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Fluorescent mechanism-based probes for aerobic flavin-dependent enzyme activity

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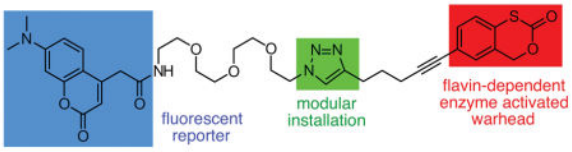
Abstract

Diversity in non-ribosomal peptide and polyketide secondary metabolism is facilitated by interaction between biosynthetic domains with discrete monomer loading and their cognate tailoring enzymes, such as oxidation or halogenation enzymes. The cooperation between peptidyl carrier proteins and flavin-dependent enzymes offers a specialized strategy for monomer selectivity, which oxidizes small molecules from within a complex cellular milieu. In an effort to study this process, we have developed fluorescent probes to selectively label aerobic flavin-dependent enzymes. Here we report the preparation and implementation of these tools to label oxidase, monooxygenase and halogenase flavin-dependent enzymes.

Graphical Abstract

Domain specific probes offer a vital tool to identify and characterize biosynthetic enzymes. We now report on the development of tools to label oxidase, monooxygenase and halogenase flavin-dependent enzymes.



Keywords

biosynthesis; synthase; polyketide; flavin dependent enzymes; molecular probes

Natural product biosynthesis occurs through a cascade of enzymatic reactions that combine monomeric building blocks into complex chemical scaffolds.[1] Over the last decade, our laboratory has participated in a global effort to develop methods that discretely label enzymatic domains responsible for fatty acid, polyketide, and non-ribosomal peptide biosynthesis.[2] A variety of tools now exist to selectively label domains containing activities of acyl and peptidyl carrier proteins,[3] ketosynthases,[4] dehydratases,[5] and

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Supporting information for this article is available on the WWW under http://

thioesterases.[6] We now report efforts to deliver probes that label aerobic flavin-dependent enzymes, or flavoenzymes, as a first step in delivering selective probes for oxidative enzymes within carrier protein dependent pathways.

Aerobic flavoenzymes are oxygen-dependent and catalyze a variety of reactions in natural product biosynthesis, including desaturations, mono-oxygenations, and halogenations.[7] These enzymes are subcategorized by their use of catalytic cycle within three major classes: oxidases,[7] monooxygenases,[8] and halogenases[9] (Supporting Figure S1). Each cycle initiates with the oxidation of reduced flavin (FlH_2) with O_2 via a radical mechanism, forming the hydroperoxy-containing flavin (Fl-OOH) at the C(4a) position depicted in Scheme 1.[10] The fate of the Fl-OOH species is resolved differently in each of the three flavoenzyme classes (Supporting Figure S1).

Mechanism-based inhibitors have been developed for the monoamine oxidase subset of flavin-dependent enzymes, where irreversible inhibitors containing *N*-propargylamine moieties, such as pargyline and clorgiline, are used to covalently react with the flavin via oxidation and Michael addition.[11] These inhibitors have been used to probe substrate specificities in the active sites of these enzymes, and fluorescent derivatives have been used as probes for targeting specific enzymatic activity.[12] However, no similar probes have yet been demonstrated for monooxygenases or halogenases.

In 1987, Latham and Walsh reported mechanism-based inhibition of an *Acinetobacter*-derived cyclohexanone monooxygenase (CHMO) using a cyclic thiocarbonate.[13] They proposed that this motif, upon oxidation with the Fl-OOH species, became an electrophilic warhead for enzyme inactivation, but further studies were not pursued. To examine the potential of these agents, we turned our attention to compound **1** as an aromatic thiocarbonate core for installation of reporter motifs.[14] Here, exposure to Fl-OOH would result in the generation of either acyl sulfoxide **2** or sulfenic anhydride **3** (Scheme 2). These reactive intermediates would then be susceptible to nucleophilic attack within the active site of the corresponding flavin-dependent enzyme, leading to covalently-linked protein **4** or the fully-crosslinked protein **5**.

