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Synthesis and evaluation of naphthoquinone-based probes for activity-based protein profiling of oxidoreductases

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Activity-based protein profiling (ABPP) has become a highly valuable proteomic technique over the past decades especially in the investigation of hydrolytic enzymes. Oxidoreductases have so far received less attention as their catalytic function usually depends on cofactors, which requires different strategies for warhead design for probe molecules. We describe the design and total synthesis of novel activity-based probes based on an α-fluoromethyl naphthoquinone warhead. Starting from inexpensive and commercially available mequinol, our synthetic route utilizes an intramolecular Friedel–Crafts acylation as the key step for the formation of an annulated aryl ring bearing the ligation handle for the linker. In total, three novel probes were synthesized in 13 to 18 sequential steps, respectively. Initial ABPP experiments with flavin-dependent reductases and murine liver revealed the promiscuous reactivity of the warhead.

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Introduction

Over the past two decades, activity-based protein profiling (ABPP) has shown its value as the key proteomic technique for the analysis of the catalytically active proteome in cells or other living organisms.[1] Pioneered by Cravatt[2] and Bogyo,[3] ABPP was originally designed for the enzyme class of hydrolases (EC 3),[2–4] but it was soon extended to other enzyme classes bearing a characteristic nucleophilic residue in the active site, which could be addressed by an electrophilic reactive group ("warhead").[5] In contrast, other enzyme classes, which are involved in various essential metabolic processes such as transferases and oxidoreductases (EC 1), have received much less attention, as they exhibit more complex catalytic activities. Oxidoreductases, in particular, strongly depend on cofactors (*e.g.* NAD(P)H, flavins, pyridoxal phosphate (PLP), heme, *etc.*) for their catalytic action, which makes the design of the warhead more challenging. So far, activity-based probes for oxidoreductases have mainly been developed to be highly specific for a subclass or even a single enzyme,[6] with notable warheads (Fig. 1) such as an alkyne for CYP450s or Fe(II)/α-ketoglutarate dependent dioxygenases,[7] a propargylamine for monoamine oxidases,[8] a vinyl ketone for specific members of the aldehyde dehydrogenase family,[9] or a bispropargylic warhead for lipoxygenases.[10] More recently, warheads have been designed, such as alkylhydrazines and aryliodonium compounds, which upon enzymatic activation lead to radical intermediates, which have been shown to address several subclasses of oxidoreductases.[11] In the past decade, a related reactivity-based protein profiling approach has been developed. In the (global) reactivity profiling of specific (nucleophilic) amino acid residues,[12] probes bearing reactive warheads are used to identify and monitor certain amino acid residues in native biological systems. Importantly, they can then also be used for the development of new targeted covalent inhibitors.[13] Together, these techniques profit from the exploration of new chemical entities as warheads.

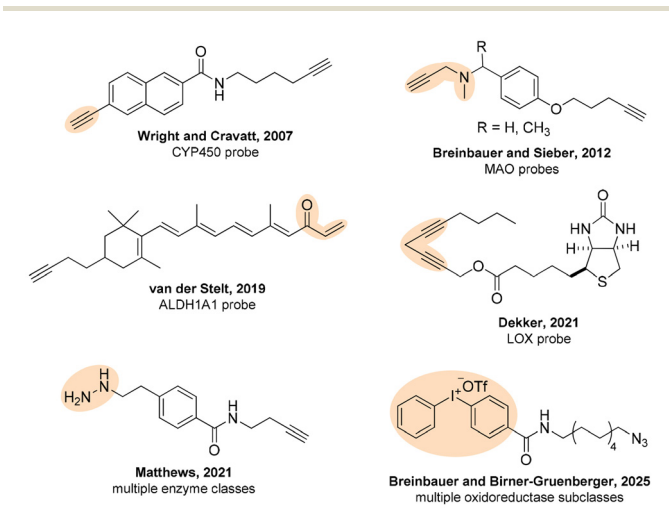

Fig. 1 Selected examples of oxidoreductase probes. The warheads undergoing activation by the respective enzymes are highlighted in orange. $^{-}$OTf, triflate.

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Here, we present our studies in which we investigated α-fluoromethylquinones as a warhead for the ABPP of oxidoreductases. Quinones are a compound class ubiquitously found in natural products, endogenous biochemicals, drugs, or environmental chemicals.[14–16] Many quinones are potent, redox-active compounds and show good electrophilic reactivity (Michael acceptors). As a result, they exhibit a variety of hazardous effects *in vivo*, besides their crucial involvement in electron transport chains of cellular respiration or photosynthesis.[16] Among other molecules (*e.g.*, GSH[14,17]), proteins can be readily modified with quinones, especially when intracellular pools of small molecule nucleophiles like GSH become depleted. Enzymes which are inhibited by quinones include oxidases such as cytochrome P450s[18] or cyclooxygenases,[19] but also reductases like NAD(P)H dehydrogenase [quinone] 1 (NQO1),[20] which plays a vital role in quinone detoxification. Certain naphthoquinone derivatives have also been described as substrates for other flavin-dependent reductases,[21] and based on these findings in 2006, Davioud-Charvet and co-workers designed and synthesized a mechanism-based inhibitor (fluoro-M_5, Fig. 2A) of the flavin-dependent glutathione reductase (GR), which was investigated for its antimalarial effects against *Plasmodium falciparum*.[22,23] They proposed a one- or two-electron activation of their inhibitor by the enzyme, which leads to the formation of a reactive quinone methide intermediate upon formal HF-elimination (Fig. 2A).[24] This intermediate is then expected to easily react with nucleophilic residues (*e.g.*, Cys) in close proximity.[25] Furthermore, these quinone methides might also be generated through photoexcitation.[26]

