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A Robust Protein Host for Anchoring Chelating Ligands and Organocatalysts

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Dedicated to Andreas Pfaltz on the occasion of his 60th birthday

In order to put the previously proposed concept of directed evolution of hybrid catalysts (proteins that harbor synthetic transition-metal catalysts or organocatalysts) into practice, several prerequisites must be met. The availability of a robust host protein that can be expressed in sufficiently large amounts, and that can be purified in a simple manner is crucial. The thermostable enzyme tHisF from Thermotoga maritima*, which constitutes the synthase subunit of a bi-enzyme complex that is instrumental in the biosynthesis of histidine, fulfills these requirements. In the present study, fermentation has been miniaturized and parallelized, as has purification of the protein by simple heat treatment. Several mutants with strategically placed cysteines for subsequent bioconjugation have been produced. One of the tHisF mutants, Cys9Ala/Asp11Cys, was subjected to bioconjugation by the introduction of a variety of ligands for potential metal ligation, of a ligand/metal moiety, and of several organocatalytic entities that comprise a flavin or thiazolium salts. Characterization by mass spectrometry and tryptic digestion was achieved. As a result of this study, a platform for performing future directed evolution of these hybrid catalysts is now available.*

Introduction

Directed evolution[1] of enantioselective enzymes as catalysts in synthetic organic chemistry, which was first demonstrated in 1997,[2] has emerged as a fundamentally new approach to asymmetric catalysis.[3] It is based on the combination of random gene mutagenesis, expression of enzyme mutants and high or medium-throughput screening for enantiomeric purity (*ee* screening) of thousands of samples.[4] Following the identification of the most enantioselective enzyme variant (hit), the respective mutant gene is used as a template for another cycle of mutagenesis/expression/*ee* screening, a process which exerts evolutionary pressure.[1,2,3] It can be repeated as many times as necessary until the desired degree of enantioselectivity has been reached. We have applied this Darwinistic approach to such enzymes as lipases,[2,5] epoxide hydrolases,[6] and monooxygenases,[7] and other academic and industrial groups have contributed many further examples.[3]

In spite of these successful developments, directed evolution of functional enzymes has clear limitations, specifically regarding the restricted range of reaction types that biocatalysts can mediate.[8] For example, no enzyme or mutant thereof can catalyze such synthetically important transformations as enantioselective transition-metal-mediated olefin-hydrogenation or hydroformylation, allylic substitution, Heck couplings, etc., which are reactions that can be performed with synthetic transition-metal catalysts in organic solvents[9] or in aqueous medium.[10] Due to this limitation, in 2001/2002 we proposed the idea of directed evolution of hybrid catalysts.[11] It had been known for decades that ligand/metal moieties can be anchored to proteins, which leads to semisynthetic catalysts (hybrid catalysts) that mediate a variety of organic transformations.[12] However, in each case only a single catalyst is obtained, and there is no reason to expect that the protein environment around the ligand/metal moiety is ideal, as needed, for example, in enantioselective transformations. In contrast, our concept foresees the generation of a library of mutant proteins and en masse chemical modification (bioconjugation) thereof, with the formation of a library of potentially enantioselective hybrid catalysts, to be followed by *ee* screening, isolation of the respective mutant gene, and repetition of the overall process (Scheme 1). Thus, we envision the utilization of directed evolution as a means to tune transition-metal catalysts in an evolutionary manner.

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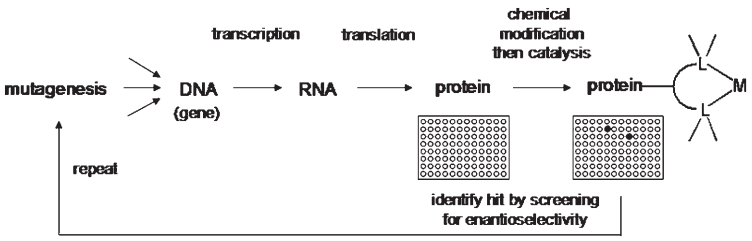

Scheme 1. Concept of directed evolution of hybrid catalysts showing the flow of genetic information from the gene to transition-metal hybrid catalysts.[11]

There are several technical problems that need to be solved before the concept can be put into practice. First, in contrast to conventional directed evolution experiments,[1–7] the mutants (clones) in a given library need to be purified because bioconjugation with introduction of a ligand/metal moiety can also occur with foreign protein, which leads to an undesired mixture of catalysts. Second, the expression system has to be very efficient to provide enough host protein for subsequent purification, bioconjugation and catalysis. Because essentially all synthetic transition-metal catalysts[9,10] have considerably lower activities than enzymes,[8] directed evolution experiments of the kind pictured in Scheme 1 require large quantities of protein. Indeed, for protein expression of many systems, the usual 1 mL reaction vessels of deep-well plates that are used in traditional directed evolution experiments are too small. In spite of the technical challenges delineated above, we were recently able to provide proof-of-principle for the first time,[13] by specifically making use of Whitesides' system in which a biotinylated diphosphine–Rh complex is anchored noncovalently to streptavidin.[14] This system has also been used by Ward et al. who performed *ee* optimization successfully by using chemical spacer modification and rational site-specific mutagenesis.[12h,14c–e] Our evolutionary approach is different. By going through three cycles of iterative CASTing (combinatorial active-site saturation test),[6b] it was possible to increase the enantioselectivity of the Rh-catalyzed hydrogenation of an α-acetamido acrylic acid ester stepwise from 23 to 65% *ee*.[13] This study, however, was extremely labor intensive, because even the best currently available expression system of streptavidin does not deliver enough protein to fulfill the requirements for efficient and extensive directed evolution. Thus, it was not possible to work with normal microtiter plates. Rather, fermentation of each clone was performed typically in 500 mL baffled flasks (125 mL culture volume). Following protein purification, asymmetric hydrogenation was carried out in 10-mL glass vessels of an in-house adapted reactor block for the Chemspeed Accelerator™ SLT100 Synthesizer.[15] Therefore, only very small mutant libraries (< 200 clones) could be handled in a slow and tedious overall process.

In order to develop a more practical variation of Scheme 1, two options appeared to be logical: 1) improve the expression system for streptavidin considerably, or 2) turn to an alternative protein as a potential host for anchoring ligand/metal moieties. Here we describe such an alternative system, based on the use of a thermostable enzyme that is easy to purify.

tHisF as a host for synthetic catalysts: parallel expression and purification

The thermostable enzyme tHisF from *Thermotoga maritima* constitutes the synthase subunit of the glutaminase–synthase bi-enzyme complex that catalyzes the formation of imidazole glycerol phosphate in histidine biosynthesis.[16a,b] Monomeric tHisF contains 253 amino acid residues and has a molecular mass of 27.7 kDa. It has a $(\beta\alpha)_8$-barrel structure, which is the most common enzyme fold found in nature.[16c,d] In the canonical $(\beta\alpha)_8$-barrel fold, the eight strands form a central parallel β sheet, the barrel, which is surrounded by the eight α helices (Figure 1). In all known $(\beta\alpha)_8$-barrels, the

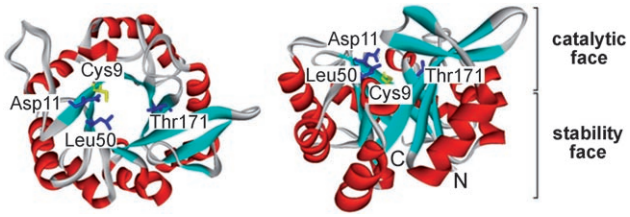

Figure 1. X-ray structure of tHisF.[16c] Left: top view of the C-terminal face of the central β barrel that contains the active site; right: side view. The central β barrel is depicted in cyan, and the surrounding α helices are depicted in red. In the present study, the positions suitable for potential bioconjugation following the introduction of cysteine are 11, 50, and 171 (blue), whereas Cys9 (yellow) was mutated to Ala.[16b] In the side view, the N and C termini of the polypeptide chain are marked.

active-site residues are located at the C-terminal ends of the β strands and in the βα loops, which connect the strands with the following α helices ("catalytic face"[16d]). In contrast, the αβ loops connect the α helices with the following β strands to form the "stability face"[16d] of the barrel.

We thought that this protein could be an ideal candidate for hosting synthetic catalysts such as ligand/metal entities or organocatalysts for several reasons.[17] First, protein expression in a 38 L fermenter in *E. coli* was already established, and it delivers approximately 12 mg of pure monomeric protein per liter of culture medium following purification.[16e] An alternative procedure, which was optimized in 3 L shaking flasks, produces 100 mg of protein per liter of culture medium.[16f] Second, the pronounced thermostability of tHisF allows a heat treatment as a straightforward way to parallelize purification, because such a process leads to the denaturation and precipitation of undesired proteins.[16,17] Third, the X-ray structure of tHisF[16c] suggests that the wide upper rim at the catalytic face of the enzyme might be a convenient site for covalent anchoring of synthetic transition-metal catalysts or organocatalysts (see next section).

As an initial step toward establishing a platform for performing directed evolution of hybrid catalysts based on tHisF, we first optimized laboratory-scale fermentation of the wild-type (WT) protein. This provided us with sufficient amounts for characterization and parallelization experiments. State-of-the-art

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fermentation protocols allowed the production of 400 to 500 mg of essentially pure tHisF within 1.5 days by using a 5 L fermentation unit (see Experimental Section). In our early attempts to miniaturize and parallelize this protocol, we studied the fermentation process more closely.[17a] Accordingly, induction with isopropyl β-D-thiogalactopyranoside (IPTG) and the purification process were optimized. A final concentration of 1.0 mM IPTG proved to be ideal for expression. Heating the crude lysates in 96-well plates at 72 °C for 30 min afforded the best results in the heat-purification. A 19 and subsequently a 24-fold fermentation unit were then developed and optimized (Scheme 2).

