Review

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# Activity-Based Protein Profiling of Oxidases and Reductases

Leo Krammer[a] and Rolf Breinbauer*[a, b]

*Dedicated to Prof. Benjamin Cravatt on the occasion of being awarded the Wolf Prize in Chemistry.*

**Abstract:** Activity-based protein profiling (ABPP) as a proteomic tool allows measuring the activity of enzyme classes or distinct proteins in their cellular context, annotating the function of uncharacterized proteins, and investigating the target profile of small molecule inhibitors. While hydrolases and other enzyme classes, which exhibit a characteristic nucleophilic residue, can be addressed by electrophilic activity-based probes, the large enzyme class of oxidoreductases has received much less attention in ABPP. In this review the state of the art of ABPP of oxidases is presented. The most frequent principle in ABPP for oxidases is the oxidative activation of the ABPP probe leading to an electrophilic group as exemplified for alkyne or amine based warheads. Alternatively, in rare cases ABPP probes can address characteristic electrophilic or nucleophilic residues of certain oxidases. Most recently, with NQO2 the first example of ABPP for a reductase has been reported.

**Keywords:** chemical proteomics · click chemistry · covalent inhibitors · drug discovery · oxidoreductases

## 1. Introduction

Over the last two decades powerful analytical tools have stimulated research in the life sciences enabling comprehensive analyses of an individual cell on the level of its genome,[1–2] protein expression profile,[3] and even of its proteome.[4–5] Progress in machine learning has made it possible that with the computer program Alpha Fold even the structure of proteins can be delineated from their amino acid sequence with great confidence.[6] However, neither gene sequence information nor quantification of protein abundance is sufficient to describe the status of the active proteome of a cell or living organism. It is even not possible to reliably predict the function of a protein from its three-dimensional structure. In order to address these limitations, activity-based protein profiling (ABPP) was introduced by Cravatt,[7] which aims to measure only the catalytically active proteins within certain enzyme classes. ABPP has become a very valuable tool in chemical proteomics,[8–13] for the investigation of the mode of action and selectivity profile of drugs[14–18] and natural products.[19–20] It has been used to determine the activity profile of proteins in fields ranging from microbiology[21–22] to plant sciences.[23] ABPP helps in the functional annotation of uncharacterized proteins[24] thereby complementing the putative annotation of proteins via sequence comparison, which implies the risk that historical functional assignment gets perpetuated but not validated.

Decisive element for the specificity of an activity-based probe is its warhead.[25–26] It typically contains an electrophilic group which in a subsequent step can be attacked by nucleophilic residues of the protein (class) of interest. Of special interests are warheads which by itself are not reactive enough but will be activated by the protein class it has been designed for and thereby transformed into a highly reactive (typically electrophilic) species, providing a higher selectivity than probes which attack nucleophilic amino acids due to their inherent electrophilicity, thereby better destined for the profiling of global proteome reactivity.[27–29] After covalent attachment of the ABPP probe to the protein, a click handle can then be ligated with various types of labels.[30] ABPP has shown its value for certain enzyme families, such as serine hydrolases, threonine hydrolases, cysteine hydrolases,[31–32] or glycosidases,[33] taking advantage of the characteristic nucleophilic group responsible for their catalytic function. There is a great need to extend the ABPP approach to other enzyme families.

Oxidoreductases (EC 1) comprise many functionally different enzymes involved in metabolic processes ranging from the synthesis and degradation of amino acids or fatty acids to the synthesis of secondary metabolites involved in hormone or pheromone signaling. More than other enzyme classes, oxidoreductases are relying on cofactors such as NADH, NADPH, flavins, PLP, heme, etc. in pursuing their catalytic function, making it more difficult – and sometimes even impossible – to annotate their function on a gene

[a] *MSc. L. Krammer, Prof. Dr. R. Breinbauer*
Institute of Organic Chemistry
Graz University of Technology
Stremayrgasse 9, A-8010 Graz, Austria
E-mail: breinbauer@tugraz.at
[b] *Prof. Dr. R. Breinbauer*
BIOTECHMED Graz
A-8010 Graz, Austria

sequence level as the encoded amino acids are not characteristic for their catalytic function. Therefore, a technique such as ABPP, which operates on the protein level, provides great opportunities to overcome these limitations and monitor individual oxidoreductases and to discover new ones. This review article describes the status quo of the ABPP of oxidoreductases providing an update to a previous review about this topic, which covered the literature until 2019.[34] In this update the literature is comprehensively covered until mid 2022. In the discussion we put emphasis on the most recent examples not covered in our previous review. We also include the first examples of ABPP of reductases, which have just recently been published.

## 2. ABBP of Oxidases by Oxidative Generation of Electrophilic Groups

The most common principle used in the ABPP of oxidases is the design of ABPP probes which by itself show little or no reactivity and through the oxidative action of an enzyme are converted to strongly electrophilic groups which are then attacked by generic nucleophilic residues of the enzyme under investigation (Scheme 1).

### 2.1 Cytochrome P450 Enzymes

#### *2.1.1 Alkyne Derived Warheads*

Cytochrome P450 enzymes are heme-dependent enzymes which catalyze a variety of chemical transformations, such as *C*-hydroxylations, epoxidations, heteroatom oxygenations, dealkylations, etc. These monooxygenases play an important role in the metabolism of drugs, xenobiotics and endogenous signaling compounds (e. g. eicosanoids).[35] The sequence homology within the 57 *CYP* genes encoded in the human genome is as low as 16%.[36] As many of these oxidases are strongly regulated by substrate binding or post-translational modifications, neither gene expression nor protein abundance reflects the active state of these oxidases. Starting from the known broad-spectrum mechanism-based P450-inhibitor 2-ethynylnaphthalene Wright and Cravatt developed in 2007 an ABPP strategy to characterize these proteins.[37] Activity-based probe **1** was developed by introducing a versatile alkyne handle, which allows selective tagging, detection, enrichment and identification of the protein-probe conjugate via Cu-catalyzed azide alkyne coupling.[38–39] The alkyne warhead[40] is first converted into a highly electrophilic ketene by a P450 oxidase, which then gets attacked by nucleophilic residues of the P450 enzyme leading to a covalent protein-probe adduct (Scheme 2). Probe **1** showed activity against P450 1A2, 3A11, 2C29 and 2D9/2D10 in mouse liver microsome, and could also be used for in vivo labelling of P450 enzymes in mice.

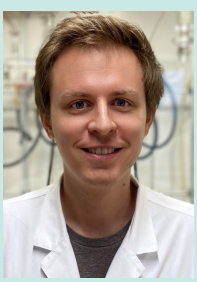
As P450 monooxygenases catalyze various oxidation reactions, the Cravatt group developed a panel of ABPP probes to study these functions.[41] Among these, the arylalkyne probe **2** (undergoing similar activation to ketene as probe **1**), showed a quite promiscuous behavior. The aliphatic alkyne **3**, which showed a strong preference for P450 2D6, accounts for a different type of P450 reactivity in which twofold hydroxylation in the allylic position should give rise to an alkynylketone, which would function as a strong Michael acceptor electrophile. It is known that the oral contraceptive 17-α-ethynylestradiol inactivates several human P450s (Scheme 2). The structurally derived probe **4** showed labelling of P450 1B1 and 3A4. With the panel of probes it was also possible to profile the inhibition profile of the known aromatase inhibitors, formestane and anastrozolole, indicating unanticipated drug-drug-interactions.