Inspired by this elegant suicide inhibitor design, we aimed to translate the underlying mechanistic principle for the design of novel activity-based probes based on a fluoromethyl naphthoquinone warhead, and to test them in initial ABPP experiments with oxidoreductases.

Results and discussion

Development and synthesis of activity-based probes

In our synthetic approach, we planned to introduce the required linker bearing a terminal azide click-handle on the phenyl ring of the naphthoquinone core *via* a stable amide bond (Fig. 2B), which should allow the linker to reach out of the active site of the enzyme, thereby facilitating an efficient introduction of the reporter tag and read-out. As late-stage introduction of an acyl linker handle at the 7-position on the fluoromethyl (or hydroxymethyl precursor) naphthoquinone core was not feasible, we had to undertake a total synthesis starting from simple and inexpensive mequinol (**4**). We planned to construct the annulated ring already bearing the desired acyl linker handle *via* an intramolecular Friedel–Crafts acylation.[27–29]

In the first step, a double hydroxymethylation at the *ortho* positions of the hydroxy group of mequinol (**4**) was carried out according to a procedure from Samuel *et al.*,[30] producing triol **5** in 59% yield after recrystallization from EtOAc (Scheme 1). The phenolic hydroxy group was then selectively methylated to

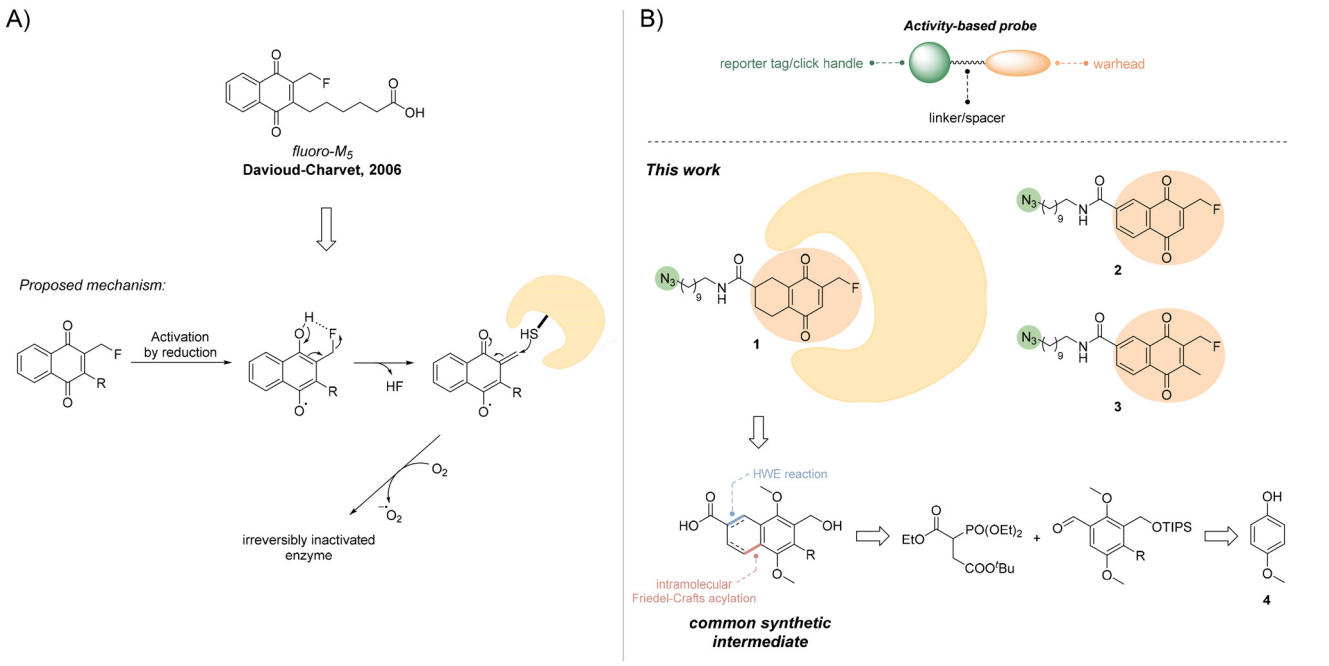
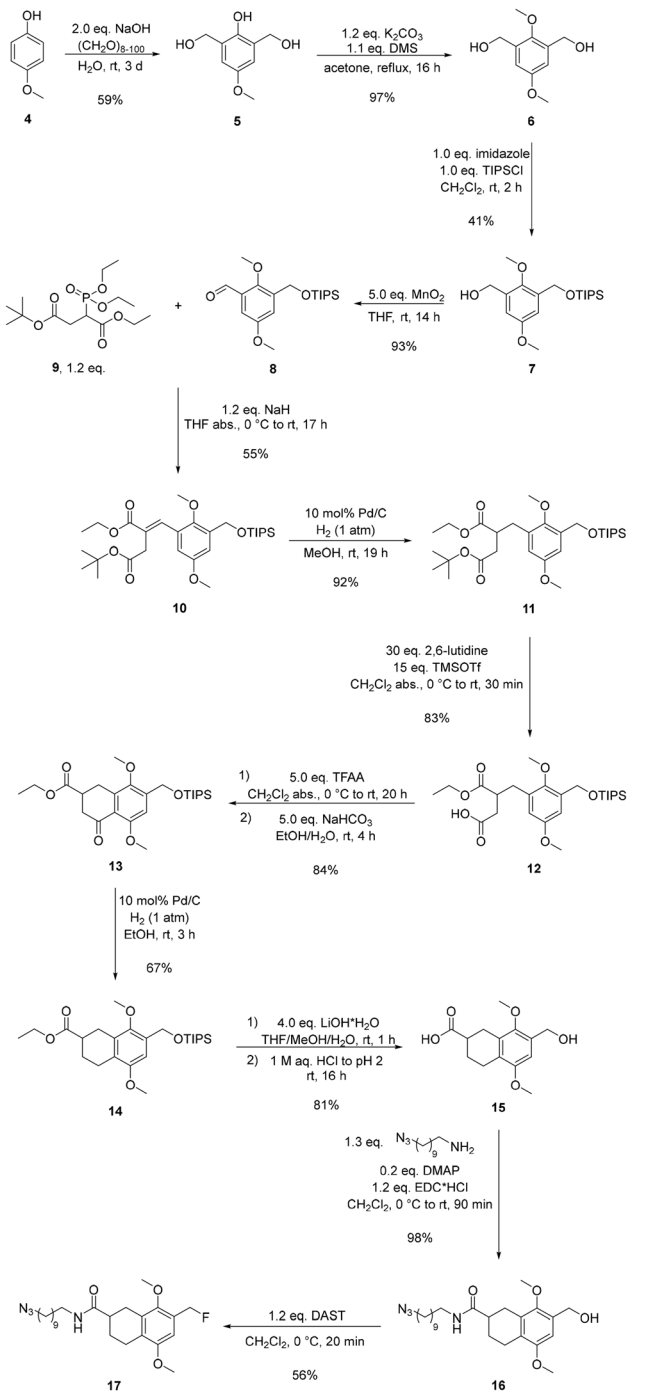


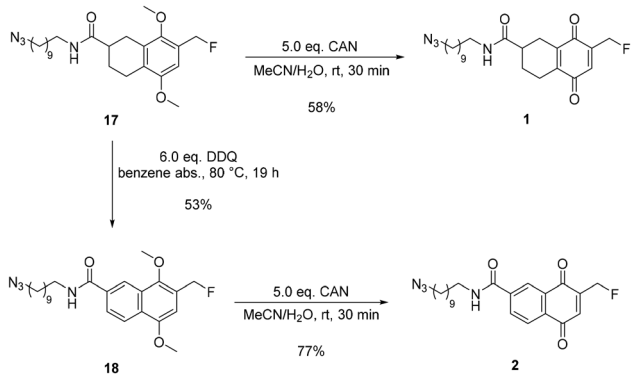
Fig. 2 (A) Structure of the mechanism-based GR inhibitor fluoro-M_5 designed by Davioud-Charvet and co-workers,[22] the proposed mechanism of activation, and irreversible binding to the enzyme; (B) general design of an activity-based probe and activity-based probe design (**1–3**) pursued in this work. Retrosynthesis of probes is depicted proceeding *via* the common hydroxymethyl-carboxylate intermediate and starting from commercially available mequinol (**4**).



Scheme 1 Multi-step synthesis of fluoromethyl intermediate **17** starting from mequinol (**4**). DAST, diethylaminosulfur trichloride; DMAP, 4-dimethylaminopyridine; DMS, dimethyl sulfate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TFAA, trifluoroacetic anhydride; THF, tetrahydrofuran; TIPS, triisopropylsilyl; TMS, trimethylsilyl.