In order to be able to produce practical amounts of tHisF in a parallel fashion, we decided to use deep 24-well plates (Scheme 2), which is a trade-off between ease of fermentation on the one hand (deep-well plates) and large fermentation volumes to obtain practical amounts of tHisF on the other. By using Luria–Bertani (LB) medium, the OD_{600} range, in which induction with IPTG resulted in good levels of expression, turned out to be narrow (see Scheme 2). The individual wells were unlikely to reach this range simultaneously, which makes the procedure shown in Scheme 2 less suitable for high-throughput. Previously, Studier reported facile and reproducible expression of proteins that were encoded on a pET vector (by making use of catabolite repression).[18] Indeed, in the case of tHisF, this auto-induction system allowed high-density parallel fermentation without the additional induction step. However, the resulting tHisF preparation contained significant amounts of impurities even after prolonged heat treatment, which cannot be tolerated in our mission (Scheme 1). This serious problem was finally solved by inducing protein expression permanently from the beginning of the fermentation (instead of the more tedious induction at a certain cell density as is shown in Scheme 2) by using a buffered medium supplemented with additional glycerol (TB medium instead of LB medium). The workup after protein expression was performed analogously to the procedure that is shown in Scheme 2, except that the ammonium sulfate precipitation step was omitted because the protein was already essentially pure (~95% as judged by SDS-PAGE). We found that this very straightforward parallel fermentation on the 12.8 mL scale (4×3.2 mL) in deep 24-well plates provides an average of 1.2 mg (0.044 mmol) of pure tHisF (as judged by SDS-PAGE). Thus, reproducible expression and purification were achieved by using this improved protocol.

The development of the optimized 24-well fermentation unit and the successful parallel purification procedure taken together constitute a potential platform for performing directed evolution of selective hybrid catalysts (Scheme 1). In this context, the parallelization in 24-well-plates offers the possibility of using robotics in order to achieve higher throughput. Moreover, further parallelization following the fermentation step, for example in a 96-well format, can be achieved easily by the use of pipetting robots. The next step in completing the platform involves reliable bioconjugation with the introduction of ligands needed for transition-metal catalysts or organocatalysts.

19-fold fermentation unit

- 19 × 40 mL sterile LB medium
- Inoculation with 0.5 mL preculture, incubation at 37 °C
- Induction with 40 µL 1 M IPTG at OD_{600} = 0.4–0.7
- Centrifugation after 6 h at 3200 *g*/10 min/4 °C in rotor for 30 tubes
- Rinsing of cell pellet with 1 mL phosphate buffer (100 mM, pH = 7.8)
- Resuspension in 400 µL phosphate buffer (10 mM, pH = 7.8 containing 2 mM DTT and 12.5 units mL^{-1} benzonase)

Deep 24-well plate

- 24 × 5 mL sterile LB medium
- Corresponds to 576 cultures per incubator
- Inoculation with 0.1 mL preculture, incubation at 37 °C
- Induction with 5 µL 1 M IPTG at OD_{600} = 0.4–0.7
- Centrifugation after 6 h at 3200 *g*/40 min/4 °C by using rotor for 4 plates
- Rinsing of cell pellet with 200 µL phosphate buffer (100 mM, pH = 7.8)
- Resuspension in 100 µL phosphate buffer (10 mM, pH = 7.8 containing 2 mM DTT and 12.5 units mL^{-1} benzonase)

↓

- Transfer into 96-well plates suitable for PCR block
- 30 min heat treatment at 72 °C in PCR block (combined cell lysis and thermoprecipitation of *E. coli* host proteins)
- Centrifugation at 3200 *g*/120 min/4 °C in rotor for plates
- Collection of supernatant and freezing at –78 °C for storage

- Yield: approximately 3 mg in 200 µL buffer
- Yield: approximately 0.3 mg in 40 µL buffer

- Immediately before chemical modification: precipitation with 400 / 80 µL saturated ammonium sulfate, centrifugation, 2 × rinsing with 200 / 40 µL ammonium sulfate, resuspension of protein precipitate in phosphate buffer (10 mM, pH = 7.0).

Scheme 2. Parallel fermentation of tHisF. Comparison of the 19-fold fermentation unit and the initial fermentation process in 24-well plates.[17a]

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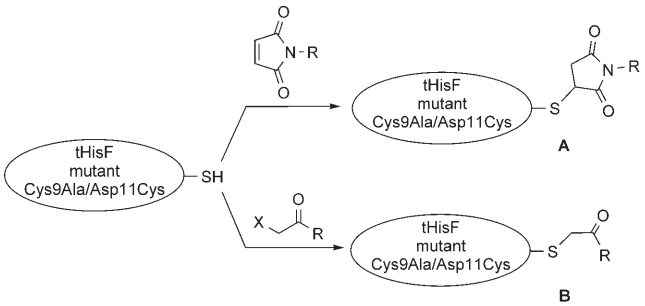
Bioconjugation of tHisF with formation of hybrid catalysts

One of the standard methods of anchoring ligands to proteins is based on S_N2 or Michael reactions at the thiol function of an appropriate cysteine.[12,19] The WT tHisF contains only one cysteine at position 9, which is located deep inside the barrel structure (Figure 1).[16c] Exploratory experiments had shown that chemical reactions at the respective thiol function occur sluggishly, if at all.[17] Therefore, we exchanged this cysteine residue for alanine (Cys9Ala) in the WT, and by considering the crystal structure of tHisF,[16a,c] we introduced cysteine at various positions that can be expected to be spatially accessible. One option was to perform site-directed mutagenesis with the introduction of cysteine at positions in the region of the lower (narrow) rim at the so-called "stability face" of the central β barrel where the flexible C and N termini are located (Figure 1). However, we chose another option, namely by focusing on the upper (relatively wide) rim at the "catalytic face" of the barrel where the natural cyclization reaction of tHisF is known to occur.[16b] This line of thought led us to believe that positions 11, 50 and 171 would be appropriate sites for the introduction of cysteine (Figure 1). Consequently, three tHisF mutants were generated as potential proteins for bioconjugation, namely Cys9Ala/Asp11Cys, Cys9Ala/Leu50Cys and Cys9Ala/Thr171Cys.

We then decided to concentrate first on mutant Cys9Ala/Asp11Cys as the host protein to which synthetic catalysts can be anchored. We chose the Michael addition to chemically modified maleimides and S_N2-reactions of α-halo carbonyl compounds (Scheme 3), which are bioconjugation reactions that are known to proceed rapidly and in high yield.[12,19]

Maleimides **1**–**9**, which harbor ligands, ligand/metals, or organocatalysts were chosen for Michael additions. In the case of bioconjugation that is based on appropriately modified α-halo carbonyl compounds, compound **10** was considered. Ligands **1**, **4**, and **10** were designed for potential transition-metal coordination (see also complex **2**), whereas **3** constitutes a potential flavin-dependent organocatalyst for possible Baeyer–Villiger reactions[20] (also sulfoxidation and/or oxidation of dihydronicotinamides). Finally, **5**, **6**, **7**, **8**, and **9** lead to potential organocatalysts for benzoin reactions that are similar to pyruvate decarboxylase[21] (Figure 2, Scheme 4, Scheme 5, Scheme 6, Scheme 7, Scheme 8, Scheme 9).

Chemical modification of mutant Cys9Ala/Asp11Cys (according to Scheme 3 by using compounds **1**–**10**) was then performed under standard conditions that have been described in the literature for other systems, to provide the respective bioconjugates.[12,19]



Scheme 3. Chemical modification of tHisF mutant Cys9Ala/Asp11Cys by means of Michael additions, that lead to bioconjugates **A**, and S_N2-reactions that provide bioconjugates **B**.

The bioconjugates were characterized by mass spectrometry (MS), and in some cases by limited proteolysis by using trypsin.[17] A few representative cases are discussed here. For example, the bioconjugate that was derived from tHisF mutant Cys9Ala/Asp11Cys and 5-iodoacetamide-1,10-phenanthroline (**10**) was characterized by a combination of ESI-TOF MS and limited proteolysis with trypsin. Trypsin is a serine protease that cleaves peptide bonds only on the C-terminal side of the basic amino acids lysine and arginine, thereby allowing for predictable selectivity. The amino acid sequence of tHisF comprises 7% lysine and 5% arginine.[16,17a] In a denaturated or highly

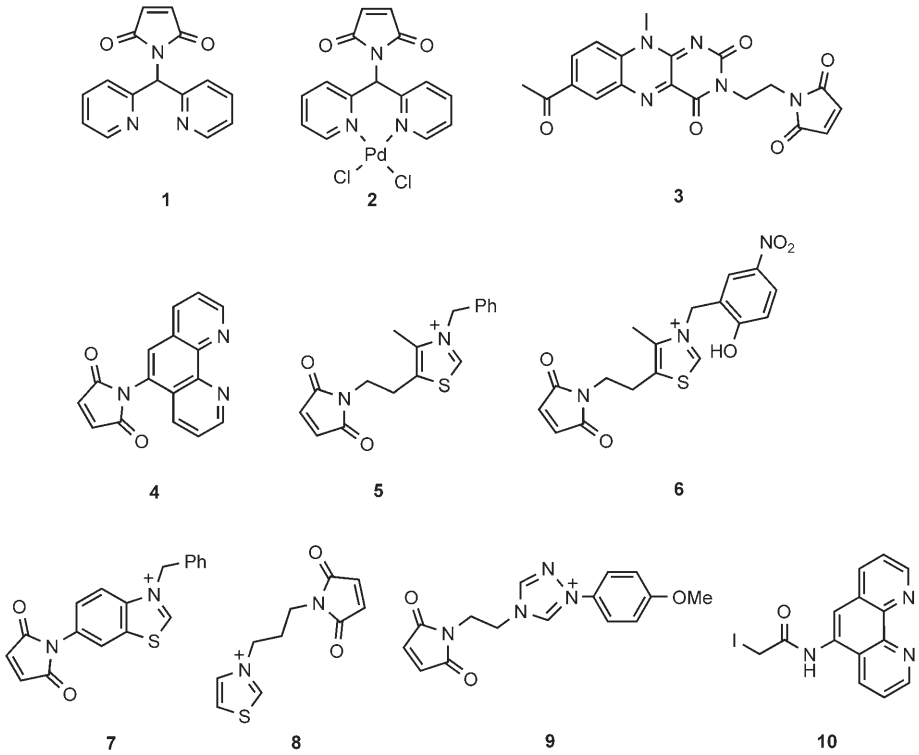


Figure 2. Compounds **1**,[11b] **2**,[11b] and **10**[22] were prepared according to literature procedures. Compounds **3**–**9** were synthesized according to the routes shown in Schemes 4–9.