Wright used the panel of P450 ABPP probes **1**–**4** to investigate the effect of high-fat diet-induced obesity on the P450 activities in the liver and lungs of mice.[42] They observed increased activity of many P450s primarily in the postnatal lung.[43]

The group of Paine prepared compound **5** to investigate the interactome of the pyrethroid deltamethrin in rat liver. In **5** the cyano group of deltamethrin was replaced by an alkyne click handle and an alkyne warhead was placed in ortho-position of the phenylether group (Scheme 2).[44] Compound **5** selectively labeled the recombinant mosquito P450 CAP6M2.

#### *2.1.2 Heterocyclic Warheads*

Sellars et al. designed the benzofuran-derived probe **6** building on knowledge about the known CYP inhibitor furanocoumarin. A P450 enzyme could convert the substrate into an epoxy-



Leo Krammer was born in 1995 in Feldbach (Austria). He completed his undergraduate studies in Chemistry at the Graz University of Technology and the University of Graz in 2017. In 2020, he received his MSc in Chemistry from the University of Vienna (Austria). He then joined the group of Prof. Rolf Breinbauer at the Graz University of Technology for his PhD studies focused on the development of small-molecule probes for activity-based protein profiling.

Rolf Breinbauer was born in 1970 in Schärding (Austria). He studied Chemistry in Vienna and Heidelberg and received his PhD at the MPI für Kohlenforschung in Mülheim/Ruhr (Germany) with Prof. M. T. Reetz. After post-doctoral studies at Harvard University with Prof. E. N. Jacobsen, he started his independent career at the MPI of Molecular Physiology in Dortmund (Germany). Since 2007 he is full professor of Organic Chemistry at Graz University of Technology (Graz/Austria).

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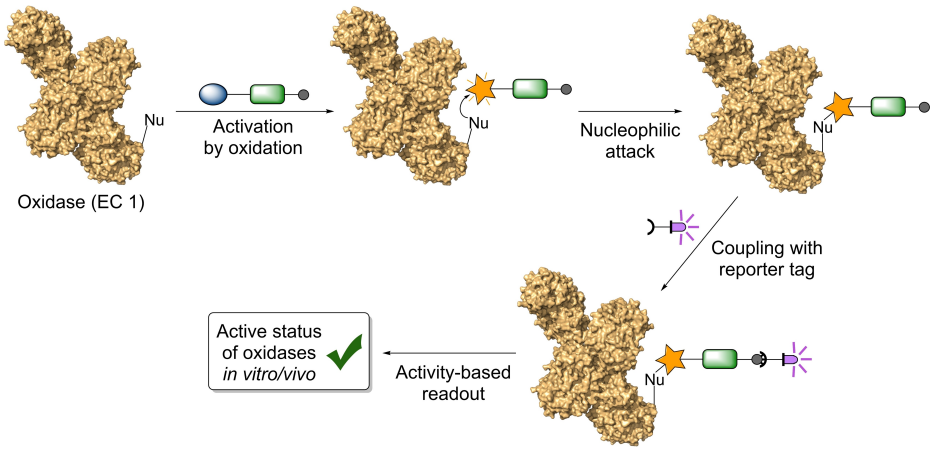
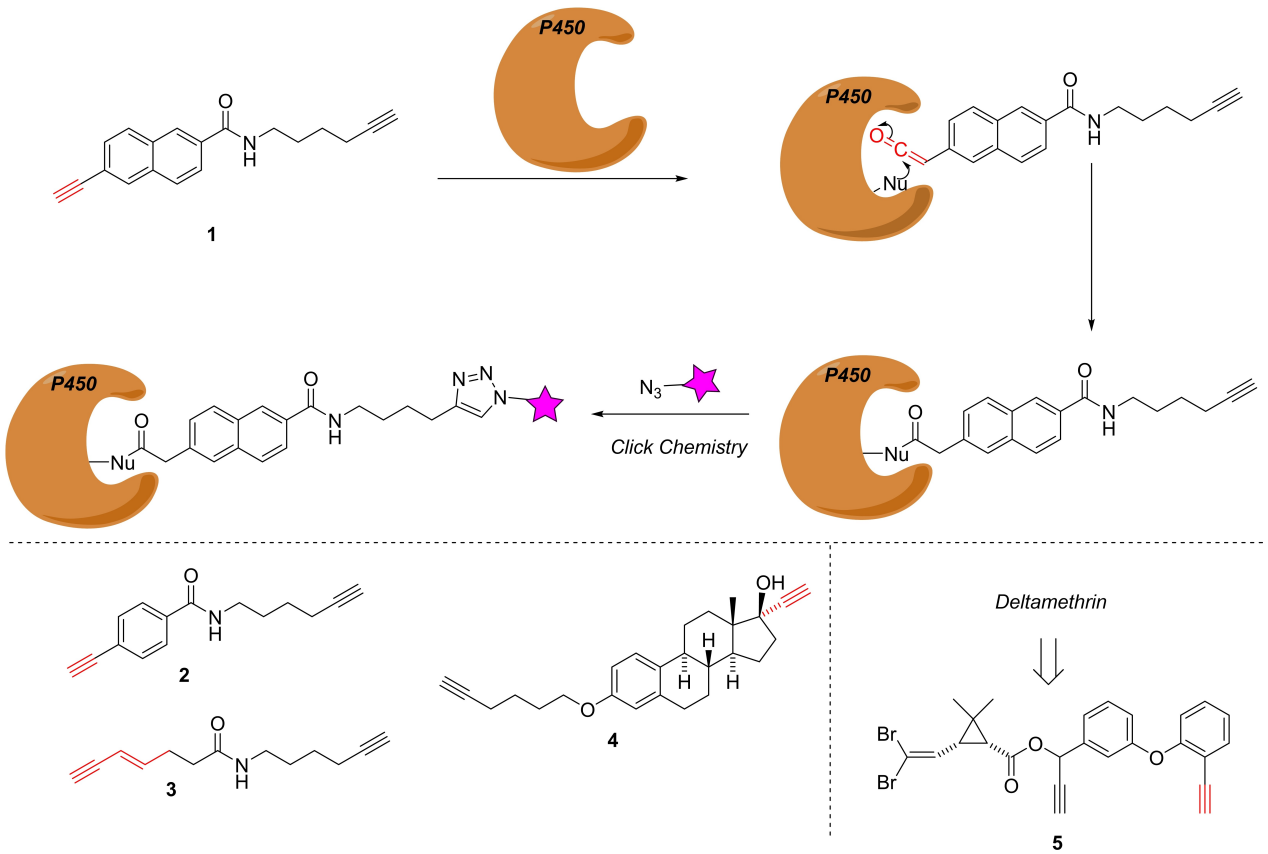



**Scheme 1.** Activation of ABPP probes via oxidative generation of electrophilic groups.




**Scheme 2.** Design of mechanism-based P450-probes. The alkyne warhead is depicted in red.

furane, which would be electrophilic enough to be attacked by protein nucleophiles (Figure 1).[45] Probe **6** exhibited the most effective NADPH-dependent binding against a panel of over-expressed CYP proteins to CYP3A4, which is also the most abundant P450 in the human body.