We began by testing the inhibitory properties of **1** with a model flavin-dependent enzyme. We chose the BpsA oxidase (Ox) domain for a convenient colorimetric readout that had been previously employed for other non-ribosomal peptide synthetase (NRPS) associated enzymes.[15] As discrete domains within NRPSs, Ox domains catalyze the desaturation or dimerization of carrier protein-tethered substrates. BpsA-Ox catalyzes the desaturation of a piperidinedione intermediate, leading to the formation of indigoidine, a blue pigment (Supporting Figure S2).[16] Assays for *in vitro* inhibition of BpsA by compound **1** revealed an IC_{50} value of 5.6 ± 3.0 μM, a K_i of 47 ± 3 nM, and a k_{inact} of 5.6 ± 0.5 ms^{-1} (Supporting Figure S3). The evidence of oxidase inhibition, along with previously demonstrated inhibition of CHMO by Latham and Walsh, highlighted the potential of these probes to target other aerobic flavoenzyme activities. We next turned our attention to elaborate **1** into a fluorescent reporter. Consistent with the proposed mechanism and structure of **1** in Scheme 2, we modified the aryl-ring in **6** (Scheme 4) to regioselectively install a reporter in a position remote from the active moiety.

The synthesis of **6** began by converting ethyl 2-hydroxy-5-iodobenzoate (**7**) to its corresponding dimethylcarbamothioate **8**. Heating **8** in bromobenzene afforded an effective Miyazaki-Newman-Kwart rearrangement,[17] providing **9** in 76% yield from **7**. After exploring multiple options for Sonogashira coupling, we found the partner hepta-1,6-diyne offered a facile route to functionalization, offering access to **10** (Scheme 3) with a terminal mono-substituted alkyne handle for reporter installation.

The conversion of **10** to **12** required considerable optimization. We found that the most effective route to install the 1,3-oxathiin-2-one motif arose by hydrolysis of **10** to afford **11**, which was immediately reduced and treated with CDI, to afford **12** in three back-to-back operations.[18]

We then explored the incorporation of a reporter tag to **12** using conventional 1,3-dipolar cycloaddition chemistry.[19] We found that the blue fluorescent immunoaffinity azide tag **13**[20] could be installed to afford **6** under either aqueous conditions with 5% molar equivalents of $CuSO_4$ and aq. sodium ascorbate or anhydrous conditions with CuI and Et*i*Pr$_2$, respectively, in toluene.

As shown in Fig. 1a, both *apo*-BpsA and *holo*-BpsA, generated *in vitro* by incubation with CoA and a 4′-phosphopantheinyl transferase, Sfp,[21] (Supporting Figure S4), were labeled with probe **6.** Furthermore, labeling of *holo*-BpsA occurred in both the presence and absence of ATP and glutamine (Fig. 1a), indicating that BpsA was labeled without substrate dependency or 4′-phosphopantetheinylation.

Next, we expanded our panel of representative oxidative flavoenzymes to include the Baeyer-Villiger cyclohexanone monooxygenase (CHMO) from *Rhodococcus* sp. HI-31, which can convert cyclohexanone to caprolactone (Scheme 1);[8] and the NRPS halogenase PltA from *Pseudomonas fluorescens* responsible for dichlorination of a pyrrolyl-loaded carrier protein (pyrrolyl-PltL) substrate.[22]

We also found that **6** prepared *in situ* from **12** effectively labeled the halogenase, PltA (Fig. 1b). While the addition of a partner enzyme SsuE, a flavin reductase compatible with PltA,[23] and NADPH increased labeling efficiency (Fig. 1b), probe **6** labeled PltA without the need for addition of its associated partner enzyme or cofactors. In addition, we were able to demonstrate that the labeling of PltA with **6** occurred in a 1:1 stoichiometry via MALDI-MS analyses (Supporting Figure S7), suggesting that PltA was modified at a single site. Multiple attempts were made to identify the modified residue in PltA using tryptic digestion followed by LC-MS-MS analysis. Unfortunately, we were not able to identify peptides bearing modifications by **6** or **1**, although superb peptide coverage was obtained. This observation was consistent with previous studies that used similar inhibitors,[13] suggesting that covalent linkages were not stable to either digestion or LC MS-MS analyses.