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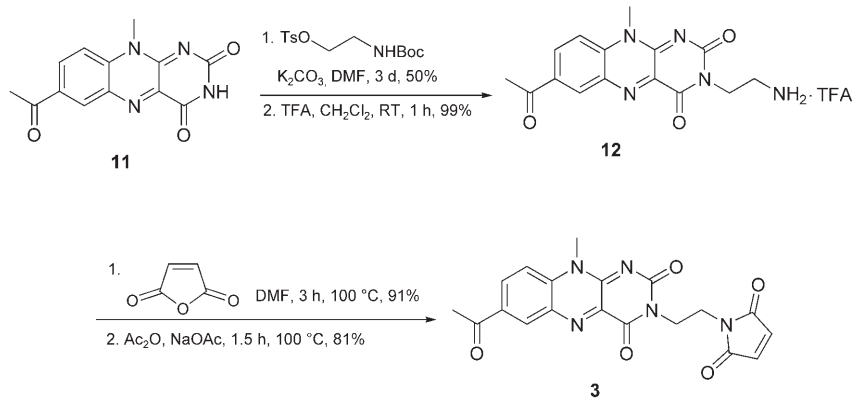
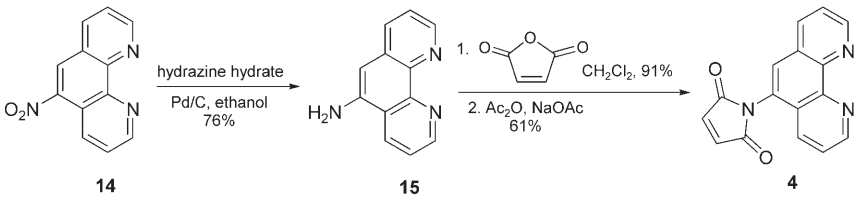
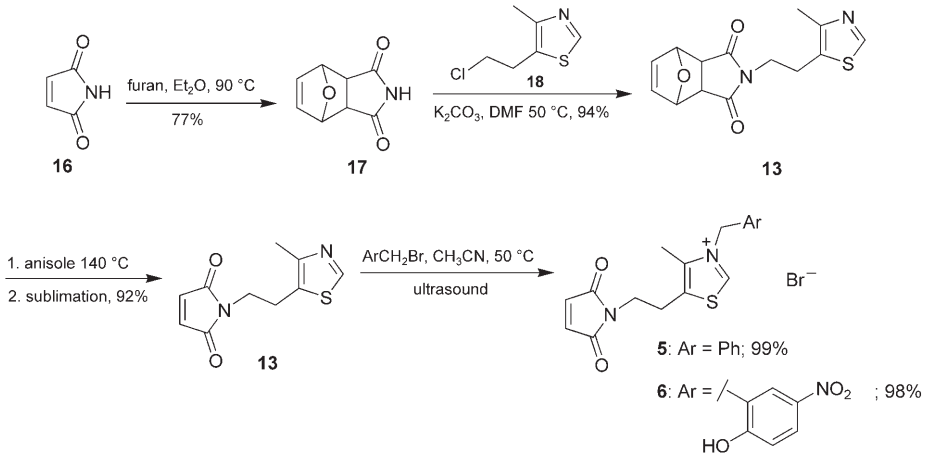


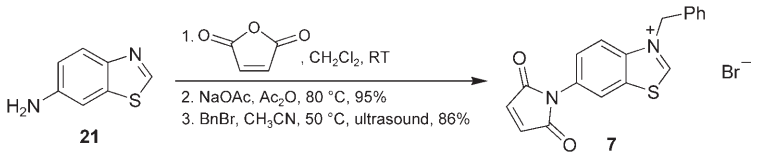
Scheme 4. Synthesis of maleimide **3**.



Scheme 5. Synthesis of maleimide **4**.



Scheme 6. Synthesis of maleimides **5** and **6**.



Scheme 7. Synthesis of maleimide **7**.

flexible protein, one would expect complete digestion by trypsin. However, it was previously shown that tHisF is accessible to trypsin digestion only at two positions due to its pronounced stability.[16b] Therefore, a combination of limited tryptic digestion and MS analysis was chosen to demonstrate that the chemical modification occurs in the desired part of the host protein. The reported cleavage positions are located at Arg27 and Lys58, though the cleavage occurs much faster at Arg27. The cysteine in position 11, which was modified with a variety of ligands is part of the N-terminal region that was cleaved off, and the remaining protein was analyzed by MS. Similar analyses were performed for bioconjugates of the other mutants Cys9Ala/Leu50Cys and Cys9Ala/Thr171Cys.[17a]

In a first set of experiments, it was shown that, upon modification of the tHisF mutant, Cys9Ala/Asp11Cys with iodoacetamide **10**, the expected mass difference is indeed detected by ESI-TOF MS (Table 1, entries 1 and 2). After tryptic digestion the mass expected from cleavage at Arg27 was observed for both the unmodified protein and the bioconjugate (Table 1, entries 3 and 4). This result clearly demonstrates that, as expected, the chemical modification of the host protein occurs within the N-terminal region that is subsequently cleaved off.

Moreover, these results show that the chemical modification of the host protein does not alter its behavior towards trypsin, a conclusion which is corroborated by following the progress of the tryptic digestion by SDS-PAGE (Figure 3). In the case of denaturation of the host protein upon chemical modification, more extensive fragmentation in the tryptic digestion would have been expected.

In a second set of experiments, the maleimide **1** was also shown to react in the expected way with the host protein. In addition to the tHisF mutant Cys9Ala/Asp11Cys, mutants Cys9Ala/Leu50Cys and Cys9Ala/Thr171Cys also turned out to be suitable for selective modification (Table 2).

In bioconjugation experiments followed by tryptic digestion, it was found in the case of mutant Cys9Ala/Asp11Cys, for example, that the mass of the resulting high-molecular-weight

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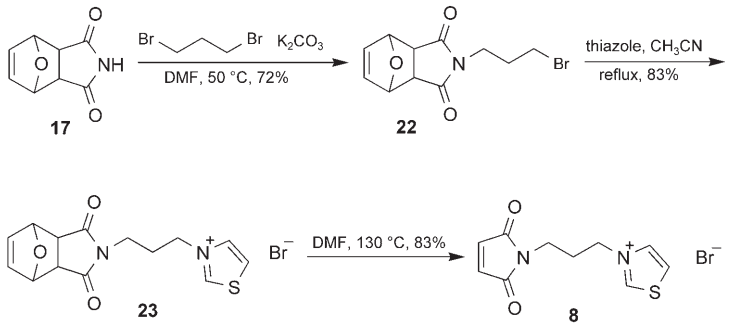
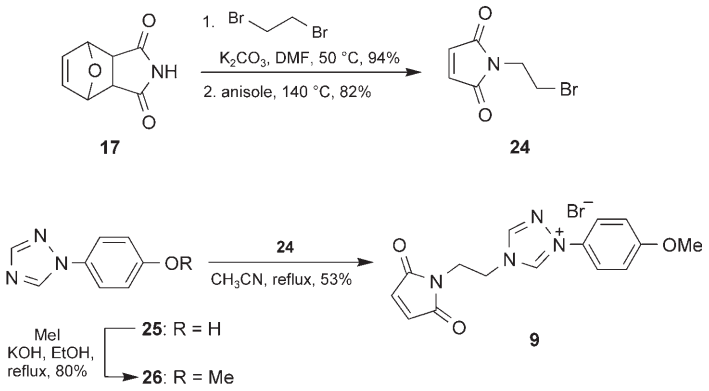


Scheme 8. Synthesis of maleimide **8**.



Scheme 9. Synthesis of maleimide **9**.

Table 1. ESI-TOF MS analysis of the bioconjugate derived from 5-iodoacetamide-1,10-phenanthroline (**10**) and tHisF mutant Cys9Ala/Asp11Cys and of the unmodified mutant as reference.

	Sample	Mass calcd	Mass from ESI-TOF-MS
1	mutant Cys9Ala/Asp11Cys	27662	27683
2	mutant Cys9Ala/Asp11Cys modified by **10**	27897 (Δ=235)	27913 (Δ=230)
3	mutant Cys9Ala/Asp11Cys unmodified; after tryptic digestion		24678
4	mutant Cys9Ala/Asp11Cys modified by **10**; after tryptic digestion	(Δ=3)	24681

fragment is the same as for the unmodified protein. This shows that chemical modification occurs within the first 27 amino acids (Table 3). In the case of the mutant Cys9Ala/Leu50Cys, the bioconjugate derived from maleimide **1** shows a higher mass than the digested unmodified mutant after tryptic digestion (Table 3). Here, the mass difference corresponds directly to the incorporated ligand, which demonstrates that in this case, tryptic digestion also leads to removal of the first 27 amino acids. Thus, cleavage in this mutant does not remove the modified region, as expected.

After the successful bioconjugation of tHisF mutant Cys9Ala/Asp11Cys with ligands **1** and **10**, we set out to study the chemical modification of the host protein with a preformed transition-metal catalyst. For that purpose we chose the Pd^{II} complex **2** and carried out bioconjugation under the same conditions. Conversion was tested by subsequent treatment with the dye 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (**27**) followed by SDS-PAGE analysis. This compound exhibits fluorescence upon Michael addition of free thiol groups to its maleimide moiety. Following UV irradiation of the gel, a signal was only obtained with the unmodified mutant; this confirms the quantitative reaction of Cys11 with **1** and **2**, respectively (Figure 4).