Tienilic acid (**7**) was used as a loop diuretic drug in the treatment of hypertension (Figure 1). After strong indications for its hepatotoxicity had emerged, it had to be withdrawn from the market in 1982. Mechanistic analyses revealed that by the action of P450 2C9 and 2C10 enzymes tienilic acid gets oxidized forming either a highly electrophilic thiophene sulfoxide[46] or via epoxidation and subsequent rearrangement a thiolactone moiety,[47] which within the protein active site are attacked at the C-2 position by a Cys-residue leading to the

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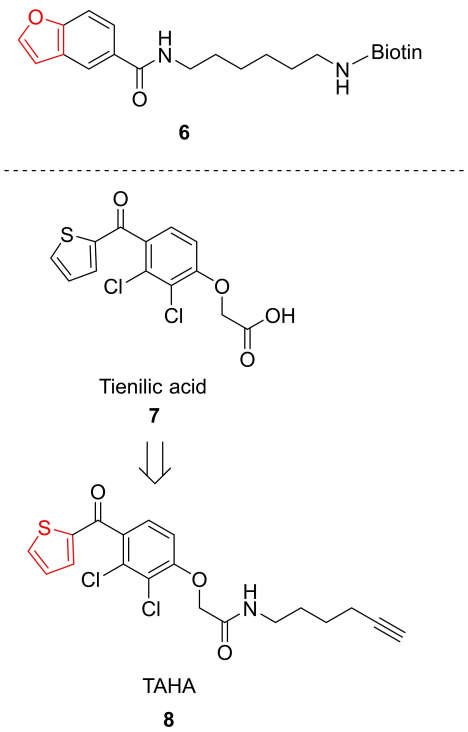


**Figure 1.** Mechanism-based P450-probes with heterocyclic warheads (depicted in red).

irreversible inhibition of the P450 enzyme. CYP2C9 is the primary metabolic enzyme for various drugs, including phenytoin (against seizures) and the widely described anti-coagulant warfarin prescribed to over 15 million people annually. Due to their narrow therapeutic indices, the latter two drugs must be dosed carefully.

Polymorphisms in the *CYP2C9* gene can affect individual drug response and an increased risk in side effects. The groups of Fowler and Dunham developed a FACS-based deep mutational scan for the activity of a variant library ("click-seq") using the TAHA probe **8** that has been derived from tienilic acid (**7**) (Figure 1).[48] 6542 CYP2C9 variants were heterologously expressed in *S. cerevisiae* yeast. Activity-based labeling with TAHA followed by attachment of a fluorophore allowed FACS-sorting of the cells according to their degree of labeling. High-throughput sequencing of the sorted cells allowed the scoring of each variant. Control experiments with 14 selected variants found that click-seq derived activity scores were well correlating with the activity measured in the gold standard LC–MS assays of microsomal preparations of the 14 CYP2C9 variants. The wealth of data produced via click-seq and a complementary screen for protein abundance (VAMP-seq) were used to establish general functional scores for certain mutations in the various regions of CYP2C9. In addition, the activity scores of 319 previously annotated human variants could be measured.

### 2.2 Monoamine Oxidases

Monoamine oxidases (MAO, EC 1.4.3.4) are flavin adenine dinucleotide (FAD)-containing enzymes, localized in the outer membrane of mitochondria, and catalyze the oxidative deamination of important neurotransmitters, including serotonin, norepinephrine and dopamine to aldehydes.[49] The human isoforms (MAO A and B) are associated with the development of cardiovascular and neurodegenerative disorders. Several MAO inhibitors are clinically used for the treatment of depression, Parkinson's disease, and other mental disorders.

In 2012 Breinbauer and Sieber developed an ABPP-approach for studying the activity of MAO enzymes,[50] which also represented the first ABPP study of a flavin-dependent oxidase. They converted the known MAO-inhibitors and anti-Parkinson-drugs pargyline and deprenyl to the MAO-ABPP probes **9a** and **9b** respectively, by attaching a click handle at the phenyl ring in *para*-position to the propargylamine warhead (Scheme 3).[50–51] Amine oxidation of **9a–b** by MAO generates the highly reactive iminium species **10a–b**, which get nucleophilically attacked by the reduced FAD cofactor. As the FAD is covalently attached to MAO via an 8α-(*S*-cysteinyl) linkage the stable protein-probe, Michael-reaction adducts **11a–b** formed (Scheme 3).

With probe **9b** and fluorescent SDS-PAGE analysis the authors showed that deprenyl reacts in human brain cancer cells quite selectively with MAO A and B. Most recently, Sieber demonstrated with probe **9a** in HeLa cells and SH-SY5Y, a model cell line for studying neurological effects, using label-free quantitative proteomics that pargyline also shows superb selectivity, as for **9a** only MAO A and ALDH1B1 are significantly enriched protein targets in both cell lines.[52] In HeLa cells also MAO B and a small number of other proteins are enriched. The excellent selectivity for these two drugs can be interpreted as a consequence of the specific reaction mechanism of this enzyme class and stands in contrast to many other CNS-drugs, exhibiting a polypharmacological profile.

Activity-based probes can also be used for the cellular imaging of MAOs.[53] The group of Yao designed a highly MAO B-selective activity-based probe, which found use for the ABPP of MAO B and live cell bioimaging of MAO B activity in cell and tissue models of Parkinson's disease.[54]

Tranylcypromine (**12**) is a clinically used drug against refractory depression (Figure 2). It has been known that tranylcypromine acts as a nonselective irreversible inhibitor of MAO A and B, which is believed to first undergo oxidation to the imine followed by spontaneous ring opening of the cyclopropane ring generating a highly reactive benzylic radical or cation intermediate.[55] As patients taking tranylcypromine often experience side effects, the group of Sieber investigated the protein target profile of tranylcypromine by preparing the probe FBP2 (**13**), which exhibits an alkyne handle suitable for click-tagging, and probe FBPP2 (**14**) bearing a diazirine photocrosslinker moiety suitable for additional photoaffinity labeling (Figure 2).[52] Through label-free quantitative proteo-