As shown in Fig. 1c, CHMO also underwent labeling under identical conditions as BpsA or PltA with probe **6**. Interestingly, the addition of cyclohexanone and cofactor, NADPH, to CHMO diminished labeling with **6**, suggesting that **6** is a weak competitive inhibitor for CHMO compared to substrate under the assayed conditions. Interestingly, all three enzymes

underwent labeling without the need of cofactors. This reactivity may be owed to relative abundance of these proteins in the C(4a)-hydroperoxide state, which is thought to predominate in the cell, as previous studies have demonstrated its relatively long-lasting stability by spectroscopic and crystallographic means.[24]

To further validate this activity, we turned to examine if these events were selective to the 1,3-oxathiin-2-one motif. As shown in Fig. 2, the labeling of all three enzymes BpsA (Fig. 2a), PltA (Fig. 2b) and CHMO (Fig. 2c) by **6** was reduced in a concentration-dependent manner when the enzymes were pre-incubated with **1**. In all examples, use of a 2 to 4 fold excess of **1** blocked labeling with **6**, suggesting a single site for inhibition for all three enzymes.

Next, we tested selectivity of the probe for each flavoenzyme *versus* bovine serum albumin (BSA), a protein known for its binding to a variety small molecules including lipids, primary metabolites, natural products, synthetic drug leads and pharmaceuticals.[25] Upon incubation with probe **6**, BpsA (Fig. 3a), PltA (Fig. 3b), and CHMO (Fig. 3c) were all preferentially labeled in the presence BSA. In all three cases, we were not able to detect any labeling of BSA, therein confirming the selectivity for flavoenzyme reactivity. Furthermore, it also indicated that the activation and reactivity of the 1,3-oxathiin-2-one motif did not occur outside the flavin cofactor pocket, such as through the release of H_2O_2 or HOCl, a concern that would have resulted in non-specific labeling.

We then applied our probe to labeling PltA in *E. coli* lysate as a model of *in vivo* applications. As shown in Fig. 4a–4c, we were able to observe labeling of PltA in wild type *E. coli* lysates to which were added 10 μM PltA, 15 μM **6** and 15 μM NADPH by Western blot analyses.[20] We were also able to conduct these experiments *in vivo*. As shown in Fig. 4d, incubation of the *E. coli* strain engineered to heterologously express PltA with 15 μM **6** also resulted in labeled PltA, indicating *in vivo* utility. Finally, we examined the role of the flavin cofactor. Under the denaturing and non-reductive conditions of SDS-PAGE, the flavin cofactors of PltA, CHMO and BPSA disassociate to discrete, green auto-fluorescent bands (Fig. S8) that lacked the blue fluorescence from the tag in **6**. In contrast other flavin-dependent probes,[12] **6** labeled PltA, CHMO and BPSA regardless of flavin covalency.

We demonstrate here the development of fluorescent-based probes containing a 1,3-oxathiin-2-one motif that can be used to selectively label aerobic flavin-dependent enzymes. This tool allows, through a modular synthetic preparation (Scheme 4), the incorporation of a variety of molecular markers to target these enzymes.[26] This development can be further modified to explore the array of oxidative enzymes associated with primary and secondary metabolism, with a focus on substrate selectivity and specificity found within modular synthases.