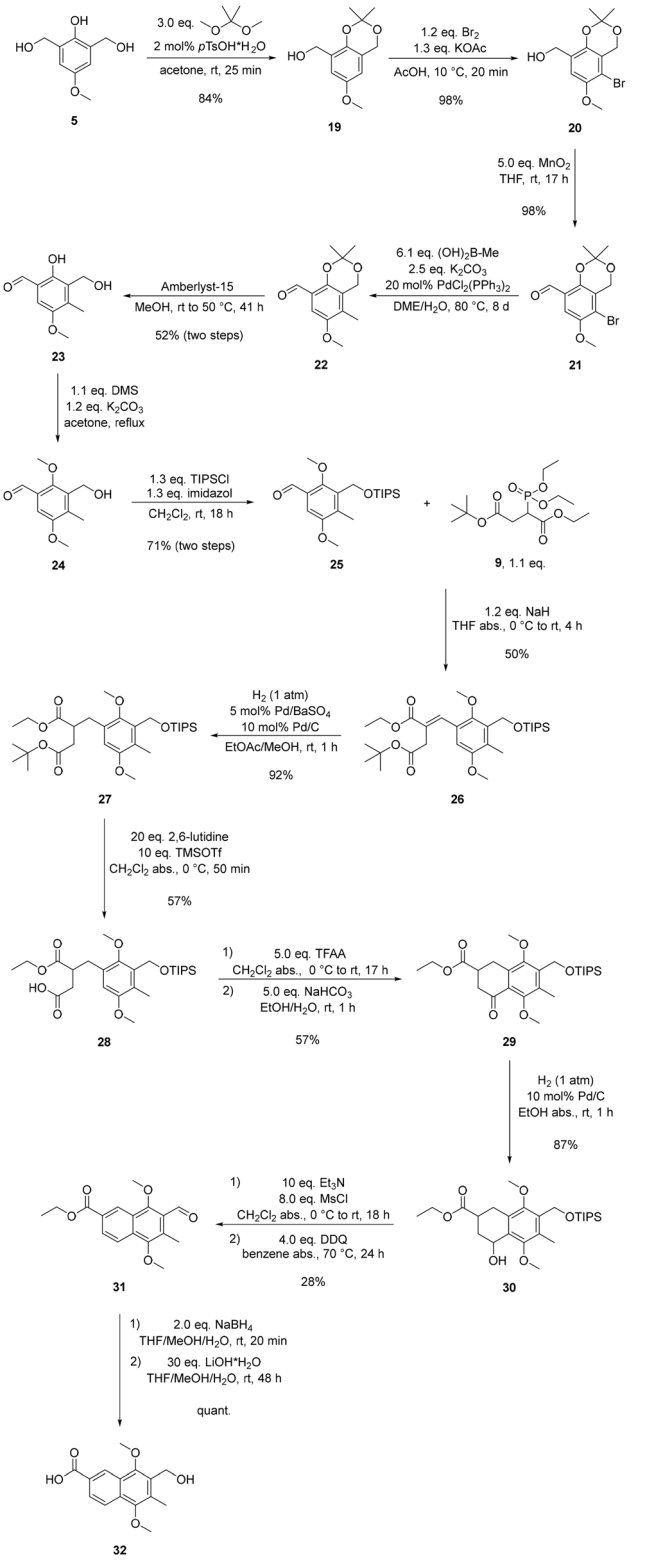
furnish diol **6** in excellent yield (97%). Selective monoprotection of the two hydroxymethyl groups as a triisopropylsilyl (TIPS) ether furnished alcohol **7** in 41% yield. TIPS was chosen because of its increased stability in acidic media compared to trimethylsilyl (TMS) or *tert*-butyldimethylsilyl (TBDMS) protecting groups,[31] which was required for later steps in the synthesis. A subsequent benzylic oxidation with MnO_2 smoothly afforded aldehyde **8** in 93% yield, which set the stage for a Horner–Wadsworth–Emmons (HWE) reaction to establish the carbon framework suitable for the planned intramolecular Friedel–Crafts reaction,[28,29] resulting in the naphthoquinone core. Aldehyde **8** was reacted with a slight excess of previously synthesized phosphonate **9** (see the SI for further information), which delivered the desired intermediate **10** in moderate yield (55%). The C=C bond formed in the HWE reaction was subsequently reduced to a simple, more flexible C–C bond *via* catalytic hydrogenation, affording **11** in very good yield (92%). The *tert*-butyl ester of **11** was selectively hydrolyzed using 2,6-lutidine and trimethylsilyl trifluoromethanesulfonate (TMSOTf)[32] in large excess. Gratifyingly, carboxylic acid intermediate **12** could be isolated in very good yield (83%) and was then subjected to an intramolecular Friedel–Crafts reaction mediated by trifluoroacetic anhydride (TFAA).[29] For the hydrolysis of the *in situ* generated TFA-enol ester, weakly basic $NaHCO_3$ in $EtOH/H_2O$ delivered the best results furnishing cyclic ketone **13** in very good overall yield (84%). Subsequent hydrogenative benzylic deoxygenation with Pd/C afforded tetraline intermediate **14** in decent yield (67%). It should be noted that this reaction required careful reaction monitoring, as too short reaction times gave only poor conversions while longer reaction times resulted in the formation of a side product in which the TIPS-protected benzyl alcohol had been hydrogenolyzed to the corresponding toluene compound. In the next step, sequential one-pot saponification of the ester and acidic deprotection of TIPS ether furnished carboxylic acid **15** in good yield (81%). Subsequently, an aliphatic linker bearing a terminal azide click handle was introduced *via* carbodiimide-mediated amidation, which provided alcohol **16** in excellent yield (98%). The essential benzylic fluoride was then introduced using the nucleophilic fluorinating reagent diethylaminosulfur trifluoride (DAST), affording fluoro-intermediate **17** in acceptable yield (56%). Compound **17** could then be readily oxidized with CAN to tetrahydronaphthoquinone-based probe **1** in 58% yield (Scheme 2). This probe features a partially saturated tetrahydronaphthoquinone system which should grant the linker a little bit more flexibility. In order to access the more rigid, fully unsaturated naphthoquinone-based probe, tetrahydronaphthalene intermediate **17** was first oxidized with dichlorodicyanobenzoquinone (DDQ) to naphthalene intermediate **18** (53% yield), which was then oxidized with CAN to eventually yield the desired naphthoquinone-based probe **2** in good yield (77%).