In addition to the above-described ligands for transition metals, various organocatalyst-based hybrid catalysts were prepared. For example, flavin **3** was used for the selective cysteine modification of the tHisF mutant Cys9Ala/Asp11Cys. This compound allows for direct quantification of the chemical modification because it has chromophoric properties. In view of future en masse paral-

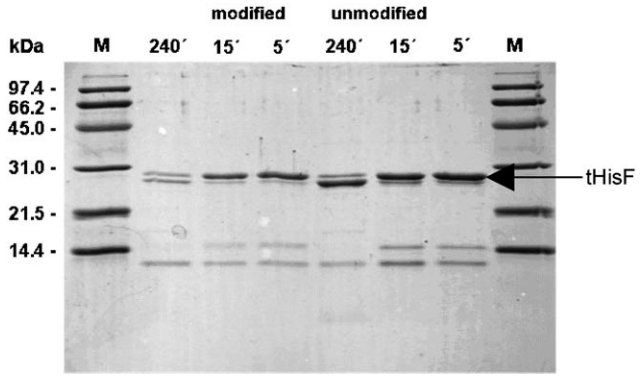


Figure 3. SDS-PAGE (12.5% acrylamide) of samples from limited proteolysis (5, 15, and 240 min) of the unmodified tHisF mutant Cys9Ala/Asp11Cys and its bioconjugate with 5-iodacetamido-1,10-phenanthroline (**10**). The observed fragmentation pattern suggests that modification does not significantly change the susceptibility of the tHisF mutant to trypsin; M: molecular weight marker.

Table 2. ESI-TOF MS analysis of tHisF mutants chemically modified by reaction with maleimide **1**. All numbers refer to mass units.

Protein	Unmodified	Modified	Δ_{obs} (Δ_{calcd}=265)
WT tHisF	27689	–	–
mutant Cys9Ala/Asp11Cys	27683	27948	265
mutant Cys9Ala/Leu50Cys	27682	27951	269
mutant Cys9Ala/Thr171Cys	27697	27962	265

Table 3. ESI-TOF MS analysis before and after tryptic digestion of chemically unmodified and modified tHisF mutants Cys9Ala/Asp11Cys and Cys9Ala/Leu50Cys; numbers refer to mass units.

Protein	Modification	Undigested	Digested	Δ
Cys9Ala/Asp11Cys	none	27683	24678	3005
	with **1**	27948	24676	3272
Cys9Ala/Leu50Cys	none	27682	24669	3013
	with **1**	27951	24933	3018

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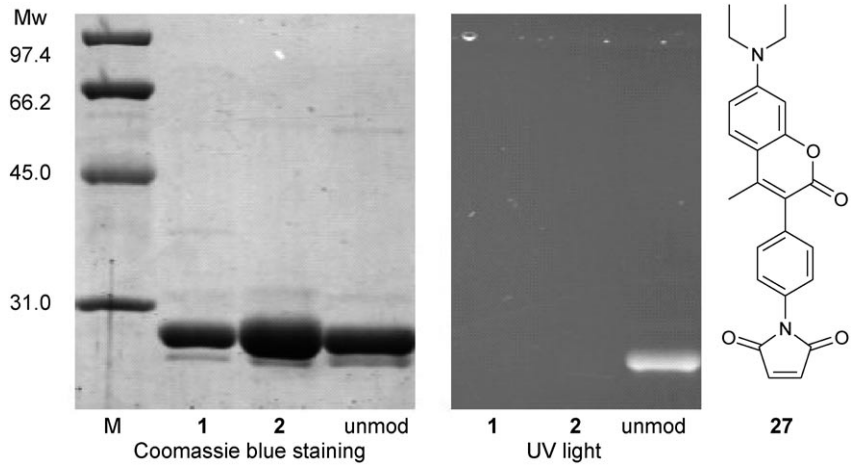

Figure 4. Modification of tHisF mutant Cys9Ala/Asp11Cys with ligand **1** and the corresponding Pd^{II} complex **2**. Quantitative modification of the host protein was confirmed by subsequent treatment with commercial dye **27** and SDS-PAGE analysis; M: molecular weight marker; unmod: unmodified mutant Cys9Ala/Asp11Cys.

lel bioconjugation of mutant libraries in the process of directed evolution experiments (Scheme 1), the course of the chemical modification process was studied more closely. In the bioconjugation experiments, 0.5, 1.0, and 2.0 equivalents of the flavin catalyst **3** were used. In order to check for possible unspecific modification of the host protein, the cysteine-free tHisF mutant Cys9Ala was studied under the same set of conditions (Figure 5).

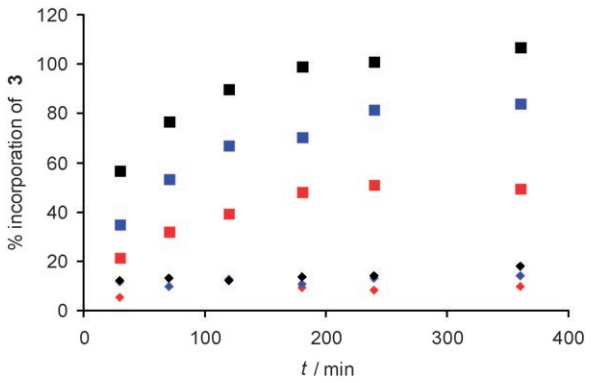

Figure 5. Progress and selectivity in the chemical modification of the tHisF mutant Cys9Ala/Asp11Cys with various amounts of flavin **3**. As a control for unspecific incorporation of **3**, the cysteine-free mutant Cys9Ala was studied under the same set of conditions. Cys9Ala/Asp11Cys with 2.0 equiv of **3** (■); Cys9Ala with 2.0 equiv of **3** (◆); Cys9Ala/Asp11Cys with 1.0 equiv of **3** (■); Cys9Ala with 1.0 equiv of **3** (◆); Cys9Ala/Asp11Cys with 0.5 equiv of **3** (■); Cys9Ala with 0.5 equiv of **3** (◆).

Two important results are apparent. First, bioconjugation is very selective, as evidenced by the extremely low degree of modification of the cysteine-free protein. Second, the results suggest that the use of stoichiometric or slightly substoichiometric amounts of the flavin **3** in an en masse chemical modification of mutant libraries is feasible without subsequent purification. This finding is important with regard to achieving maximum throughput in future screening processes.

The selectivity of this particular bioconjugation was confirmed by MALDI-TOF MS analysis (Table 4 and Figure 6). The observed mass difference with respect to the unmodified protein is in accord with the expected difference for the bound ligand within the error range of the method.

Figure 6 clearly shows that only a very small fraction of the protein remains unmodified and that double chemical modification is negligible. In analogous experiments, it was shown that the phenanthroline **4**, which bears a maleimide linker also has the desired reactivity towards the host protein and therefore appears to be suitable as a ligand for organocatalysis (base catalysis) or for transition-metal-based catalysis.[17a]

Table 4. Summary of data from MALDI-TOF analysis of bioconjugates that were derived from tHisF mutant Cys9Ala/Asp11Cys and ligands **3** and **4**; numbers refer to mass unit.

Protein	Peak maximum	$\Delta_{obs.}$	Δ_{calcd}
tHisF mutant Cys9Ala/Asp11Cys unmodified	27 665	–	–
tHisF mutant Cys9Ala/Asp11Cys modified by **3**	28 046	381	393
tHisF mutant Cys9Ala/Asp11Cys modified by **4**	27 958	293	275

Another class of organocatalysts considered in this study comprises thiazolium and triazolium moieties that promote reactions such as the benzoin reaction or Stetter-type transformations.[21] The thiazolium catalysts **5**, **6**, **8**, and triazolium catalyst **9** proved to be suitable for analogous modification of the cysteine thiol moiety of the tHisF mutant Cys9Ala/Asp11Cys, as shown by titration with Ellman's reagent.[23] The thiazolium derivative **7** was not suitable for bioconjugation experiments under the standard conditions due to poor solubility.

Conclusions

Although we recently provided proof-of-principle regarding the concept of directed evolution of enantioselective hybrid catalysts as outlined in Scheme 1,[11] the system that was employed had some flaws which prevented systematic investigations.[13] In that study, Whitesides' system,[14] which is comprised of a biotinylated diphosphine–Rh complex that is anchored noncovalently to streptavidin and is in principle well

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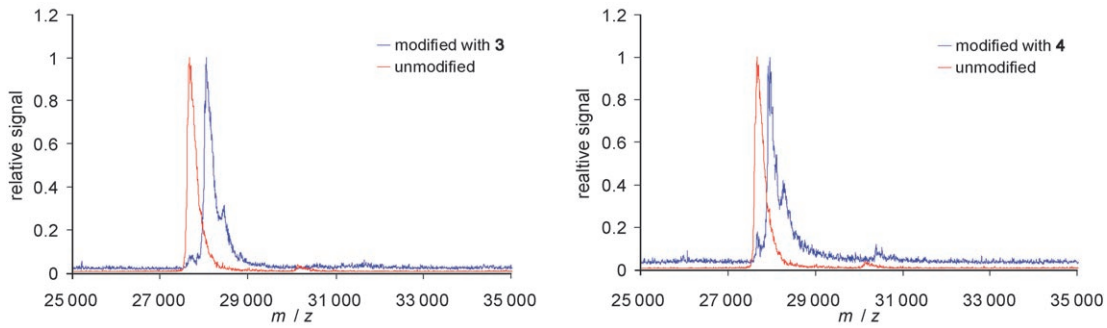

Figure 6. MALDI-TOF spectra of tHisF mutant Cys9Ala/Asp11Cys after modification with **3** and **4** (blue). The spectrum of the unmodified protein is shown in red for comparison.

suited for our goals, has the disadvantage that all currently known expression systems of the host streptavidin do not deliver enough protein to perform extensive directed evolution.[13] For that reason, an alternative host had to be found. In the present study we have developed a viable alternative. It is based on the thermophilic enzyme tHisF, which in nature catalyzes a crucial step in the biosynthesis of histidine.[16] Important for our application of tHisF as a protein for hosting synthetic catalysts is the fact that it is robust and can be expressed in large amounts in *E. coli*. We have shown here that fermentation can be miniaturized and carried out in parallel in a 24-well format, and that parallel purification is possible.

