) with protease inhibitor cocktail (Roche). The protein concentration was determined by BCA assay. The lysates (25 µL) were then mixed with 100 µL substrate mix (1.5 mg/mL 4-methylumbelliferyl-α-D-galactopyranoside in McIlvaine pH 4.6 + 0.1% BSA). After incubation for 1 hr at 37 °C, the reaction was quenched with 2.5 mL 0.3 M glycine, pH 10.6 and fluorescence was measured with a fluorimeter LS55 (Perkin Elmer) using λ_{ex} 366 nm and λ_{em} 445 nm. All samples were corrected for background fluorescence (sample without enzyme) and residual enzyme activity was calculated as compared to control samples from cells incubated without inhibitors. Displayed values represent mean values from duplicate experiments and error bars indicate standard deviation (SD).

References

1. Ovaa, H.; van Swieten, P. F.; Kessler, B. M.; Leeuwenburgh, M. A.; Fiebiger, E.; van den Nieuwendijk, A. M. C. H.; Galardy, P. J.; van der Marel, G. A.; Ploegh, H. L.; Overkleeft, H. S. *Angew. Chem. Int. Ed.* **2003**, *42*, 3626.
2. Devaraj, N. K.; Hilderbrand, S.; Upadhyay, R.; Mazitschek, R.; Weissleder, R. *Angew. Chem. Int. Ed.* **2010**, *49*, 2869
3. Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430*, 873.
4. Rossin, R.; Renart Verkerk, P.; van den Bosch, S. M.; Vulders, R. C. M.; Verel, I.; Lub, J.; Robillard, M. S. *Angew. Chem. Int. Ed.* **2010**, *49*, 3375.
5. Soriano del Amo, D.; Wang, W.; Jiang, H.; Besanceney, C.; Yan, A.; Levy, M.; Liu, Y.; Marlow, F. L.; Wu, P. *J. Am. Chem. Soc.* **2010**, *132*, 16893.
6. Hong, V.; Steinmetz, N. F.; Manchester, M.; Finn, M. G. *Bioconjug. Chem.* **2010**, *21*, 1912.
7. Späte, A.-K.; Busskamp, H.; Niederwieser, A.; Schart, V. F.; Marx, A.; Wittmann, V. *Bioconjugate Chem.* **2014**, *25*, 147.
8. Cole, C. M.; Yang, J.; Šečkutė, J.; Devaraj, N. K. *Chembiochem* **2013**, *14*, 205.
9. Niederwieser, A.; Späte, A.-K.; Nguyen, L. D.; Jüngst, C.; Reutter, W.; Wittmann, V. *Angew. Chem. Int. Ed.* **2013**, *52*, 4265.

10. Vocadlo, D. J.; Hang, H. C.; Kim, E. J.; Hanover, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 9116.
11. Homann, A.; Qamar, R. U.; Serim, S.; Dersch, P.; Seibel, J. *Bellstein J. Org. Chem.* **2010**, *6*, doi:10.3762/bjoc.6.24.
12. Kamber, D. N.; Nazarova, L. A.; Liang, Y.; Lopez, S. A.; Patterson, D. M.; Shih, H.-W.; Houk, K. N.; Prescher, J. A. *J. Am. Chem. Soc.* **2013**, *135*, 13680.
13. Geurink, P. P.; Florea, B. I.; Li, N.; Witte, M. D.; Verasdonck, J.; Kuo, C.-L.; van der Marel, G. A.; Overkleeft, H. S. *Angew. Chem. Int. Ed.* **2010**, *49*, 6802.
14. Willems, L. I.; Verdoes, M.; Florea, B. I.; van der Marel, G. A.; Overkleeft, H. S. *ChemBioChem* **2010**, *11*, 1769.
15. Gieselmann, V. *Biochim. Biophys. Acta* **1995**, *1270*, 103.
16. Witte, M. D.; Kallemeijn, W. W.; Aten, J.; Li, K.-Y.; Strijland, A.; Donker-Koopman, W. E.; van den Nieuwendijk, A. M. C. H.; Bleijlevens, B.; Kramer, G.; Florea, B. I.; Hooibrink, B.; Hollak, C. E. M.; Ottenhof R.; Boot, R. G.; van der Marel, G. A.; Overkleeft, H. S.; Aerts, J. M. F. G. *Nat. Chem. Biol.* **2010**, *6*, 907.