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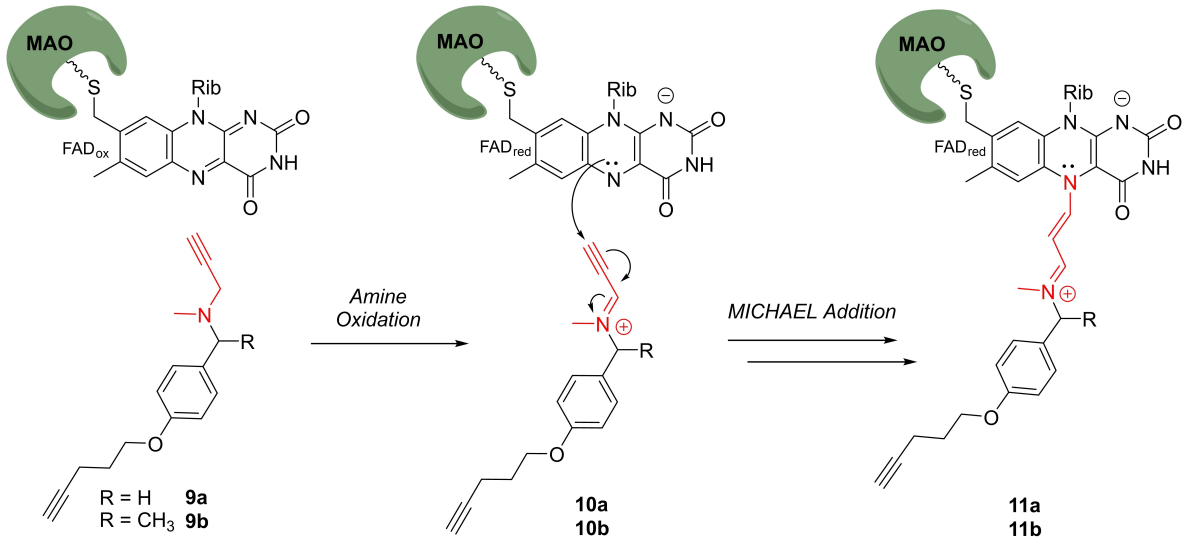


**Scheme 3.** Design of mechanism-based MAO-probes **9a** and **9b** derived from pargyline and deprenyl respectively. The warhead is depicted in red (Rib = Adenosine diphosphate ribosyl).

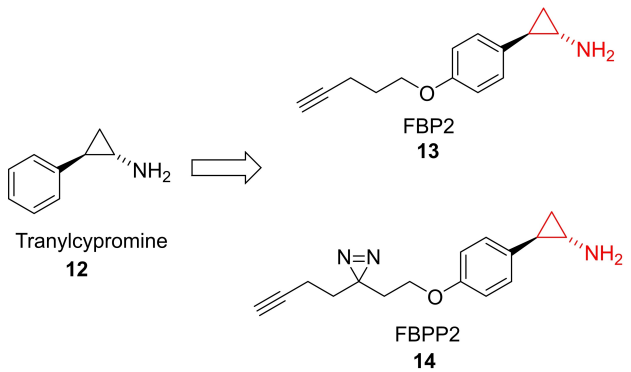


**Figure 2.** Design of tranylcypromine-derived probes FBP2 (**13**) and FBPP2 (**14**). The warhead is depicted in red.

mics experiments with HeLa cells and SH-SY5Y using avidin beads for target enrichment they found that FBP2 (**13**) showed significant labelling of MAO A but not of MAO B in both cell lines, and in addition multiple off-targets.

These findings were corroborated with studies using probe FBPP2 (**14**). Importantly, they were able to show that tranylcypromine shows significant trapping in lysosomes due to its primary amine group.

Interestingly, in these studies no evidence was reported for binding to lysine-specific demethylase-1 (LSD-1), which is in accordance with previous studies by Dekker et al. with a structurally similar tranylcypromine-derived ABPP probe, who also could not observe an activity-dependence for in vitro LSD-1 labeling although tranylcypromine had been previously described as an irreversible inhibitor of LSD-1.[56]

### 2.3 Flavin Monooxygenases

The group of Burkart developed probe **15**, which was designed to act as a universal probe for aerobic flavoenzymes (which comprise oxidases, monooxygenases and halogenases). In **15**, a cyclic thiocarbonate acts as a latent warhead (Scheme 4).[57] In contrast to the MAO-probes discussed above, **15** does not require flavin covalency to the protein of interest, thereby taking advantage of a common characteristic for the catalytic activity of flavoproteins that upon oxidation of reduced flavin the hydroperoxy-containing flavin **16** is formed, which in the presence of **15** would produce the electrophilic acyl sulfoxide **17** or sulfenic anhydride **18**. These highly electrophilic intermediates would react with nucleophilic residues in the active site of the corresponding flavin-dependent enzyme leading to covalent adduct **19**. In proof of concept experiments it could be shown that probe **15** was able to label in an *E. coli* strain heterologously expressed NRPS halogenase PltA. So far, no studies applying compound **15** to full proteomes or utilizing the probe for the detection of flavoproteins at natural abundance have been published.

### 2.4 Ammonia Monooxygenase

Ammonia monooxygenases (AMO) are Cu-dependent multimeric transmembrane enzymes used by autotrophic bacteria[58] and archaea[59] to oxidize ammonia to hydroxylamine, which is further oxidized to nitrate by hydroxylamine dehydrogenase (HAO) for completing the energy delivering nitrification process. The group of Hyman has introduced 1,7-octadiyne as an ABPP probe to analyze AMO in the nitrifying bacteria *Nitrosomonas europea*.[58] One alkyne functions as an inhibitor of AMO in a covalent manner, probably by oxidation to a

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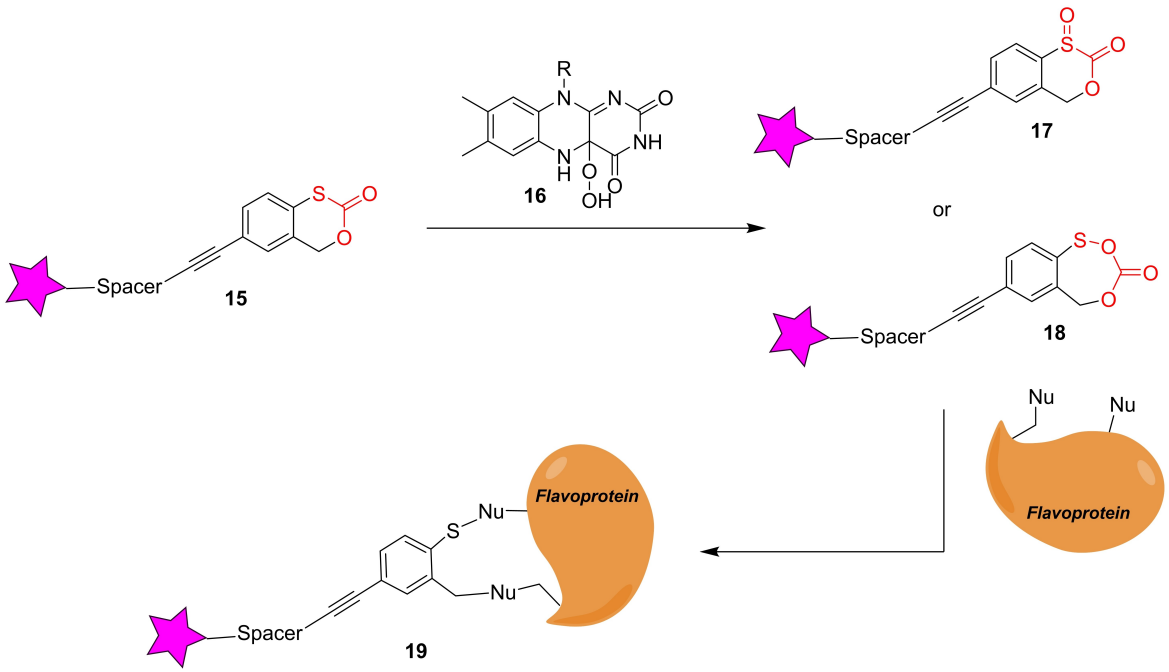
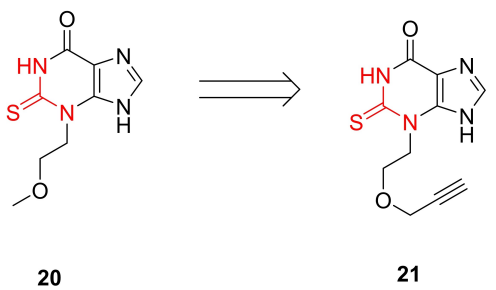



**Scheme 4.** Labeling of flavin-dependent monoxygenases with the thiocarbonate probe **15**. The warhead is depicted in red.

highly electrophilic ketene moiety.[60] The second alkyne group was used as a click handle for fluorescent labeling and affinity enrichment. It could be shown that 1,7-octadiyne labeled AmoA as its main target.