We have also demonstrated that bioconjugation with the introduction of ligands, ligand/metal entities, and organocatalysts is likewise easily achieved. Thus, a technological platform for future directed evolution of these interesting hybrid catalysts has been established. One may anticipate that directed evolution by using iterative CASTing[6b] will provide a means to control activity and enantioselectivity.

Experimental Section

General remarks: All chemicals were purchased from Aldrich, Fluka, or Acros and were used without further purification. Media components were purchased either from Applichem (Darmstadt, Germany) or Invitrogen.

Site-specific mutagenesis: The mutants tHisF-Cys9Ala/Asp11Cys, Cys9Ala/Leu50Cys, and Cys9Ala/Thr171Cys were produced by PCR-based methods by using the plasmid pET11c_tHisF-C9A as template.[16b] The mutant tHisF-Cys9Ala/Asp11Cys was generated by a standard PCR reaction by using the oligonucleotide 5′-TGATGA A-GA CAT ATG CTC GCT AAA AGA ATA ATC GCG GCT CTC **TGT** GTG AAA-3′ (new codon in bold) as the 5′ primer, and the T7 terminator as 3′ primer. By using the *Nde*I and *Bam*HI restriction sites, tHisF-Cys9Ala/Asp11Cys was cloned into pET11c.

The mutant tHisF-Cys9Ala/Leu50Cys was generated by overlap–extension PCR.[24] To produce the first megaprimer, oligonucleotide **A** (5′-CTC GTT TTT **TGC** GAT ATC ACC-3′; new codon in bold) was used as the 5′ primer, and oligonucleotide **B** (5′-CCC CAA GCT TTC A-CA ACC CCT CCA GTC TCA-3′; primer that introduced a *Hind*III restriction site at the end of the gene) was used as the 3′ primer. To produce the second megaprimer, oligonucleotide **C** (5′-TAT ACG CAT GCT CGC TAA AAG AAT AAT CGC-3′; primer that introduced a *SpH*I restriction site at the start of the gene) was used as the 5′ primer, and oligonucleotide 5′-GGT GAT ATC **GCA** AAA AAC GAG-3′ (new codon in bold) was used as the 3′ primer. The two megaprimers were purified and mixed with oligonucleotides **B** and **C** in a PCR that yielded the full-length tHisF-Cys9Ala/Leu50Cys gene.

The mutant tHisF-Cys9Ala/Thr171Cys was also generated by overlap–extension PCR. To produce the first megaprimer, oligonucleotide 5′-ATC CTG CTC **TGC** AGT ATC GAC-3′ (new codon in bold) was used as the 5′ primer, and oligonucleotide **B** was used as the 3′ primer. To produce the second megaprimer, oligonucleotide **C** was used as the 5′ primer, and oligonucleotide 5′-GTC GAT ACT **GCA** GAG CAG GAT-3′ (new codon in bold) was used as the 3′ primer. Again, the two megaprimers were purified and mixed with the oligonucleotides **B** and **C** in a PCR that yielded the full-length tHisF-Cys9Ala/Thr171Cys gene.

By using the *SpH*I and *Hind*III restriction sites, the two variants tHisF-Cys9Ala/Thr171Cys and tHisF-Cys9Ala/Thr171Cys were cloned into the plasmid pDS56/RBSII/*SpH*I.[25]

Parallel fermentation and purification of WT tHisF: For production of WT tHisF, LB medium that contained carbenicillin (100 mg L^{-1}) was inoculated with *E. coli* BL21(DE3) cells that had been transformed with pET11c-thisF,[16c] and treated according to Scheme 2 by using standard procedures. Alternatively, an improved and more straightforward protocol was followed by using permanent induction with IPTG (1 mM) and fermentation in 24-well plates (analogous to Scheme 2). In the latter protocol, a fresh overnight LB culture (20 μL) was added to TB medium (3.2 mL) in each well of the plate. Fermentation was carried out in a shaking incubator at 37 °C and 900 rpm (maximal amplitude attainable) for 7 h. Fermentation of each clone was performed in four wells, followed by centrifugation (4 °C, 15 min, Eppendorf centrifuge 5810R, rotor A-4-62, 4000 rpm). Cells were resuspended in buffer (200 μL per well, Tris-HCl, 100 mM, pH 8), and clones were pooled. Further concentration was achieved by a second centrifugation step and resuspension in a lysis buffer (400 μL) that was suitable for the next step: phosphate buffer or Tris-HCl (10 mM), pH 7.0–9.0. The resulting suspensions were stored at −78 °C, overnight. For combined lysis/thermoprecipitation of *E. coli* cells/proteins, the thawed cells were heated for 30 min at 75 °C, centrifuged (4 °C, 45 min, 14000 rpm, Eppendorf centrifuge 5810R, rotor F-45-30-11) and the clear supernatant was collected. This procedure was also applied for the fermentation and purification of tHisF mutants.

Large-scale protein expression and purification of WT tHisF and its mutants: Production of larger quantities of tHisF and the Cys9Ala/Asp11Cys mutant was achieved by using *E. coli* BL21-

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(DE3)pLysS cells that contained pET plasmids with the corresponding genes.[16] For production of the mutants Cys9Ala/Leu50Cys and Cys9Ala/Thr171Cys *E. coli* W3110*trp*EA2 cells[26] were used; these contained the pDS/RBSII/*Sph*I plasmid with the corresponding genes as well as the repressor plasmid pDM.[25] An overnight LB culture (100 mL) supplemented with the suitable antibiotics—that is, carbenicillin (100 mg L^{-1}) and chloramphenicol (34 mg L^{-1}) for the pET/pLysS-system, and ampicillin (150 mg L^{-1}) and kanamycin (75 mg L^{-1}) for the pDS/pDM-system—was used to inoculate a 5 L fermenter (Labfors HT, Infors AG, Switzerland) that contained TB medium supplemented with the same antibiotics. The culture was incubated at 37 °C with constant air supply and continuously stirred (700 rpm). Expression was induced at an OD_{600} between 0.4 and 0.6 by the addition of IPTG to a final concentration of 1 mM. After another 5 h of incubation, the cells were harvested by centrifugation (4 °C, 15 min, 6000 rpm, Sorvall Evolution Superspeed, rotor SLC 6000), washed with Tris-HCl (100 mM; pH 7.4) buffer, and centrifuged again (4 °C, 25 min, 8000 rpm, Sorvall Evolution Superspeed, rotor SLC 6000); the cell pellet was frozen. For cell lysis the pellet was resuspended in Tris-HCl buffer (10 mM; 150 mL, pH 7.4), supplemented with benzonase (20 μL; Boehringer). After sonication (Bandelin, 4×1 min, 40% pulse, on ice), the crude lysate was heated to 75 °C for 35 min, centrifuged (4 °C, 45 min, 14000 rpm, Eppendorf Centrifuge 5810R, rotor F-45-30-11), and the clear supernatant was collected. The supernatant was desalted by using a PD-10 desalting column (GE Healthcare) either with Tris-HCl buffer (10 mM; pH 7.2) or another buffer that was suitable for the subsequent experiment. In order to obtain protein of very high purity, the remaining impurities such as DNA were removed by anion-exchange chromatography with a stepwise gradient (5 mL, 5 mg mL^{-1} sample per run, Fractogel EMD DEAE (Merck) 16× 50 mm, starting buffer: 10 mM Tris–HCl pH 7.2, elution buffer: 10 mM Tris-HCl pH 7.2 with 1 M NaCl). Fractions that contained tHisF were pooled and desalted via a PD-10 desalting column (GE Healthcare). SDS-PAGE (12.5% acrylamide) of the protein showed single-band purity for all of the studied mutants.

General procedure for chemical modification of tHisF or mutants: A tHisF solution with a concentration of up to 5 mg/mL was mixed with a solution of the modification reagent in acetonitrile (**1**, **4**), DMF (**3**, **10**), or aqueous buffer (**5**, **6**, **8**, **9**). In the case that acetonitrile or DMF was used, the final concentration of the organic solvent was 5 vol%. To achieve selective modification, a buffered solution in the neutral pH range (pH 7.0–7.5) was used. The modification reactions were incubated in a shaking incubator for 14–24 h at 25 °C. Excess modification reagent was removed by gel-filtration (PD-10 desalting columns, GE Healthcare). Modifications with dye **27** were performed at room temperature for 16 h by using the ligand in excess.

Tryptic digestion of tHisF or mutant: A solution of tHisF (0.25 mg) in phosphate buffer (50 mM, 1 mL, pH 7.0) was incubated at 25 °C with trypsin solution (1 μL, 2 mg mL^{-1}), which was prepared from tablets (Sigma–Aldrich). For limited proteolysis, samples were taken after the respective digestion time and directly treated with a SDS-containing loading dye for subsequent analysis by SDS-PAGE.