Overall, this study identifies an oxidatively-activated warhead for probing aerobic flavin-dependent activity.[27] The appendage of 1,3-oxathiin-2-one motif onto a protein or related biomolecule offers a tool to selectively probe biomolecular interactivity. With this advance, the inhibitor motif may also be positioned as a tool for crosslinking flavoenzyme domains

with cognate partner domains such as carrier proteins, to study crucial protein•protein interactions that guide substrate processing.[28]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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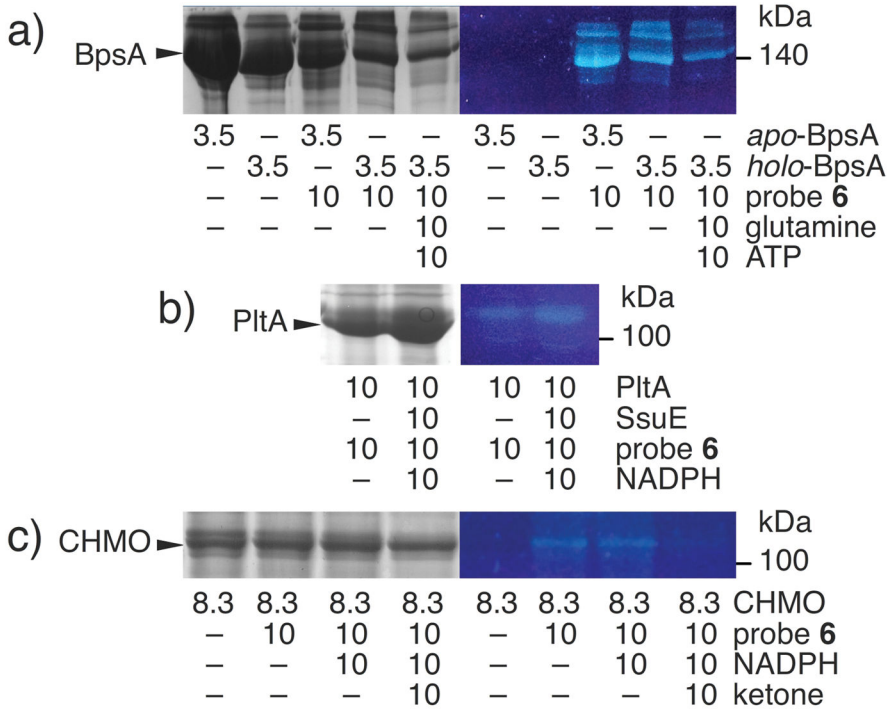


Figure 1.

In-gel fluorescent SDS-PAGE analysis of protein labeling. Coomassie stained (left) and fluorescent (right) SDS-PAGE gels depicting the labeling of BpsA, PltA, or CHMO with probe **6. a**) The labeling of *apo*-BpsA or *holo*-BpsA with and without the presence of glutamine and ATP. **b**) The labeling of PltA with and without the presence of SsuE and NADPH. **c**) The labeling of CHMO with and without the presence of cyclohexanone (ketone) and NADPH. Concentrations are provided in μM. Full images of the gels are provided in Supporting Figure S5.

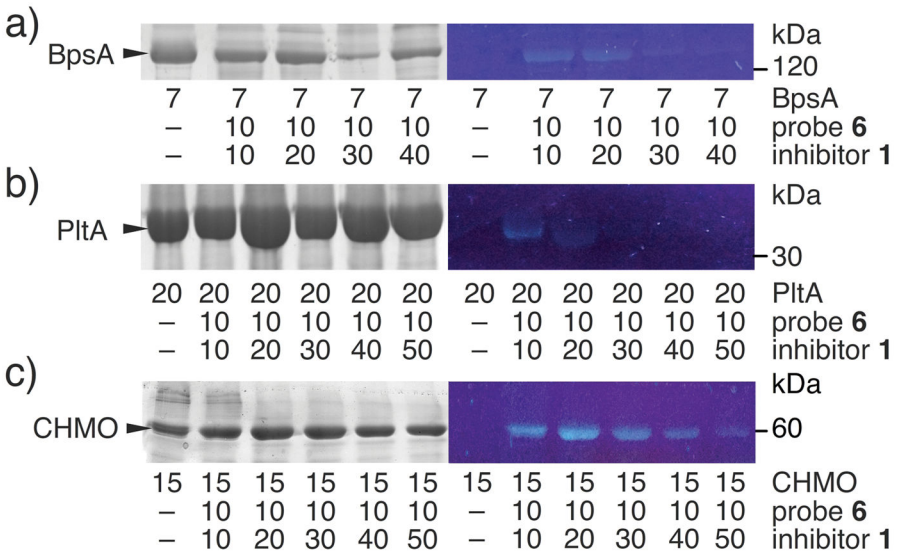


Figure 2.