### 2.5 Myeloperoxidase

Myeloperoxidase (MPO, EC 1.11.1.7) is a heme peroxidase in neutrophils that catalyzes the synthesis of hypochlorous acid (HOCl) from chloride anions and $H_2O_2$. Ahn from Pfizer converted the 2-thioxanthine (**20**), a known mechanism-based inactivator of MPO,[61] into the ABPP probe **21** (Figure 3). This compound allowed them to study the protein profile of a structurally similar drug candidate.[62] A limitation is that compound **21** alone was unreactive towards soluble human and mouse liver proteomes, but it labeled several protein targets from the same liver proteomes in the presence of MPO and $H_2O_2$. This result suggests that under artificially high MPO levels probe **21** is converted into an activated species, which is released from the enzyme and can attack other proteins.



**20** **21**

**Figure 3.** Design of the MPO-probe **21**. The warhead for covalent attachment is depicted in red. It is assumed that the Fe=O unit of activated MPO converts the thiourea unit of **20** and **21** into a radical, which could form a thioether bond with the methyl group of the heme cofactor.

### 2.6 Lipoxygenases

Lipoxygenases (LOX) are Fe-containing dioxygenases, which catalyze the stereospecific insertion of molecular oxygen ($O_2$) into polyunsaturated fatty acids, such as arachidonic acids. An important mammalian representative is 15-lipoxygenase-1 (15-LOX-1) playing a role in the biosynthesis of leukotrienes, lipoxins, 15-HPETE, 15-HETE and eoxins. 15-LOX-1 has gained attention as a drug target as its activity is associated with allergic airway diseases, atherosclerosis, cancer, and various CNS diseases. The group of Dekker has designed the ABPP probe N144 (**22**), which mimics the natural polyunsaturated fatty acid substrate but contains a bispropargylic warhead (Scheme 5).[63] Single-electron oxidation of the bis-propargylic carbon atom results in a highly reactive allene radical, which leads to the covalent attachment of the probe. For this original design Dekker et al. used a biorthogonal oxidative Heck reaction[64] instead of the commonly used azide-alkyne click chemistry.

As the two-step labelling approach of the original probe N144 (**22**) proved to be inconvenient and not compatible with cellular imaging studies, the Dekker lab developed the probe

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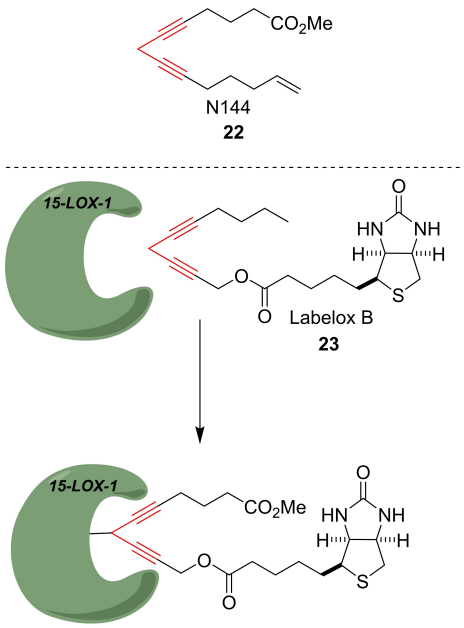



**Scheme 5.** Labeling of LOX-enzymes with bispropargylic probes N144 (**22**) and Labelox B (**23**). The warhead is depicted in red.

Labelox B (**23**) for the one-step activity-based labeling of proteins with LOX activity.[65] Similar to the previously described probe N144, Labelox B also showed non-competitive inhibition of 15-LOX-1. Labelox B (**23**) is sufficiently cell-permeable and allowed labeling of endogenous LOX in intact cells. Comparing the localization of active LOXs labeled with Labelox B and LOX isoenzymes labeled with immunostaining using confocal microscopy Dekker et al. observed that lipopolysaccharides (LPS) stimulate the activation of LOX isoenzymes at the nuclear envelope and distinct locations in the nucleus.

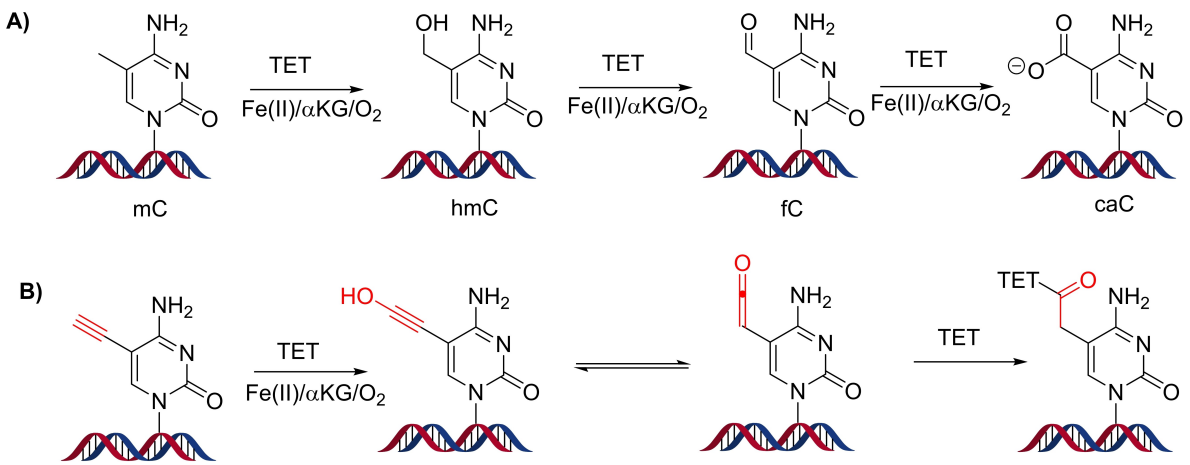
Furthermore, they showed that 15-LOX-1 contains a bromodomain-like region that binds to acetylated histones. The Labelox B probe was also used to establish an ABPP-based ELISA-assay with which the selectivity of inhibitors known from literature could be assessed.