p-Quinones like naphthoquinone can act as direct Michael acceptors for nucleophiles,[33] which, for example, leads to the formation of thiodione from the reaction of menadione with glutathione.[34] As this direct reaction between protein and warhead might interfere with the desired enzyme-imposed activation of the probe before it should react with a nucleophile, we designed a probe with an additional methyl group blocking the β-position of the quinone moiety (Fig. 2B). Taking advantage of the already synthesized diol **5**, the pheno-

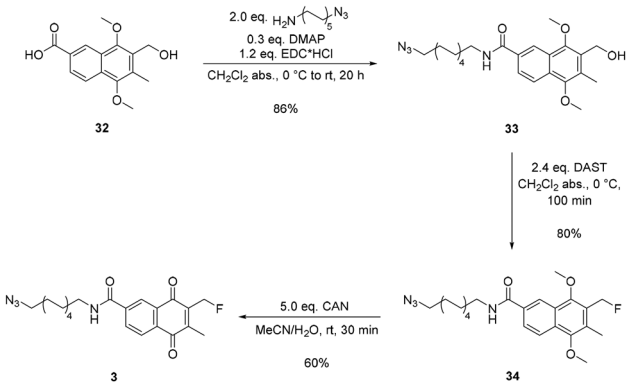


Scheme 2 Final steps towards (tetrahydro)naphthoquinone-based probes **1** and **2**. CAN, ceric ammonium nitrite; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

lic hydroxy group was protected together with one of the benzylic hydroxy groups as a ketal (**19**, 84% yield; Scheme 3), which also led to desymmetrization of the molecule. Next, a bromo-substituent was selectively introduced at the *para*-position to the free hydroxymethyl substituent, furnishing alcohol **20** in excellent yield as described by Tietze and co-workers.[35] After benzylic oxidation with MnO_2 to aldehyde **21** (98% yield), the previously installed bromide was converted to a methyl group *via* a Suzuki-coupling reaction with methylboronic acid to give crude aldehyde **22**, which was then deprotected using acidic Amberlyst-15 beads in MeOH, from which diol **23** could be isolated in 52% yield over these two steps. Methylation of the phenolic hydroxy group followed by the protection of the remaining benzylic alcohol with TIPSCl afforded **25** in 71% yield (2 steps). This aldehyde was then used in a HWE reaction with phosphonate reagent **9** – analogous to the synthesis of **10** – furnishing α,β-unsaturated ester **26** in 50% yield. For the subsequent reduction of the double bond, we found that the previously used conditions (Pd/C in MeOH) led to an increased formation of hydrogenolyzed side products. Much to our delight, this could be circumvented by using Pd/C (10 mol%) in combination with Pd/$BaSO_4$ (5 mol%) in EtOAc/MeOH. Pd/$BaSO_4$, also known as the Rosenmund catalyst,[36] has a reduced Pd-activity caused by the $BaSO_4$ support and has found extensive application in the literature, especially for the reduction of acyl chlorides to aldehydes.[37] With this optimization, the hydrogenation smoothly afforded the desired intermediate **27** (92%). This intermediate was then subjected to the previously established selective cleavage of the *tert*-butyl ester with TMSOTf and 2,6-lutidine as well as the intramolecular Friedel–Crafts acylation to deliver intermediate **29**. In the next step, we aimed to remove the benzylic ketone moiety *via* hydrogenation analogously to the synthesis of **14**. However, with the additional methyl group, the reaction stopped at secondary alcohol **30**, which was obtained in very good yield (87%). With alcohol **30** in hand, we then decided to make use of an elimination/oxidation reaction cascade that provided fully unsaturated aldehyde **31** (28% yield). After a subsequent aldehyde



Scheme 3 Multi-step synthesis of carboxyl intermediate **32** starting from **5**. AcOH, acetic acid; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DME, dimethoxyethane; Et, ethyl; Me, methyl; Ms, mesyl; quant., quantitative yield; TFAA, trifluoroacetic acid anhydride; THF, tetrahydrofuran; TIPS, triisopropylsilyl; TMS, trimethylsilyl.