Competition experiments. Coomassie stained (left) and fluorescent (right) SDS-PAGE gels depicting the competition of **1** and **6** in labeling of: **a)** BpsA; **b)** PltA; or **c)** CHMO. Proteins were treated with **1** for 30 min then subjected to labeling with **6**. Concentrations are provided in μM. Full images of the gels are provided in Supporting Figure S6.

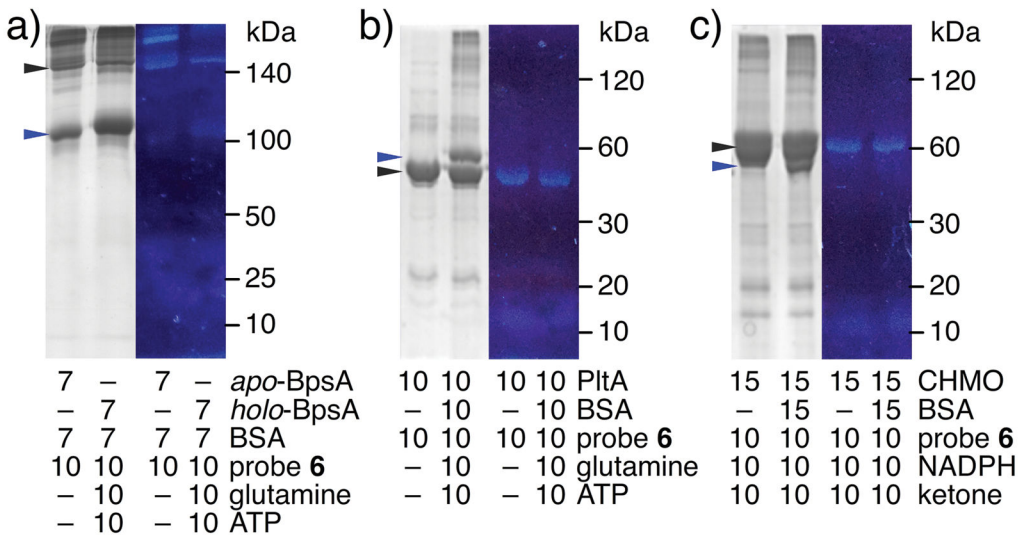


Figure 3.

Selectivity analyses. Coomassie stained (left) and fluorescent (right) SDS-PAGE gels depicting the selective labeling of flavoenzymes BpsA, PltA, or CHMO (black arrows) in the presence of BSA (blue arrows). Gels depicting the labeling of: **a)** *apo*-BpsA/*holo*-BpsA; **b)** PltA; or **c)** CHMO. Concentrations are provided in μM.