### 2.7 Ten-Eleven Translocation Enzymes

Ten-eleven translocation (TET) enzymes are Fe(II)/α-ketoglutarate dependent dioxygenases playing an important role in epigenetic demethylation of 5-methylcytosine (mC). TET enzymes can oxidize mC to 5-hydroxymethylcytosine (hmC), which can be further oxidized to 5-formylcytosine (fC) and 5-carboxycytosine (caC) (Scheme 6A).[66] Building on earlier work by Stubbe for the mechanism-based inhibition of thymine hydroxylase with 5-ethynyluracil,[67] the group of Kohli developed 5-ethynyl-dC based probes which were incorporated into double stranded DNA (Scheme 6B).[68] Through the action of the TET enzymes the alkynyl moiety gets oxidized to a ketene intermediate, which because of its highly electrophilic nature traps the TET enzyme forming enzyme-DNA complexes, which could be observed via SDS-PAGE electrophoresis. The suitability of this concept was demonstrated with lysates from HEK293Tcells transfected with TET2.

The group of Kleiner applied this principle for RNA-mediated activity-based protein profiling by feeding HEK293T cells with 5-ethynylcytidine, which gets incorporated into cellular RNA.[69] Protein-RNA enrichment and quantitative mass spectrometry proteomics showed that the Fe(II)-α-KG-dependent dioxygenase ALKBH1 is the major m5C-oxidizing enzyme in HEK293T cells, where it is largely responsible for the production of $hm^5C$ and $f^5C$ of within RNA.

In this context also work by the group of Schofield should be mentioned in which a probe was designed for the affinity based labeling of Fe(II)/α-ketoglutarate dependent oxygenases




**Scheme 6.** A) Stages of oxidation of mC-containing DNA by TET-enzymes; b) labeling of TET-enzymes oligonucleotides containing 5-ethynyl-dC probes. The warhead is depicted in red.

in general (Figure 4).[70] The probe features a chelating group to achieve selective interaction with the Fe-dependent enzymes and a photoaffinity label for covalent linkage. A biotin affinity tag allows the enrichment of the labeled proteins. When using this affinity-based probe in profiling 2-OG oxygenases in a human cell line two histone lysyl demethylases (JARID1C, FBXL11) and the collagen lysyl hydroxylase LH3 emerged as clearly enriched proteins even at an endogenous level.

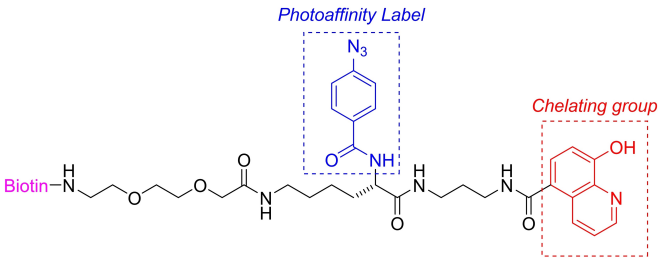


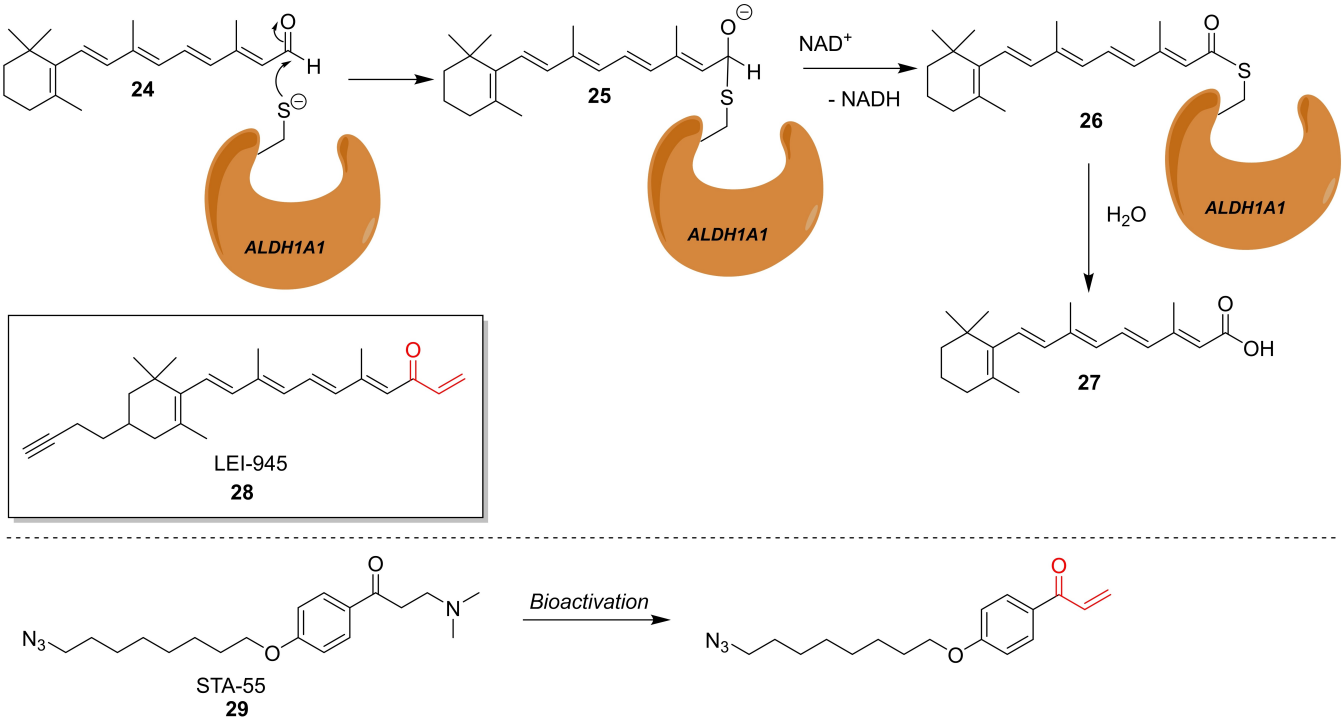

**Figure 4.** Design of an affinity based-probe for Fe(II)/α-ketoglutarate dependent oxygenases.

## 3. ABBP of Oxidases by Probes with Existing Electrophilic Groups

A subset of oxidoreductases uses highly nucleophilic amino acid residues for their catalytic mechanism, which could also be addressed by an ABPP-probe in which an electrophilic group is already installed.

### 3.1 Aldehyde Dehydrogenase

*All-trans* retinoic acid (ATRA, **27**) is the bioactive form of vitamin A. The crucial step in its biosynthesis is the oxidation of retinaldehyde (**24**) catalyzed by three retinaldehyde dehydrogenases (ALDH1A1, ALDH1A2 and ALDH1A3). The mechanism involves the nucleophilic attack of a Cys of the dehydrogenase at the carbonyl forming a thioacetal (**25**), which is oxidized to the thioester adduct **26** with $NAD^+$. Hydrolysis releases ATRA (**27**) (Scheme 7). By analyzing available structural information of ALDH1A1 the group of van der Stelt rationally designed ABPP-probe LEI-945 (**28**), which features a vinyl ketone warhead as a Michael acceptor addressing the reactive Cys-residue and an attached alkyne handle for click chemistry-tagging.[71] It was shown that compound **28** quite selectively binds to the three retinaldehyde dehydrogenases. Importantly, it does not react with other proteins known to have a "hyperreactive" cysteine,[72] but




**Scheme 7.** Oxidation of retinal to *all-trans* retinoic acid (**27**) via ALDH1A1 and the ABPP-probes LEI-945 (**28**) and STA-55 (**29**). The warhead is depicted in red. In STA-55 the vinylketone warhead is formed in situ after intracellular elimination of the dimethylamino group.

shows only cross reactivity with other proteins involved in retinal/retinoic acid biochemistry. Probe **28** enabled the comparative profiling of retinaldehyde dehydrogenase activity in a series of cancer cells.