17. Stubbs, K. A. *Carbohydr. Res.* **2014**, *390*, 9.
18. Ferraz, M. J.; Kallemeijn, W. W.; Mirzaian, M.; Herrera Moro, D.; Marques, A.; Wisse, P.; Boot, R. G.; Willems, L. I.; Overkleeft, H. S.; Aerts, J. M. *Biochim. Biophys. Acta* **2014**, *1841*, 811.
19. Gold, H.; Mirzaian, M.; Dekker, N.; Ferraz, M. J.; Lugtenburg, J.; Codée, J. D. C.; van der Marel, G. A.; Overkleeft, H. S.; Linthorst, G. E.; Groener, J. E. M.; Aerts, J. M.; Poorthuis, B. J. H. M. *Clin. Chem.* **2013**, *59*, 547.
20. Carlson, J. C. T.; Meimetis, L. G.; Hilderbrand, S. C.; Weissleder, R. *Angew. Chem. Int. Ed.* **2013**, *52*, 6917.
21. Kallemeijn, W. W.; Li, K.-Y.; Witte, M. D.; Marques, A. R. A.; Aten, J.; Scheij, S.; Jiang, J.; Willems, L. I.; Voorn-Brouwer, T. M.; van Roomen, C. P. A. A.; Ottenhoff, R.; Boot, R. G.; van den Elst, H.; Walvoort, M. T. C.; Florea, B. I.; Codée, J. D. C.; van der Marel, G. A.; Aerts, J. M. F. G.; Overkleeft, H. S. *Angew. Chem. Int. Ed.* **2012**, *51*, 12529.
22. Sweeney, J. B. *Chem. Soc. Rev.* **2002**, *31*, 247.
23. Serrano, P.; Llebaria, A.; Delgado, A. *J. Org. Chem.* **2005**, *70*, 7829.
24. Palakurthy, N. B.; Mandal, B. *Tetrahedron Lett.* **2011**, *52*, 7132.
25. Jat, J. L.; Paudyal, M. P.; Gao, H.; Xu, Q.-L.; Yousufuddin, M.; Devarajan, D.; Ess, D. H.; Kürti, L.; Falck, J. R. *Science* **2014**, *343*, 61.
26. Bercier, A.; Plantier-Royon, R.; Portella, C. *Carbohydr. Chem.* **2007**, *342*, 2450.
27. Win-Mason, A. L.; Jongkees, S. A. K.; Withers, S. G.; Tyler, P. C.; Timmer, M. S. M.; Stocker, B. L. *J. Org. Chem.* **2011**, *76*, 9611.
28. Janda, K. D.; Lo, L. C.; Lo, C. H.; Sim, M. M.; Wang, R.; Wong, C. H.; Lerner, R. A. *Science* **1997**, *275*, 945.
29. Kurogochi, M.; Nishimura, S.-I.; Lee, Y. C. *J. Biol. Chem.* **2004**, *279*, 44704.
30. Kwan, D. H.; Chen, H.-M.; Ratananikom, K.; Hancock, S. M.; Watanabe, Y.; Kongsaeree, P. T.; Samuels, A. L.; Withers, S. G. *Angew. Chem. Int. Ed.* **2011**, *50*, 300.
31. Komatsu, T.; Kikuchi, K.; Takakusa, H.; Hanaoka, K.; Ueno, T.; Kamiya, M.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2006**, *128*, 15946.
32. Geurink, P. P.; Prely, L. M.; van der Marel, G. A.; Bischoff, R.; Overkleeft, H. S. *Top. Curr. Chem.* **2012**, *324*, 85.
33. Sumranjit, J.; Chung, S. J. *Molecules* **2013**, *18*, 10425.
34. Loenarz, C.; Schofield, C. J. *Nat. Chem. Biol.* **2008**, *4*, 152.
35. Hausinger, R. P. *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 21.
36. Rose, N. R.; McDonough, M. A.; King, O. N. F.; Kawamura, A.; Schofield, C. J. *Chem. Soc. Rev.* **2011**, *40*, 4364.