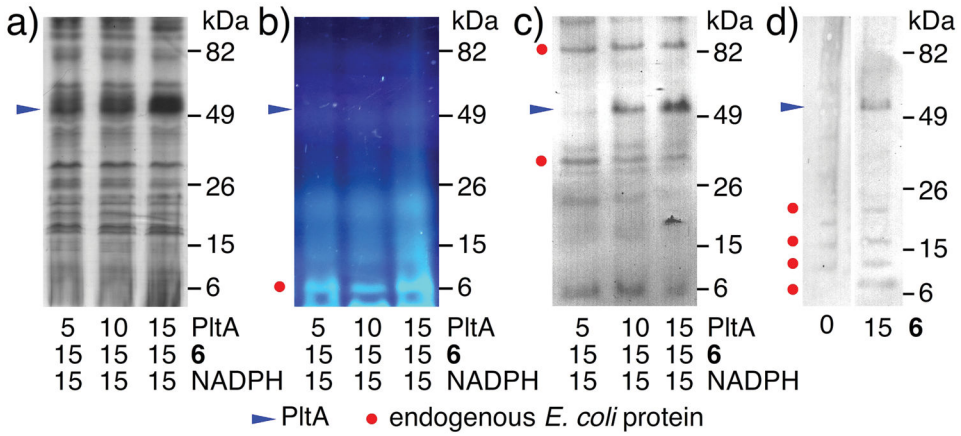
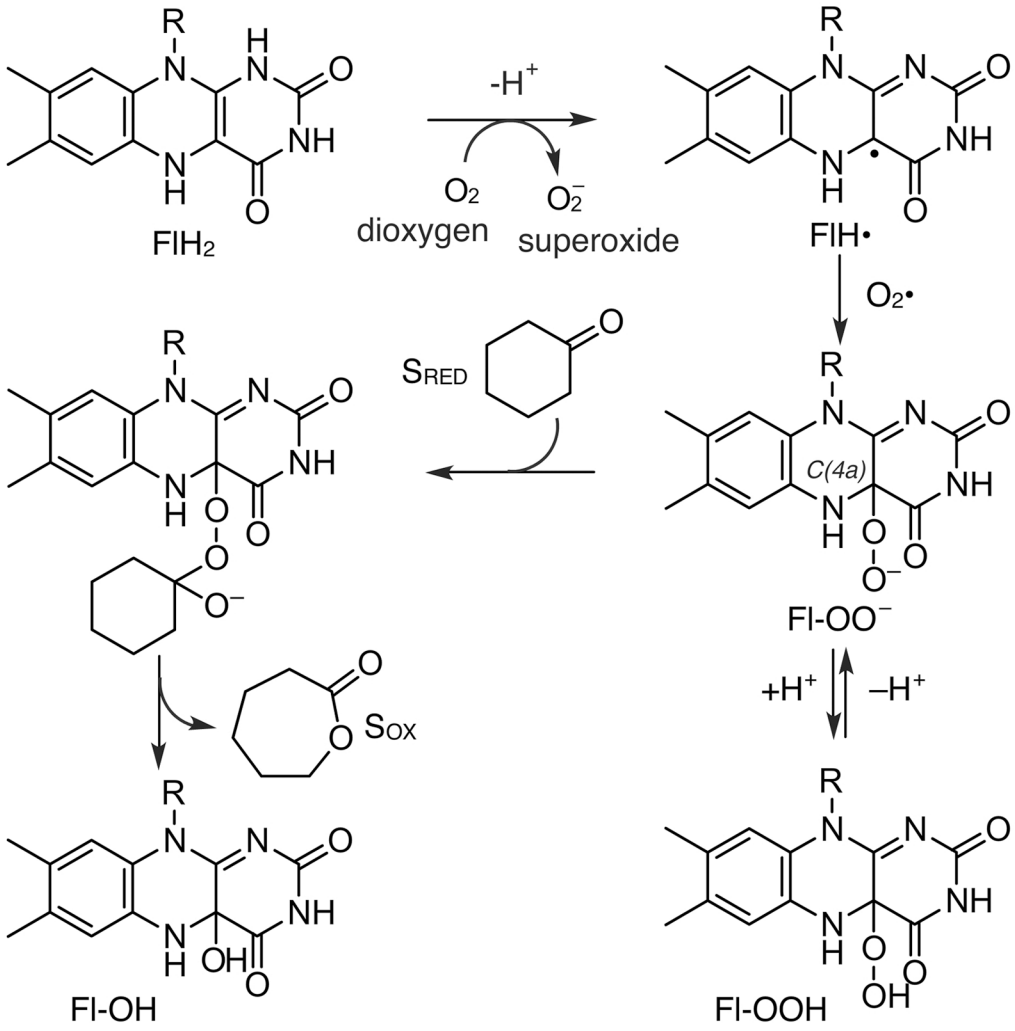