ESI-TOF MS analyses: A sample of protein or modified protein (20 μL) was added to a water/acetonitrile (1:1) mixture (300 μL) followed by glacial acetic acid (6 μL). The final protein concentration was 0.5 mg mL^{-1}. Alternatively, formic acid instead of glacial acetic acid was used. The sample was injected with a flow-rate of 600 μL min^{-1} in the ESI source. The Micromass LCT ESI-TOF MS was calibrated with myoglobin as standard. Parameters: capillary: 3000 V, polarity: positive, sample cone: 40 V, extraction cone: 3.0 V, desolvation temperature: 250 °C, source temperature: 120 °C, MCP: 2600 V, RF lens: 600 V, mass range: 400–2500, cone gas flow: 1 L $h,^{-1}$ desolvation gas flow: 326 L $h.^{-1}$

Synthesis of modification reagents

Synthesis of modification reagent 3

tert-*Butyl* N-*(2-hydroxyethyl)carbamate*: This compound was prepared according to a modified procedure of Callahan.[27] Ethanolamine (5.18 g, 84.8 mmol, 5.08 mL) was dissolved in CH_2Cl_2 (180 mL), Et_3N (8.57 g, 84.8 mmol, 11.9 mL) was added and di-*tert*-butyl dicarbonate (18.5 g, 84.8 mmol) was added slowly. The initially turbid reaction mixture cleared up after approximately 10 min. The mixture was stirred for 4 h at room temperature, then washed with saturated aq NaCl (80 mL) and then with HCl (1 M, 100 mL). The organic phase was dried over $MgSO_4$, then concentrated to afford a colorless oil; yield: 13.4 g (83.2 mmol, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 5.2$ (br s, 1 H), 3.65 (t, $J = 4.9$ Hz, 2 H), 3.24 (t, $J = 4.9$ Hz, 2 H), 2.9 (br s, 1 H),1.40 ppm (s, 9 H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 156.4$, 61.9, 42.8, 30.7, 27.9 ppm.

tert-*Butyl* N-*[2-(tosyloxy)ethyl]carbamate*: *tert*-Butyl *N*-(2-*h*ydroxyethyl)carbamate (13.4 g, 83.3 mmol, 1.0 equiv) and triethylamine (18.9 g, 187 mmol, 26.3 mL, 2.25 equiv) were dissolved in CH_2Cl_2 (120 mL), and cooled in an ice/water-bath. A solution of tosyl chloride (17.44 g, 91.5 mmol, 1.1 equiv) in CH_2Cl_2 (120 mL) was added slowly to the cold solution. The mixture was stirred at room temperature for 60 h. It was washed successively with 10% (*w/v*) aq citric acid (3×70 mL) and saturated aq NaCl (70 mL), and then dried ($MgSO_4$). The crude product was treated with of EtOAc/hexanes (20 mL, 1:1) and crystallized at −20 °C. White crystals were isolated; yield: 18.73 g (59.3 mmol, 71%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.71$ (d, $J = 8.4$ Hz, 2 H), 7.27 (d, $J = 8.3$ Hz, 2 H), 4.8 (br s, 1 H), 3.99 (t, $J = 5.0$ Hz, 2 H), 3.30 (br d, $J = 4.9$ Hz, 2 H), 2.37 (s, 3 H), 1.33 ppm (s, 9 H).

*3-(*N*-Boc-2-aminoethyl)-7-acetyl-10-methylisoalloxazine*: 7-Acetyl-10-methylisoalloxazine (**11**; 1.20 g, 4.44 mmol) was prepared according to the procedure of Levine,[28] and was dissolved in DMF (180 mL). K_2CO_3 (920 mg, 6.66 mmol) and *tert*-butyl *N*-[2-(tosyloxy)ethyl]-carbamate (7.00 g, 22.2 mmol) were added and the mixture was stirred at room temperature in the dark for 3 days. The color changed from yellow to greenish-brown. Then the solvent was removed in vacuo. The brown residue was resuspended in EtOAc (50 mL) and H_2O (50 mL), and then it was extracted with EtOAc (5×50 mL) and CH_2Cl_2 (5×50 mL). Evaporation of the solvent gave the red crude product. Purification by flash chromatography (SiO_2, EtOAc/MeOH, 15:1) afforded desired product as a yellow solid; yield: 910 mg (2.20 mmol, 50%). ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta =$ 8.78 (d, $J = 1.9$ Hz, 1 H), 8.41 (dd, $J = 1.9$ and 9.0 Hz, 1 H), 8.09 (d, $J =$ 9.0 Hz, 1 H), 6.85 (t, $J = 5.4$ Hz, 1 H), 4.02–4.10 (m, 5 H), 3.26 (d, $J =$ 5.6 Hz, 2 H), 2.78 (s, 3 H), 1.39 ppm (s, 9 H); ^{13}C NMR (100 MHz, [D_6]DMSO): $\delta = 196.8$, 159.4, 156.0, 155.1, 150.2, 139.2, 136.4, 134.4, 134.1, 133.3, 132.7, 117.4, 78.0, 41.3, 38.0, 32.3, 28.6, 27.2 ppm; HRMS (ESIpos-ICR): *m/z* calcd for $C_{20}H_{23}N_5O_5Na$: 436.15968; found: 436.15988 $[M+Na]^+$.

*3-(2-Aminoethyl)-7-acetyl-10-methylisoalloxazine-trifluoracetate (**12**)*: 3-(*N*-Boc-2-aminoethyl)-7-acetyl-10-methylisoalloxazine (500 mg, 1.21 mmol) was dissolved in CH_2Cl_2 (30 mL) and TFA (30 mL) and stirred at room temperature. After 2 h the solvent was removed in vacuo. The resulting residue was treated with MTBE (15 mL) and a yellow precipitate formed. The clear supernatant was discarded. The yellow solid was dried in vacuo to give the pure desired prod-

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uct; yield: 510 mg (1.21 mmol, 99%). ^{1}H NMR (300 MHz, [D$_4$]MeOH) δ=8.74 (d, J=1.9 Hz, 1H), 8.52 (dd, J=1.9 and 9.1 Hz, 1H), 8.07 (d, J=9.1 Hz, 1H), 4.42 (t, J=5.8 Hz, 2H), 4.16 (s, 3H), 3.38 (t, J=5.8 Hz, 2H), 2.75 ppm (s, 3H); ^{13}C NMR (75.5 MHz, [D$_4$]MeOH) δ=198.2, 162.3, 158.2, 152.0, 139.9, 138.2, 136.8, 136.6, 135.8, 134.3, 118.6, 40.9, 40.0, 33.5, 27.6, 27.1 ppm; HRMS (ESIpos-ICR): m/z calcd for $C_{15}H_{16}N_5O_3$: 314.124764; found: 314.124627 [M+H]$^+$.

3-(2-Maleiamidoethyl)-7-acetyl-10-methylisoalloxazine: 3-(2-Aminoethyl)-7-acetyl-10-methylisoalloxazine trifluoracetate (**12**; 510 mg, 1.21 mmol) and maleic anhydride (597 mg, 6.08 mmol) were dissolved in DMF (20 mL) and stirred for 3 h at 100 °C. The mixture was cooled to room temperature, and the solvent was removed in vacuo. The yellow residue was washed with MTBE (3×15 mL) in order to remove excess maleic anhydride; yield: 455 mg (1.11 mmol, 91%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=14.6 (br s, 1H), 9.1 (br s, 1H), 8.76 (d, J=1.9 Hz, 1H), 8.38 (dd, J=1.9 and 9.0 Hz, 1H), 8.07 (d, J=9.0 Hz, 1H), 6.29 (d, 12.4 Hz, 1H), 6.21 (d, J=12.4 Hz, 1H), 4.07 (t, J=6.2 Hz, 2H), 4.02 (s, 3H), 3.48 (q, J=6.2 and 12.2 Hz, 2H), 2.74 ppm (s, 3H); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): δ=196.8, 165.9, 159.6, 155.1, 150.2, 139.1, 136.5, 134.5, 134.2, 132.9, 131.8, 117.5, 37.0, 32.3, 27.2 ppm; HRMS (ESIpos-ICR): m/z calcd for $C_{19}H_{17}N_5NaO_6$: 434.107100; found: 434.107268 [M+Na]$^+$.

*3-(2-Maleimidoethyl)-7-acetyl-10-methylisoalloxazine (**3**)*: 3-(2-Maleinamidoethyl)-7-acetyl-10-methylisoalloxazine (330 mg, 0.802 mmol) and NaOAc (1.30 g) were suspended in Ac_2O (20 mL), and stirred for 80 min in a 100 °C oil bath. The mixture was cooled to room temperature, and Ac_2O was removed in vacuo. The greenish-yellow residue was resuspended in H_2O (15 mL) and stirred for 10 min at room temperature. The crude product was collected by filtration as a yellow solid. In addition, the filtrate was extracted with CH_2Cl_2 (4×25 mL). The collected organic phase was dried ($MgSO_4$), and the crude product was purified by flash chromatography (SiO_2, EtOAc/MeOH, 19:1); yield: 256 mg (0.651 mmol, 81%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=8.76 (d, J=1.8 Hz, 1H), 8.38 (dd, J=1.9 and 9.0 Hz, 1H), 8.05 (d, J=9.0 Hz, 1H), 6.99 (s, 2H), 4.10 (t, J=5.3 Hz, 2H), 4.01 (s, 3H), 3.76 (t, J=5.2 Hz, 2H), 2.73 ppm (s, 3H); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): δ=196.8, 171.4, 159.5, 154.9, 150.1, 138.4, 136.6, 134.9, 134.6, 134.2, 133.5, 133.0, 117.6, 35.7, 32.5, 27.2 ppm; HRMS (ESIpos-ICR): m/z calcd for $C_{19}H_{15}N_5NaO_5$: 416.096536; found: 416.096849 [M+Na]$^+$.

Synthesis of modification reagent 4: Compound **4** was prepared according to a modified literature procedure.[29]

5-Amino-1,10-phenanthroline (15): 5-Nitro-1,10-phenanthroline (**14**; 1.2 g, 5.3 mmol), 10% Pd/C (200 mg) and hydrazine hydrate (1.0 mL) were stirred in EtOH (50 mL) and incubated in a 75 °C oil bath for 4 h. The reaction mixture was filtered, the filtrate was concentrated to a final volume of 10 mL, and the product was crystallized at 4 °C. The precipitate was washed with H_2O (2×10 mL) and dried in vacuo to afford the desired product as a yellow solid; yield: 770 mg (3.94 mmol, 74%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=9.05 (dd, J=1.6 and 4.2 Hz, 1H), 8.6–8.7 (m, 2H), 8.02 (dd, J=1.6 and 8.1 Hz, 1H), 7.33 (dd, J=4.2 and 8.3 Hz, 1H), 7.50 (dd, J=4.2 and 8.2 Hz, 1H), 6.86 (s, 1H), 6.13 ppm (s, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ=149.7, 146.5, 145.2, 143.0, 140.9, 133.0, 131.1, 130.9, 123.5, 122.4, 122.2, 102.1 ppm; MS (EI): m/z (rel.%): 195 (100), 168 (12), 140 (8).