37. Hamada, S.; Kim, T. D.; Suzuki, T.; Itoh, Y.; Tsumoto, H.; Nakagawa, H.; Janknecht, R.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2852.
38. Rose, N. R.; Woon, E. C. Y.; Kingham, G. L.; King, O. N. F.; Mecinovic, J.; Clifton, I. J.; Talib-Hardy, J.; Oppermann, U.; McDonough, M. A.; Schofield, C. J. *J. Med. Chem.* **2010**, *53*, 1810.

39. Hamada, S.; Suzuki, T.; Mino, K.; Koseki, K.; Oehme, F.; Flamme, I.; Ozasa, H.; Itoh, Y.; Ogasawara, D.; Komaarashi, H.; Kato, A.; Tsumoto, H.; Nakagawa, H.; Hasegawa, M.; Sasaki, R.; Mizukami, T.; Miyata, N. *J. Med. Chem.* **2010**, *53*, 5629.
40. Tschank, G.; Brocks, D. G.; Engelbart, K.; Mohr, J.; Baader, E.; Guenzler, V.; Hanauske-Abel, H. M. *Biochem. J.* **1991**, *275*, 469.
41. Rotili, D.; Altun, M.; Kawamura, A.; Wolf, A.; Fischer, R.; Leung, I. K. H.; Mackeen, M. M.; Tian, Y.-m.; Ratcliffe, P. J.; Mai, A.; Kessler, B. M.; Schofield, C. J. *Chem. Biol.* **2011**, *18*, 642.
42. Geurink, P. P.; Klein, T.; Prely, L.; Paal, K.; Leeuwenburgh, M. A.; van der Marel, G. A.; Kauffman, H. F.; Overkleeft, H. S.; Bischoff, R. *Eur. J. Org. Chem.* **2010**, 2100.
43. Breder, A.; Chinigo, G. M.; Waltman, A. W.; Carreira, E. M. *Angew. Chem. Int. Ed.* **2008**, *47*, 8514.
44. Kresze, G.; Schulz, G.; Walz, H. *Liebigs Ann. Chem.* **1963**, *666*, 45.
45. Corey, E. J.; Weinshenker, N. M.; Schaaf, T. K.; Huber, W. *J. Am. Chem. Soc.* **1969**, *91*, 5675.
46. Kende, A. S.; Koch, K.; Smith ,C. A. *J. Am. Chem. Soc.* **1988**, *110*, 2210.
47. Cappon, J. J.; Baart, J.; van der Walle, G. A. M.; Raap, J.; Lugtenburg, J. *Recl. Trav. Chim. Pays-bas* **1991**, *110*, 158.
48. For reviews, see: a) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, *77*, 383; b) Fonovic, M.; Bogyo, M. *Expert Rev. Proteomics* **2008**, *5*, 721; c) Schmidinger, H.; Hermetter, A.; Birner-Gruenberger, R. *Amino Acids* **2006**, *30*, 333.
49. Lo, L. C.; Pang, T. L.; Kuo, C. H.; Chiang, Y. L.; Wang, H. Y.; Lin, J. J. *J. Proteome Res.* **2002**, *1*, 35.
50. Kumar, S.; Zhou, B.; Liang, F.; Wang, W. Q.; Huang, Z.; Zhang, Z. Y. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7943.
51. Walls, C.; Zhou, B.; Zhang, Z. Y. *Methods Mol. Biol.* **2009**, *519*, 417.
52. Wright, A. T.; Song, J. D.; Cravatt, B. F. *J. Am. Chem. Soc.* **2009**, *131*, 10692.
53. Krysiak, J. M.; Kreuzer, J.; Macheroux, P.; Hermetter, A.; Sieber, S. A.; Breinbauer, R. *Angew. Chem. Int. Ed.* **2012**, *51*, 7035.
54. For reviews, see: a) S Stubbs, K. A. *Carbohydr. Res.* **2014**, *390*, 9; b) Witte, M. D.; van der Marel, G. A.; Aerts, J. M. F. G.; Overkleeft, H. S. *Org. Biomol. Chem.* **2011**, *9*, 5908.
55. Corey, E. J.; Tramontano, A. *J. Am. Chem. Soc.* **1981**, *103*, 5599.
56. Wessel, D.; Flügge, U. I. *Anal. Biochem.* **1984**, *138*, 141.