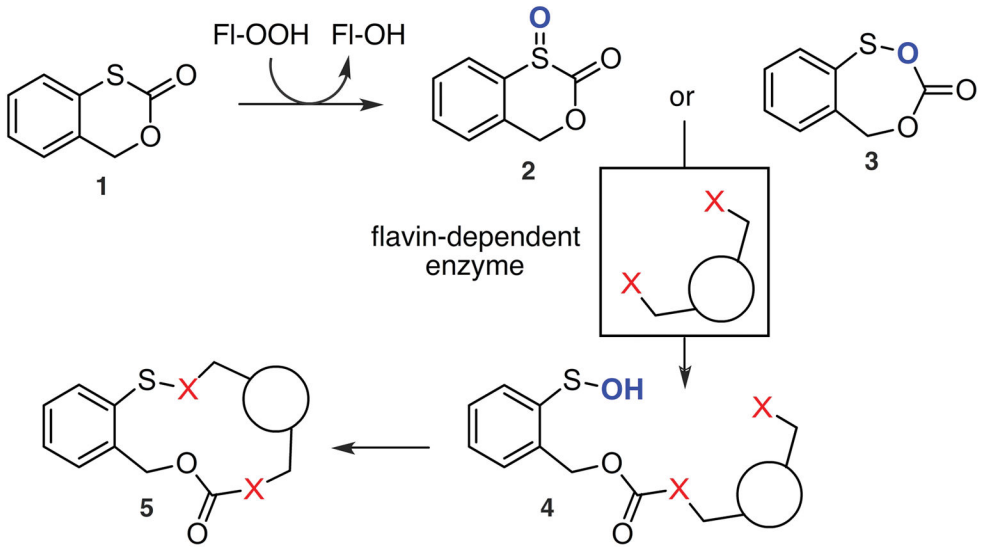
Figure 4.

Labeling in PltA in lysate and live cells. **a)–c)** Images of SDS-PAGE gels depicting 20 μL *E. coli* lysate containing of **1** mg/mL total protein doped with 5–15 μM PltA. Samples were incubated with **6** and NADPH for 30 min prior to analysis via SDS-PAGE. **a)** Coomassie-stained gel depicting total protein in the sample. **b)** Fluorescent image with excitation at λ_{max} at 365 nm prior Coomassie staining. **c)** Western blot from the gel in a) generated after development with an anti-IAF tag XRI-TF35 mAb (primary) and development using AP-labeled anti-mouse mAb (secondary). d) A 5 mL sample of the *E. coli* strain engineered to overexpress PltA (8×10^8 cells/mL) was treated with either 0 or 15 μM probe **6** for 30 min at 25 °C. The cells were lysed and subjected to SDS-PAGE and Western blotting as in c). Western blot controls for wild type *E. coli* and PltA expression levels are provided in Supporting Figure S9. Concentrations are provided in μM.