The group of van der Stelt later designed the more general ABPP probe STA-55 (**29**) based on the pan-ALDH inhibitor Aldi-2.[73] STA-55 (**29**) was used for the in situ selectivity profiling of three known ALDH-inhibitors by competitive ABPP4.

## 4. ABBP of Oxidases with Highly Nucleophilic Groups

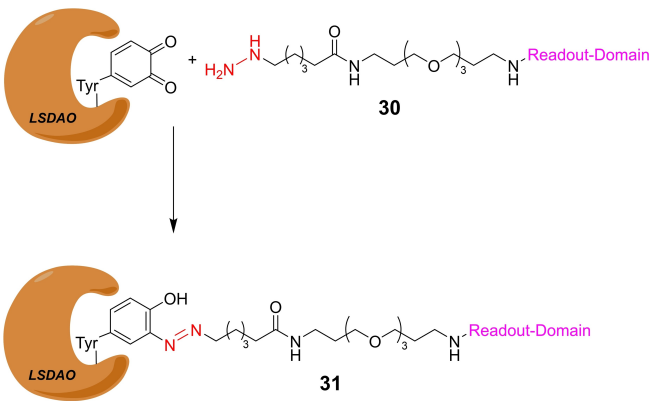
If oxidoreductases use cofactors or amino acid residues which are by themselves oxidized, it should be possible that these electrophilic units can be attacked by highly nucleophilic residues.

### 4.1 Amine Oxidases

Quinone-dependent amine oxidases utilize a 1,2-benzoquinone cofactor together with copper ions to oxidize aliphatic amines into aldehydes.

The group of Jakobsche has developed ABPP probe **30** with a hydrazine nucleophile to visualize lentil seedling diamine oxidase enzyme (LSDAO) in *in vitro* experiments (Scheme 8).[74] The hydrazine group in **30** attacks one carbonyl of the 1,2-benzoquinone group formed from the tyrosine residue, which results in hydrazine condensation. Tautomeric shift ultimately produces the azophenol conjugate **31**.

As will be discussed in Chapter 5.2, hydrazine nucleophiles can also undergo oxidative activation forming alkyl radicals thereby leading to a different reactivity behaviour addressing a series of other oxidases as well as even a reductase.[75]




**Scheme 8.** ABPP labeling for the visualization of LSDAO. The warhead is depicted in red.

## 5. ABBP of Reductases

The warhead design for activity-based probes targeting reductases is far more difficult compared to oxidases, since oxidation of functional groups usually generates more electrophilic groups (e.g. amine to iminium by MAO, alkyne to ketene by cytochrome P450, etc.) as discussed above. Therefore, until most recently this research field was completely unexplored.

### 5.1 Glutathione Reductases

In 2021, the group of Davioud-Charvet reported the development of (pro-)activity-based protein profiling probes, designed for the reaction with glutathione reductases (GRs), NADPH-dependent reductases found throughout all biological kingdoms.[76]

In their proof-of-concept study using *in vitro* conditions, probes were designed and tested based on the potent antimalarial drug plasmodione (**32**) (Scheme 9), which was assumed to possibly target NAD(P)H-dependent flavoenzymes (e.g. GR) in malarial parasites. First, in order to mimic the metabolism of plasmodione in the malaria parasite cell, 3-benzylmenadione probe **33** was oxidized at the benzylic position in solution with molecular oxygen under irradiation with 350 nm light, leading to a benzophenone-like structure. Upon photoirradiation with 365 nm light this 3-benzoylmenadione intermediate **34** was then activated to ketyl radical **35**, thereby mimicking the single electron reduction step presumably carried out by the flavoprotein in the cell. In the presence of the enzyme of interest (e.g. purified *h*GR) in a buffer solution under exclusion of oxygen, the highly reactive intermediate **35** eventually cross-links in two different ways leading either to the benzophenone-like protein adduct **36** or the benzoxanthone adduct **37** allowing the pull down of labeled proteins via click tagging.

It will be interesting to see if the probe design can be modified that the reductive potential of an endogenous reductase is sufficient to activate the probe.

### 5.2 Quinone Reductases

In order to overcome the current bias in ABPP of using electrophilic probes addressing nucleophilic amino acid residues, the group of Matthews developed reverse polarity (RP)-activity based protein profiling (RP-ABPP), in which super-nucleophilic hydrazine-based probes are used to profile electrophilic enzyme cofactors, transient intermediates, and labile regulatory modifications.[75,77] Interestingly, when testing their probes in quantitative proteomics experiments with HEK293T and MDA-MB-231 cell lines they found that some of the highest enriched protein hits using SILAC (stable isotopic labelling by amino acids in cell culture) were oxidoreductases. This suggested that first the hydrazine group