5-Maleiamido-1,10-phenanthroline: 5-Amino-1,10-phenanthroline (**15**; 400 mg, 2.05 mmol) were suspended in CH_2Cl_2 (20 mL), and maleic anhydride (1.00 g, 10.2 mmol) was added. The reaction mixture was heated to reflux for 3.5 h. After cooling to room temperature, the solid was filtered off and washed with CH_2Cl_2; yield: 550 mg (1.88 mmol, 91%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=10.7 (br s, 1H), 9.13 (dd, J=1.5, 4.2 Hz, 1H), 9.05 (dd, J=1.6, 4.2 Hz, 1H), 8.73 (dd, J=0.8, 8.3 Hz, 1H), 8.44 (dd, J=1.4, 8.1 Hz, 1H), 8.22 (s, 1H), 7.7–7.9 (m, 2H), 6.73 (d, J=12.0 Hz, 1H), 6.39 ppm (d, J=12.0 Hz, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ=167.3, 165.0, 150.3, 149.8, 146.1, 144.2, 136.4, 132.9, 132.3, 131.6, 130.3, 128.3, 125.0, 124.0, 123.2, 120.7 ppm.

5-Maleimido-1,10-phenanthroline (4): 5-Maleiamido-1,10-phenanthroline (380 mg, 1.29 mmol) was added to a suspension of NaOAc (2 g) in Ac_2O (20 mL) and incubated for 1 h in a 100 °C oil bath. After cooling to room temperature, the mixture was poured into ice-water (80 mL) and stirred until the Ac_2O had decomposed completely. After extraction with CH_2Cl_2 (3×100 mL), the organic phase was washed with H_2O (3×100 mL), dried over $MgSO_4$, and concentrated to 10 mL. The addition of hexane (50 mL) provoked precipitation of the product, which was isolated as a white solid; yield: 216 mg (0.785 mmol, 61%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=9.15–9.25 (m, 2H), 8.21 (dd, J=1.7, 8.1 Hz, 1H), 7.90 (dd, J=1.7, 8.3 Hz, 1H), 7.55–7.65 (m, 2H), 7.69 (s, 1H), 6.97 ppm (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=169.9, 151.9, 151.3, 147.2, 146.7, 136.7, 135.1, 131.6, 128.0, 127.8, 126.5, 126.3, 123.9, 123.7 ppm.

Synthesis of modification reagent 5 and 6

*7-Oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboximide (**17**)*: This compound was prepared according to a modified literature procedure.[30] A solution of maleimide (5.00 g, 51.5 mmol) and furan (19.0 mL, 257 mmol) in Et_2O (175 mL) was heated in an autoclave for 24 h at 90 °C. The white precipitate that formed upon cooling was filtered off and dried under vacuum to give the desired product; yield: 6.59 g (39.9 mmol, 77%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=11.1 (br s, 1H), 6.53 (s, 2H), 5.12 (s, 2H), 2.85 ppm (s, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ=178.2, 136.8, 80.7, 48.8 ppm.

*5-(2-Chloroethyl)-4-methyl-thiazole (**18**)*: This compound was prepared according to a literature procedure.[31]

*Protected thiazole **19***: 7-Oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboximide (**17**; 1.00 g, 5.91 mmol) and K_2CO_3 (4.09 g, 29.6 mmol) were mixed under argon, and DMF (20 mL) was added. A solution of 5-(2-chloroethyl)-4-methyl-thiazole (**18**; 1.44 g, 8.87 mmol) in DMF (10 mL) was added dropwise to this suspension. The reaction mixture was heated to 50 °C for 3 h, cooled to room temperature, and concentrated in vacuo to give a viscous yellow oil. The crude product was taken up in H_2O (20 mL) and EtOAc (30 mL). The aqueous phase was extracted with EtOAc (3×30 mL). The combined organic phase was dried over $MgSO_4$. Purification by flash chromatography (SiO_2, hexanes/EtOAc, 4:6→2:8) gave a colorless viscous oil that crystallized upon standing to give a white solid; yield: 1.60 g (5.51 mmol, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.51 (s, 1H), 6.45 (s, 2H), 5.19 (s, 2H), 3.62 (t, 2H), 2.97 (t, 2H), 2.77 (s, 2H), 2.35 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=176.2, 150.7, 150.2, 136.9, 126.9, 81.2, 47.8, 39.7, 24.5, 15.1 ppm; MS (EI): m/z (%): 290 (13.4), 222 (44.1), 125 (100), 112 (20.7), 110 (21.3). HRMS: (ESIpos-ICR): m/z calcd for $C_{14}H_{14}N_2NaO_3S$: 313.061735; found: 313.061605 [M+Na]$^+$.

*Maleimido thiazole **20***: A solution of protected thiazole **19** (500 mg, 1.72 mmol) in anisole (4 mL) was heated under argon to 140 °C for 1 h. The reaction mixture was cooled down and was concentrated in vacuo to give a light-brown solid. Sublimation (0.04 mbar, from 110 to 170 °C in 30 min, then 170 °C for 15 min) afforded the desired product as a white solid; 353 mg (1.59 mmol, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.51 (s, 1H), 6.63 (s, 2H), 3.67 (t, 2H), 3.02 (t, 2H), 2.31 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ=170.7, 150.6, 150.3, 134.6, 127.0, 38.8, 25.4, 15.1 ppm; MS (EI) m/z (%): 222 (55.5),

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125 (100), 112 (48.0), 110 (42.5); HRMS: (EI): *m/z* calcd for $C_{10}H_{10}N_2O_2S$: 222.046303; found: 222.046005 $[M]^+$.

Thiazolium salt ***5***: A solution of maleimido thiazole **20** (300 mg, 1.35 mmol) and benzyl bromide (322 μL, 2.70 mmol) in anhydrous acetonitrile (1 mL) was heated to 50 °C under sonication, overnight. After cooling to room temperature, anhydrous Et_2O (50 mL) was added to provoke precipitation of a light-brown solid. The hygroscopic solid was triturated in Et_2O under argon and rinsed with dry Et_2O. After drying in vacuo in a dessicator with P_2O_5, the desired thiazolium salt was obtained as a beige powder; yield: 528 mg (1.34 mmol, 99%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=10.23$ (s, 1H), 7.5–7.1 (m, 5H), 6.96 (s, 2H), 5.81 (s, 2H), 3.67 (t, 2H), 3.20 (t, 2H), 2.19 ppm (s, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta=171.1$, 158.4, 143.1, 135.7, 134.9, 133.4, 129.6, 129.1, 127.7, 56.2, 37.8, 25.6, 11.5 ppm; MS (ESIpos): *m/z* (%): 312.9 (100); HRMS: (ESIpos-ICR): *m/z* calcd for $C_{17}H_{17}N_2O_2S$: 313.100527; found: 313.100764 $[M]^+$.

Thiazolium salt ***6***: Maleimido thiazole **20** (220 mg, 1.25 mmol) and 2-hydroxy-5-nitrobenzyl bromide (436 mg, 1.88 mmol) were dissolved in anhydrous acetonitrile (2 mL) under an argon atmosphere. Upon sonication of the solution for 2 h at 30 °C, a white precipitate formed. Anhydrous Et_2O (60 mL) was added, and the white solid was triturated. The solid was filtered off under an argon atmosphere and rinsed with Et_2O (2×20 mL). The white solid was dried in vacuo over P_2O_5; yield: 557 mg (1.23 mmol, 98%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta=11.87$ (s, 1H), 10.08 (s, 1H), 8.30 (d, $J=3.0$ Hz, 1H), 8.21 (dd, $J=3.0$ and 9.1 Hz, 1H), 7.12 (d, $J=9.2$ Hz, 1H), 6.94 (s, 2H), 5.75 (s, 2H), 3.65 (t, $J=6.3$ Hz, 2H), 3.20 (t, $J=6.3$ Hz, 2H), 2.34 ppm (s, 3H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta=171.0$, 162.7, 159.0, 143.3, 139.9, 134.9, 134.5, 127.6, 127.4, 119.9, 116.5, 52.2, 37.6, 25.4, 11.6 ppm; MS (ESIpos, *m/z* (%): 374.0 (100). HRMS (ESIpos-ICR): *m/z* calcd for $C_{17}H_{16}N_3O_5S$: 374.080516; found: 374.080402 $[M-Br]^+$.

Synthesis of modification reagent 7

6-Maleimido-benzothiazole: 6-Aminobenzothiazole (**21**; 500 mg, 3.33 mmol) and maleic anhydride (327 mg, 3.33 mmol) in a mixture of anhydrous CH_2Cl_2 (10 mL) and anhydrous Et_2O (10 mL) were stirred for 3 h at room temperature. The yellow solid was filtered off and dried in vacuo. Anhydrous NaOAc (5.46 g, 66.6 mmol) and Ac_2O (30 mL) were added. The suspension was heated to 80 °C under argon for 4 h. After cooling to room temperature, Ac_2O was removed in vacuo, and the remaining solid was partitioned between EtOAc (100 mL) and H_2O (50 mL). The aqueous phase was extracted with EtOAc (2×50 mL). The combined organic phase was washed with saturated aq $NaHCO_3$, and dried over $MgSO_4$. Removal of the solvent gave the pure desired product; yield: 730 mg (3.17 mmol, 95%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=8.99$ (s, 1H), 8.16 (d, $J=8.9$ Hz, 1H), 7.92 (d, $J=2.1$ Hz, 1H), 7.46 (dd, $J=2.1$, 8.8 Hz, 1H), 6.84 ppm (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) $\delta=169.8$, 155.6, 152.8, 134.7, 129.1, 124.8, 124.4, 120.0 ppm; MS (EI) *m/z* (%): 230 (100), 174 (12.8), 160 (18.1). HRMS: (EI): *m/z* calcd for $C_{11}H_6N_2O_2S$: 230.015003, found: 230.015255 $[M]^+$.