Scheme 4 Final steps towards naphthoquinone-based probe **3**. CAN, ceric ammonium nitrite; DAST, diethylaminosulfur trichloride; DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

reduction and ester saponification executed as a one pot reaction, carboxylic acid **32** could be isolated in quantitative yield. Similar to the synthesis of probe **2**, the final steps (Scheme 4) comprised the introduction of the azide linker *via* EDC-mediated amide bond formation (86% yield), benzylic fluorination with DAST (80% yield), and oxidation with CAN (60% yield) to eventually furnish the desired methylated naphthoquinone probe **3**.

ABPP experiments with recombinant enzymes

With all desired probes in hand, we first tested the non-methylated probes **1** and **2** in *in vitro* ABPP experiments with NQO1 (Fig. 3), which was chosen as a model enzyme due to its involvement in the metabolism of various quinones such as coenzyme Q[38] or Vitamin K3,[39] which is structurally related to our probes. NQO1 was incubated with and without probes (40 μM, 1 h) in a reduced (NADH added prior to probe addition) and an oxidized state (no NADH added).[27] In the FAD-dependent NQO1, electrons are sequentially transferred from NAD(P)H to the flavin cofactor and then from the reduced flavin to the substrate.[40] As the naphthoquinone warheads should be activated following a reduction by the enzyme in its active and reduced state (Fig. 2A), a significant difference in observed labeling between reduced and oxidized NQO1 was therefore expected. Following reduction, alkylation, and click reactions with a Dibac-atto633 dye (synthetic details are given in the SI), SDS-PAGE was performed and the labeled proteins were visualized. In addition, we performed control experiments with denatured NQO1 (Fig. 3A). Here, a more or less complete suppression of activity-based labeling should be expected as a protein usually loses its native secondary, tertiary, and/or quaternary structure and hence also its bioactivity. Indeed, labeling of NQO1 was observed with both probes **1** and **2**. However, similar labeling intensities were observed without NADH addition, which already indicated a potentially promiscuous reactivity of these probes. Unfortunately, the control experiments with denatured enzyme confirmed that the probes might simply be too reactive, as labeling even further intensified, presumably due to the exposure of additional nucleophilic residues usually not accessible in the native protein. The high reactivity also became apparent when we tested tetrahydronaphthoquinone probe **1** in an ABPP experiment with fresh murine liver (Fig. S1).[27,41] Here, the gel experiments indicated that the full proteome was unspecifically labeled by the probe. One might therefore hypothesize

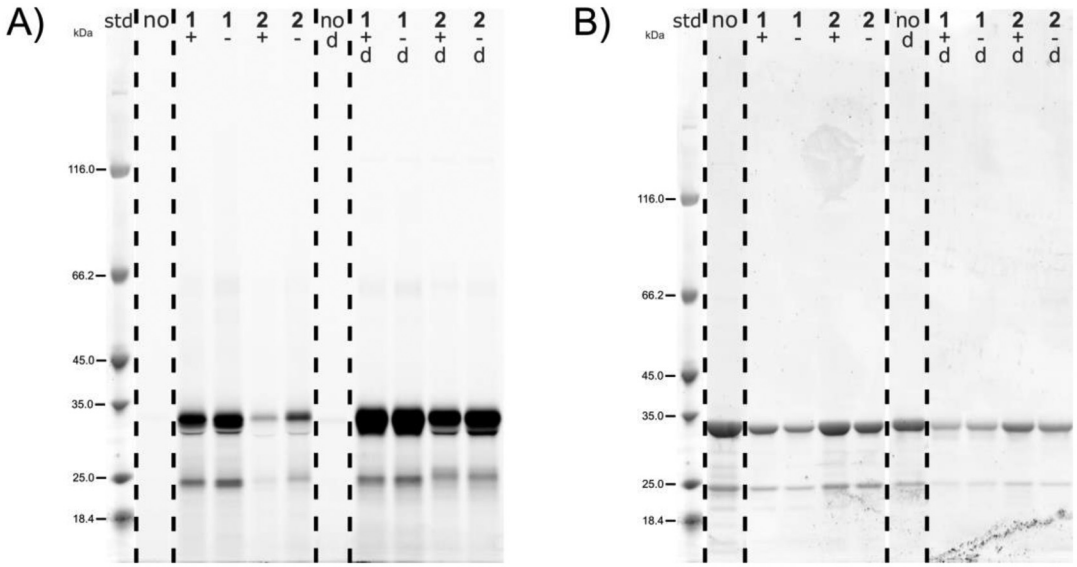


Fig. 3 (A) Fluorescence SDS-PAGE analysis of the labeling of intact or denatured (d) NQO1 with probes **1** and **2** (40 μM, 1 h; no, no probe added) with (+) or without (–) NADH added. Protein bands were visualized by fluorescence staining. (B) Total protein stain of activity-based gel. Proteins were visualized with Krypton fluorescent protein stain. Pierce™ Unstained Protein MW Marker (Thermo Fisher Scientific) was used as a protein standard (= std).