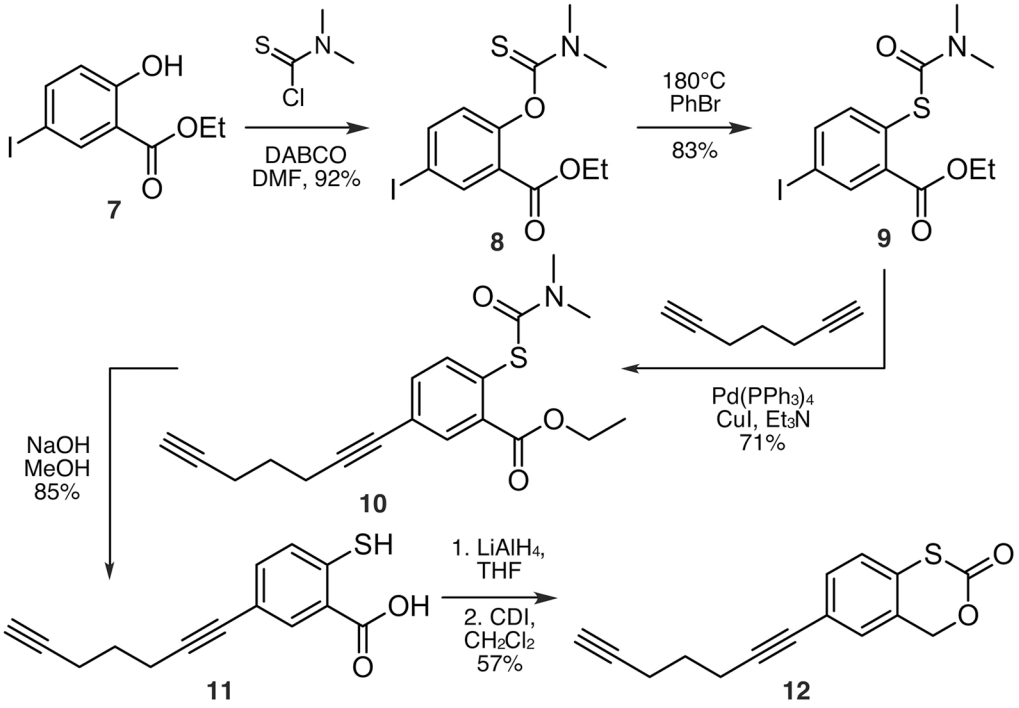
Scheme 1.

Action of a flavin-dependent enzymes as illustrated by the Baeyer-Villiger oxidation of cyclohexanone (S_{RED}) to caprolactone (S_{OX}).[10]



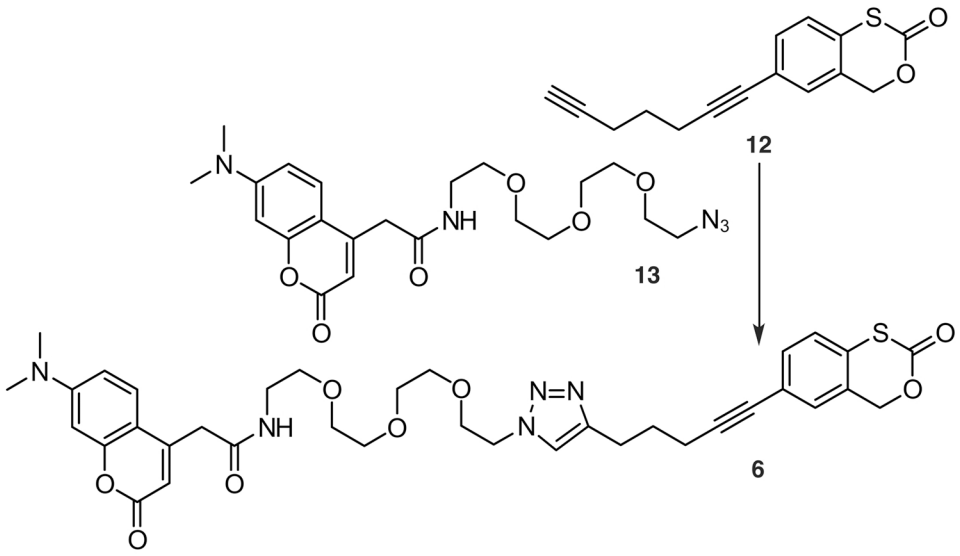
Scheme 2.

Proposed mechanism for the inhibition of flavin-dependent enzymes by **1**. The sphere represents a flavin-dependent enzyme, X (red) denotes nucleophilic residues within a flavin-dependent enzyme (in box) and O (blue) denotes oxygen atoms incorporated by the action of a flavin-dependent enzyme.



Scheme 3.

Probe **12** as prepared in 6 steps in an overall yield of 26% from ethyl 2-hydroxy-5-iodobenzoate (**7**).



Scheme 4.

Reporter-labeling of probe **12** was achieved in aqueous (0.05 eq. $CuSO_4$ in 0.10 eq. sodium ascorbate) and anhydrous conditions (CuI, EtN*i*Pr$_2$, toluene).