*3-Benzyl-6-maleimido-benzothiazolium bromide (**7**)*: 6-Maleimido-benzothiazole (446 mg, 1.94 mmol) and benzyl bromide (461 μL, 3.87 mmol) were mixed with anhydrous acetonitrile (2 mL) and sonicated at 50 °C, overnight. After it had cooled, the beige precipitate was filtered off, rinsed with EtOAc, and dried in vacuo to give the desired product (677 mg, 1.69 mmol, 86%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta=10.80$ (s, 1H), 8.56 (d, $J=1.9$ Hz, 1H), 8.41 (d, $J=9.1$ Hz, 1H), 7.54 (dd, $J=2.0$ and 9.1 Hz, 1H), 7.6–7.4 (m, 5H), 7.29 (s, 2H), 6.16 ppm (s, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta=169.8$, 166.7, 139.3, 135.4, 133.1, 132.8, 131.8, 129.53, 129.47, 128.7, 128.5, 123.3, 118.2, 55.8 ppm.

Synthesis of modification reagent 8

Protected N-*(3-bromopropyl) maleimide* ***22***: 1,3-Dibromopropane (2.50 mL, 24.2 mmol) was added dropwise to a suspension of 7-oxabicyclo[2.2.1]hept-5-ene-2,3-dicarboximide (**17**; 2.00 g, 12.1 mmol) and K_2CO_3 (8.37 g, 60.6 mmol) in anhydrous DMF (120 mL). The suspension was stirred at 50 °C for 1.5 h, allowed to cool to room temperature, and concentrated in vacuo. The residue was taken up in H_2O (20 mL) and EtOAc (50 mL). The aqueous phase was extracted with EtOAc (3×20 mL). The combined organic phase was dried over $MgSO_4$. Removal of the solvent gave the crude product (3.12 g) as a brown oil that crystallized upon standing. Purification by flash chromatography (SiO_2, hexanes/EtOAc, 7:3) gave the desired product (2.49 g, 8.71 mmol, 72%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.45$ (s, 2H), 5.20 (s, 2H), 3.57 (t, $J=6.8$ Hz, 2H), 3.27 (t, $J=6.8$ Hz, 2H), 2.78 (s, 2H), 2.07 ppm (tt, $J_1=J_2=6.8$ Hz , 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=176.5$, 136.9, 81.4, 47.8, 38.0, 31.0, 29.0 ppm; MS (EI) *m/z* (%): 287 (4.0), 285 (4.1), 219 (0.84), 217 (0.85), 138 (5.1), 110 (5.1), 68 (100). HRMS (EI): *m/z* calcd for $C_{11}H_{12}BrN_1NaO_3$: 307.989288; found: 307.989677 $[M+Na]^+$.

Protected thiazolium bromide ***23***: A solution of the protected *N*-(3-*b*romopropyl) maleimide **22** (500 mg, 1.75 mmol) and thiazole (248 μL, 3.50 mmol) in anhydrous acetonitrile (1 mL) was refluxed for 20 h under argon in a flask equipped with a cold finger. After cooling to room temperature and addition of anhydrous Et_2O (20 mL), a white precipitate that had formed during the reaction was filtered off and dried in vacuo over P_2O_5; yield: 538 mg, (1.45 mmol, 83%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta=10.19$ (dd, $J=1.3$, 2.3 Hz, 1H), 8.58 (dd, $J=1.3$, 3.7 Hz, 1H), 8.37 (d, $J=3.6$, 2.5 Hz, 1H), 6.57 (s, 2H), 5.15 (s, 2H), 4.52 (t, $J=7.1$ Hz, 2H), 3.39 (t, $J=7.1$ Hz, 2H), 2.96 (s, 2H), 2.12 ppm (tt, $J_1=J_2=7.0$ Hz, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta=176.9$, 160.0, 137.3, 136.8, 127.3, 80.7, 52.4, 47.6, 35.0, 28.3 ppm; MS (ESIpos, *m/z* (%)): 291 (49), 223 (100), 138 (33). HRMS (ESIpos): *m/z* calcd for $C_{14}H_{15}N_2O_3S$: 291.079793; found: 291.079869 $[M-Br]^+$.

Thiazolium bromide ***8***: Protected thiazolium bromide **23** (200 mg, 0.539 mmol) in anhydrous DMF (1 mL) was heated under an argon atmosphere to 135 °C for 1.5 h. After cooling to room temperature, the solvent was removed in vacuo to yield the desired product (135 mg, 0.445 mmol, 83%) as a beige solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta=10.17$ (dd, $J=1.3$ and 2.1 Hz, 1H), 8.58 (dd, $J=1.2$ and 3.7 Hz, 1H), 8.36 (dd, $J=2.3$ Hz and 3.7 Hz), 7.07 (s, 2H), 4.55 (t, $J=7.3$ Hz, 2H), 3.45 (t, $J=6.5$ Hz, 2H), 2.15 ppm (tt, $J_1=J_2=7.0$ Hz, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta=171.5$, 160.0, 137.4, 135.0, 127.2, 52.5, 34.3, 29.3 ppm; MS (ESIpos, *m/z* (%)): 223 (100). HRMS (ESIpos): *m/z* calcd for $C_{10}H_{11}N_2O_2S$: 223.053572; found: 223.053503 $[M-Br]^+$.

Synthesis of modification reagent 9

Protected N-*(2-bromoethyl) maleimide*: Dibromoethane (1.04 mL, 12.1 mmol) was added dropwise to a suspension of protected maleimide **17** (1.00 g, 6.06 mmol) and K_2CO_3 (4.19 g, 30.3 mmol) in anhydrous DMF (60 mL). The mixture was heated to 50 °C for 1.5 h, cooled to room temperature, and the solvent was removed in vacuo. The residue was taken up in H_2O (10 mL) and EtOAc (30 mL). The aqueous phase was extracted with EtOAc (2×20 mL). The combined organic phase was dried over $MgSO_4$. Evaporation of the solvent gave the desired product as a pale brown solid; yield: 1.55 g (5.70 mmol, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.46$

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(s, 2H), 5.22 (s, 2H), 3.83 (t, $J=6.9$ Hz, 2H), 3.42 (t, $J=6.8$ Hz, 2H), 2.82 ppm (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=175.3$, 136.2, 80.6, 47.1, 39.8, 26.5 ppm; MS (EI) *m/z* (%): 273 (2.0), 271 (2.0), 205 (0.7), 203 (0.7), 138 (1.6), 110 (3.8), 68 (100); HRMS (ESIpos-ICR): *m/z* calcd for $C_{10}H_{10}BrNNaO_3$: 293.973638; found: 293.973775 $[M]^+$.

N-*(2-Bromoethyl) maleimide (**24**)*: A solution of protected *N*-(2-bromoethyl)maleimide (800 mg, 2.94 mmol) in anisole (5 mL) was heated to 140 °C for 2 h. The mixture was allowed to cool to room temperature and the solvent was removed in vacuo to give a pale brown solid. Sublimation (0.06 mbar, from 50 °C to 90 °C over 30 min) afforded the desired product as a white solid; yield: 492 mg (2.41 mmol, 82%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.768$ (s, 2H), 3.95 (t, $J=6.6$ Hz, 2H), 3.54 ppm (t, $J=6.6$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=169.8$, 133.9, 38.8, 27.8 ppm; MS (EI) *m/z* (%): 205 (26.9), 203 (28.1), 110 (100); HRMS (EI): *m/z* calcd for $C_6H_6BrNO_2$: 202.958205; found: 202.957976 $[M]^+$.

*4-Methoxyphenyl-1,2,4-triazole (**26**)*: MeI (1.4 mL, 22.3 mmol) was added dropwise to a stirred solution of 4-hydroxyphenyl-1,2,4-triazole (**25**; 3.00 g, 18.6 mmol) and KOH (1.25 g, 22.3 mmol) in ethanol (100 mL). After complete addition of MeI, the mixture was heated to reflux for 14 h, then cooled to room temperature, and filtered. The filtrate was concentrated in vacuo and the remainder was taken up in CH_2Cl_2 (30 mL) and KOH (1 N, 20 mL). The aqueous phase was extracted with CH_2Cl_2 (2×20 mL). The combined organic phase was washed with saturated aq Na_2SO_3 and KOH (1 N), dried over $MgSO_4$, and the solvent was removed in vacuo to give the desired product as a pale orange solid; yield: 2.60 g (14.8 mmol, 80%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=8.38$ (s, 1H), 8.01 (s, 1H), 7.50 (m, 2H), 6.94 (m, 2H), 3.79 ppm (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=159.8$, 152.8, 141.2, 130.6, 122.3, 115.2, 56.0 ppm. MS (EI) *m/z* (%): 175 (100), 121 (56.5), 106 (14.2), 80 (18.1); HRMS (EI): *m/z* calcd for $C_9H_9N_3O$: 175.074560; found: 175.074697 $[M]^+$.

*Triazolium bromide **9***: A solution of 4-methoxyphenyl-1,2,4-triazole (**26**; 309 mg, 1.77 mmol) and *N*-(2-bromoethyl) maleimide (**24**; 300 mg, 1.47 mmol) in anhydrous acetonitrile (2 mL) was heated to reflux for 36 h, cooled to room temperature, and anhydrous Et_2O (40 mL) was added to provoke precipitation. The precipitate was filtered off and dried in vacuo. In order to remove traces of 4-methoxyphenyl-1,2,4-triazole, the precipitate was dissolved in MeOH (2 mL) and added dropwise to anhydrous Et_2O (50 mL) while it was stirred. The light-brown precipitate was filtered off and dried in vacuo over P_2O_5 to give the desired product; yield: 296 mg (0.781 mmol, 53%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta=$ 11.00 (s, 1H), 9.51 (s, 1H), 7.80 (m, 2H), 7.25 (m, 2H), 7.06 (s, 2H), 4.51 (t, $J=5.4$ Hz, 2H), 4.01 (t, $J=5.4$ Hz, 2H), 3.86 ppm (s, 3H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta=171.3$, 161.0, 145.6, 141.7, 135.2, 128.3, 122.7, 115.7, 56.2, 47.4, 37.3 ppm; MS (ESIpos, *m/z* (%)): 299 (100); HRMS (ESI-ICR): *m/z* calcd for $C_{15}H_{15}N_4O_3$: 299.113863; found: 299.114122 $[M-Br]^+$.

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