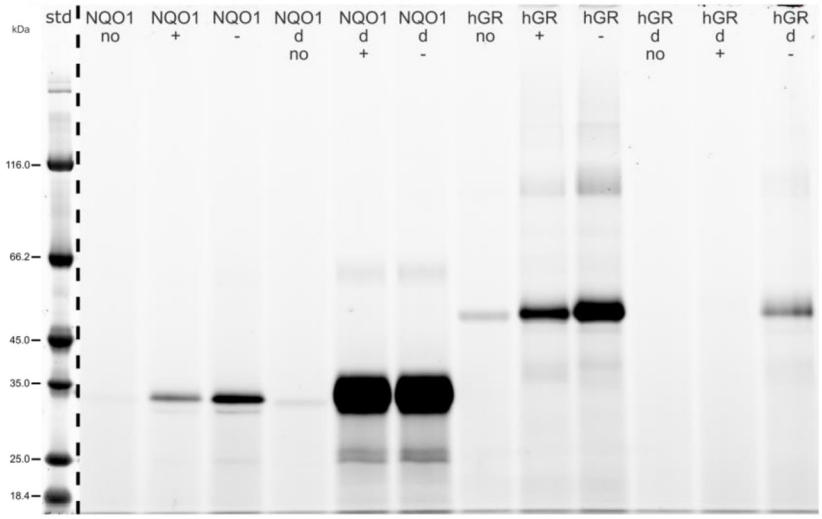


Fig. 4 Fluorescence SDS-PAGE analysis of the labeling of intact or denatured (d) NQO1 and hGR with probe **3** (40 μM, 1 h; no, no probe added) with (+) or without (–) NAD(P)H added. Protein bands were visualized by fluorescence staining. Pierce™ Unstained Protein MW Marker (Thermo Fisher Scientific) was used as a protein standard (= std).

that probes **1** and **2** act as simple bioalkylating agents upon activation to their respective quinone methide form either upon bioreduction[42] or addition of nucleophiles.[43]

We then subjected probe **3** – bearing the additional methyl group on the naphthoquinone core – to ABPP experiments with NQO1 and human glutathione reductase (hGR), one of