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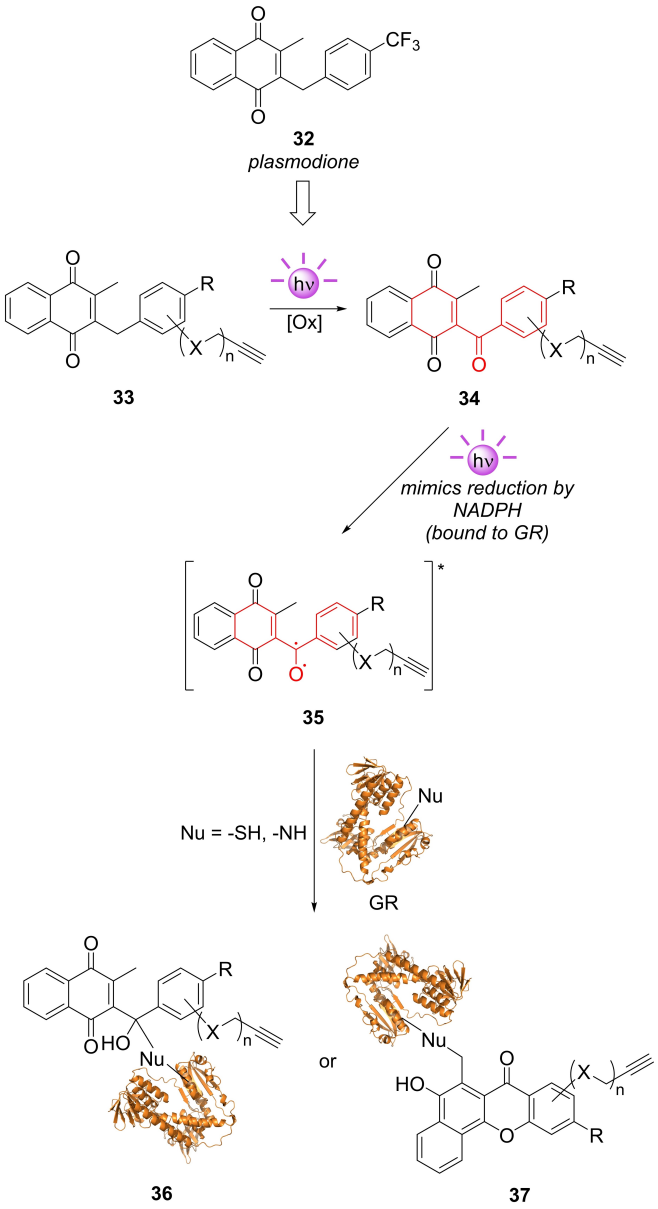
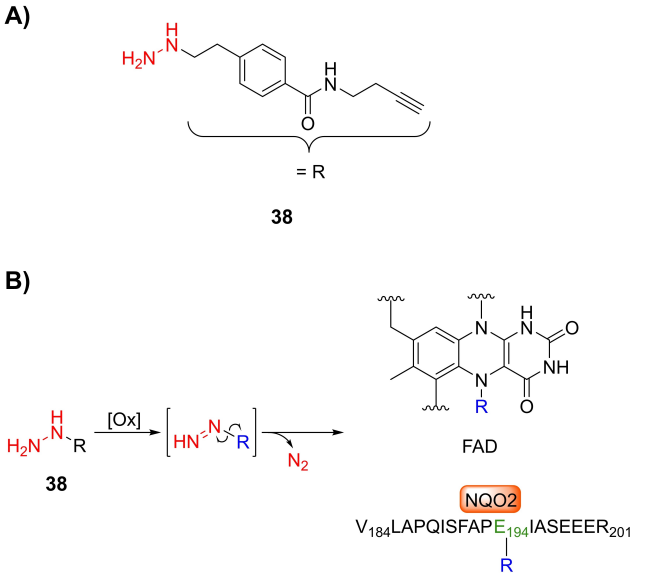



**Scheme 9.** (Pro-)ABPP probes based on plasmodione and mode of action with GR. The active warhead is depicted in red.




**Scheme 10.** (A) ABPP probe (**38**) targeting NQO2 (among others). The warhead is depicted in red, the active labeling species is depicted in blue. (B) **38** labels FAD and an amino acid residue of NQO2 upon activation as an alkyl radical.

in **38** might have undergone oxidative activation to a diazo group, which subsequently undergoes spontaneous fragmentation to an alkyl radical (Scheme 10).

Using gel-based profiling and MS analysis they were able to confirm the radicaloid mode of labeling for enzymes belonging to structurally and functionally diverse enzyme classes, such as FAD-dependent enzymes (monoamine oxidases (MAO A and MAO B, lysine-specific histone demethylase 1A (LSD1), Fe(II)/2-oxoglutarate dependent enzymes (nucleic acid dioxygenase (ALKBH1), fat mass and obesity-associated dioxygenase (FTO)), heme enzymes (prostaglandin E synthase 2 (PTGES2), holocytochrome c-type synthase (HCCS), heme oxygenase 2 (HMOX2) and $NAD^+$-dependent enzymes (ALDH2). This indicates that the hydrazine probes **38** can function as quite general probes for the ABPP of oxidases.

Notably, among the enriched proteins also a reductase was targeted: the noncovalent flavoenzyme NQO2, a quinone reductase which uses reduced dihydronicotinamide riboside to transfer electrons to its FAD cofactor in an initial phase. NQO2 plays an important role in phase II drug metabolism[78–79] and has also been associated with several disease states, such as schizophrenia or Parkinson's disease.[80] By LC–MS analysis they confirmed that one of their activity-based probes (Scheme 10, B) formed adducts with FAD located in the active site of NQO2, which is in accordance to other FAD-containing enzyme classes like MAO and related monoamine oxidase inhibitors (e.g. phenelzine). In addition, they were able to demonstrate that the reactive radical species, which is formed during oxidative fragmentation of NQO2 probe **38**, could also bind directly to the protein (residue E194) in close vicinity of the active site (Scheme 10, B).

Even though these two ABPP approaches do not involve the generation of the reactive species via enzyme-mediated reduction per se, the groups of Davioud-Charvet and Matthews have presented possible ways towards future progress in this still largely unexplored research field.

## 6. Modified Cofactors

The group of Sieber has introduced a new concept in which structurally modified cofactors are offered to enzymes and which after covalent linkage can be used for protein profiling.

They designed a pyridoxal analogue containing an alkyne-click tag in the 2'-position which is taken up by cells and phosphorylated to form the PLP-analogue **39** (Figure 5),[81–82] which was accepted by PLP-dependent enzymes. Reduction with $NaBH_4$ led to the formation of stable clickable PLP-cofactor/enzyme-conjugates. With this approach they could access 73% of the current *S. aureus* PLPome and identified many more putative PLP-dependent enzymes.[81] Similarly, they could study the PLPome in human HEK293 cells and used it for target screening of Vitamin B6 antagonists.[82] This work is of special significance as PLP-dependent enzymes are responsible for a broad range of essential cellular processes, but are evolutionary diverse, which makes its classification via sequence homology challenging.

## 7. Summary

Over the last 15 years ABPP of oxidases has seen considerable progress, although it has not reached the level of maturity as already accomplished for ABPP of hydrolytic enzymes. The mechanistic rationale for most probes involves the oxidation of a functional group to a highly electrophilic species (e.g. alcohol to aldehyde, amine to iminium ion, etc.), which is then attacked by a nucleophilic residue of the enzyme. Several probes, such as alkyne-based probes **1**–**3** for P450s, **15** for aerobic flavoproteins, and **29** for aldehyde dehydrogenases are rather general. These compounds follow the general aim in ABPP of providing a universal probe, which reacts with most members of the enzyme family to study. This approach can be used for metabolic profiling and comparative ABPP addressing the target-specificity of small molecule inhibitors.

On the other hand, the quite specific probes **8** for CYP2C9, **9a**–**b** for MAO, and **28** for retinaldehyde dehydrogenases allowed to investigate the mode of action and target profile of the underlying drug structure or the profiling of enzyme variants.

Only most recently, the still unexplored field of ABPP of reductases has received attention. With NQO2 the first example for labeling of a reductase could be achieved. The concept of (pro-)activity-based protein profiling probes might bear fruits in this respect in the future.

It can be expected, that in the next years new pan-family probes as well as protein-specific probes will be developed filling the toolbox for ABPP of oxidoreductases, which will stimulate research in Chemical Biology and Drug Discovery.

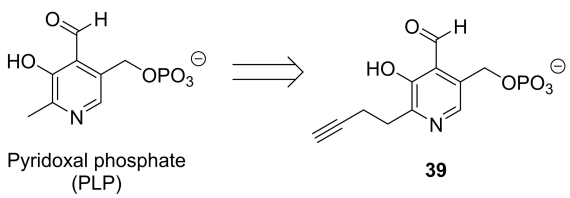



**Figure 5.** Design of the clickable PLP-cofactor **39**, which allows the profiling of the PLPome.

## Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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