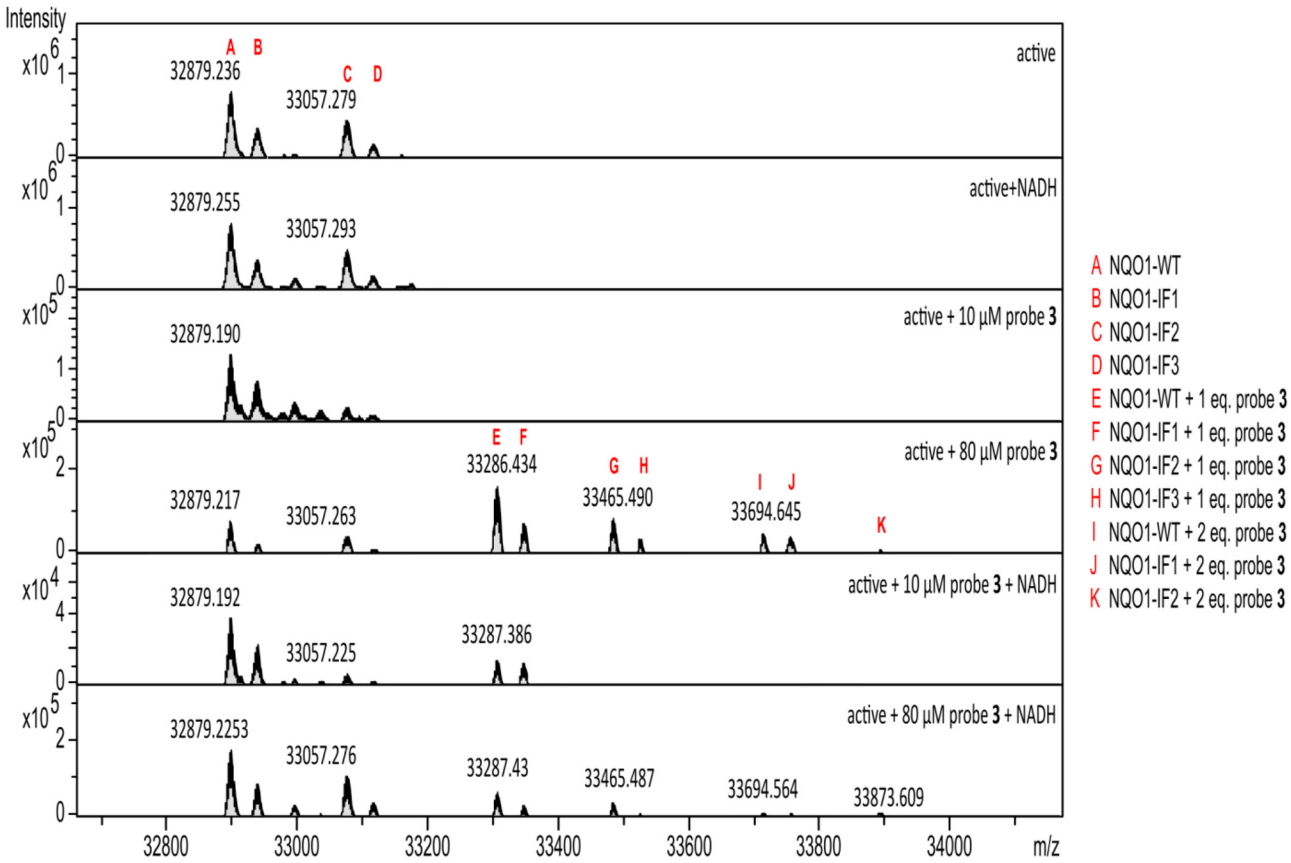


Fig. 5 Deconvoluted mass spectra of labelling experiments with active (non-denatured) NQO1 and probe **3** (10 or 80 μM), with and without addition of NADH (70 μM). Mass peaks are labelled with the corresponding modification (A–K). eq., equivalent; IF, isoform; WT, wild type.

the originally investigated targets of fluoro-M_5 in the studies of Davioud-Charvet and co-workers.[22] However, the results were similar to probes **1** and **2**, as for denatured NQO1, again a drastic increase in labeling was observed (Fig. 4) and labeling occurred even without added NADH. In the experiments with hGR, labeling was also more pronounced without added NADPH, which is not in line with the originally proposed mechanism of activation of our probe design (Fig. 2A). Moreover, denaturation of hGR prior to incubation with the probes – either by addition of sodium dodecyl sulfate (SDS), heat, or both heat and SDS addition – led to precipitation of the hGR protein in the utilized hGR-buffer, which resulted in a comparatively reduced transfer of protein to the gel, as can be seen in the total protein stain (Fig. S2) of the activity-based gel in Fig. 4.

To gain further insight into the reactivity of probe **3**, control experiments with **3** and the peptide thiol nucleophile glutathione (GSH) were conducted in the presence and absence of NADH (Fig. S3). GSH was chosen as the model nucleophile due to the high intrinsic nucleophilicity of the thiol side chain coupled and its high cellular concentrations. Therefore, its presence is highly relevant for the reaction with quinone methides due to the relatively low abundance of cysteine residues in wild-type proteins.[44] Here, the results greatly coincided with the gel-based experiments as the adduct of **3** and GSH (thereby formally releasing HF) readily formed even without NADH addition (Fig. S4 and S5). Furthermore, HPLC-MS analyses of labeling experiments with active (non-denatured) NQO1 and probe **3** confirmed the proposed enzyme-probe adducts (Fig. 5).[27] Here, it was also shown that more than one probe molecule can be attached to the enzyme, which further suggests that also probe **3** acts as a nonspecific alkylating probe. However, mass spectra from experiments with inactive (denatured) NQO1 (Fig. S6 and 7) unfortunately did not resolve properly, which can be attributed to an exceptional increase in enzyme isoforms caused by the large number of modifications.

Taken together, the ABPP experiments demonstrated pronounced inherent reactivity of the described α-fluoromethylquinone moieties, which especially could be observed in the experiments with denatured enzyme, and suggest that these specific warheads appear to be unsuitable to serve as warheads for ABPP-probes.

Conclusions

In summary, we have achieved the total synthesis of three novel probes based on a fluoromethyl (tetrahydro)naphthoquinone warhead. The probes could be synthesized in 13, 14, and 18 sequential steps, respectively, starting from an inexpensive and commercially available starting material. Our initial ABPP experiments showed a pronounced inherent reactivity of the α-fluoromethylquinone moiety. These observations were then further validated in control experiments with GSH as well as HPLC-MS analyses of labeling experiments with NQO1 and probe **3**. While the most plausible cause for the increased reactivities of the probes would be the presence of Michael acceptor sites or a base-catalyzed quinone methide release, other side reactions cannot be ruled out at this stage. While quinone methide-based probes have already found applications in bioorganic settings,[45] our employed α-fluoromethylquinone motifs might eventually be disqualified as selective warheads for ABPP. However, they might potentially be used as reactive groups in the field of targeted covalent inhibitors.

Author contributions

L. K. designed the probes and performed the synthetic work and spectroscopic analyses. L. K. and B. D. carried out all enzyme experiments and collected the data. L. K. and R. B. wrote the manuscript. R. B. conceived and supervised the project.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: experimental details and procedures including biological and synthetic methods, characterization data, whole-protein gel images, and NMR spectra. See DOI: **https://doi.org/10.1039/d5ob01320h**.

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