DOI: 10.1002/anie.201307386

Proteins

Mild Bioconjugation Through the Oxidative Coupling of *ortho*-Aminophenols and Anilines with Ferricyanide**

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Abstract: *Using a small-molecule-based screen, ferricyanide was identified as a mild and efficient oxidant for the coupling of anilines and o-aminophenols on protein substrates. This reaction is compatible with thiols and 1,2-diols, allowing its use in the creation of complex bioconjugates for use in biotechnology and materials applications.*

The synthetic modification of proteins is a critical aspect of chemical biology and biomaterials science. Synthetically modified proteins are used to study biochemical function,[1] modulate pharmacokinetics,[2] and construct new materials with applications in drug delivery[3,4] and targeted imaging.[5] Many of these applications require consistent, well-defined modifications that do not perturb the native protein structure or function. The precise modification of proteins, however, presents a significant chemical challenge as the modification must occur at ambient temperature, near neutral pH, and in the presence of a wide variety of unprotected functional groups.

A large number of methods have been developed for the site-selective modification of proteins.[6] These methods either rely on natural amino acids found in low abundance, typically targeting the nucleophilic thiolate side chain of cysteine,[6a,7] or they rely on engineered artificial amino acids.[6c] The latter approach can result in completely site-selective modification, but it depends critically on bioorthogonal[8] chemical reactions that can modify the functional groups with exquisite selectivity. Ketones,[9] azides,[10] strained alkenes,[11] alkynes,[12] and anilines[13] are commonly targeted in these reactions. These strategies are particularly useful for preparing proteins that are modified in multiple locations for use in biophysical and materials applications. In our own work,[14] the modification of proteins at two distinct locations is required for varied applications from light harvesting[15] to drug delivery[4] and water remediation.[16] Each of the complex biomolecule targets in these studies requires bioorthogonal methods that are compatible with cysteine chemistry.

Recent work in our group has explored the oxidative coupling reaction of aniline side chains with electron-rich aromatic coupling partners such as *o*-aminophenols.[13,17] Previous studies demonstrated the use of sodium periodate as an oxidant for highly efficient and rapid coupling. However, the ability of periodate to oxidize other moieties on proteins, notably cysteines and 1,2-diols found in glycans, may limit the scope of these coupling reactions. Additionally, the periodate-mediated coupling forms a mixture of two products, and complicates analysis and may prevent its use in applications such as antibody–drug conjugates which require well-defined linkages.[3] Herein, we report a substantially milder oxidant, potassium ferricyanide, which is capable of coupling anilines and *o*-aminophenols on protein substrates without oxidizing thiols or 1,2-diols (Figure 1a). Notably, a single, stable reaction product is obtained with this oxidant. We demonstrate the use of this reaction in conjunction with thiol maleimide chemistry as well as on a complex glycoprotein substrate. These optimized reaction conditions should allow the broad adoption of this coupling chemistry to virtually any bioconjugation target.

Our efforts to identify milder reaction conditions began with an HPLC-based assay to screen the ability of different oxidants to couple *p*-toluidine to electron-rich coupling partners, such as 2-amino-*p*-cresol. Reactions were run with 0.1 mM of each coupling partner and 1 mM oxidant at near-neutral pH (6.5–7.2) to mimic the conditions compatible for use on most biomolecules. Reactions were quenched with excess tris(2-carboxyethyl)phosphine (TCEP, 5–10 mM), and an internal standard, *p*-toluenesulfonic acid (*p*-TsOH, 0.2 mM), was added for reliable quantitation. Many oxidants screened (Figure 1b) were capable of coupling the small molecules, and more importantly generated the compound **1** as the sole product. The oxidants formed the [A + B] product with varying levels of conversion, requiring anywhere from 15 minutes to 18 hours to reach completion (Figure 1b,c and Figure S1 in the Supporting Information). We found Ag^{I}, Ce^{IV}, Cu^{II}, and Fe^{III} were particularly promising and potentially suitable for protein substrates.

We next determined the applicability of these oxidants for a protein substrate containing one aniline side chain (*p*-aminophenylalanine, *p*AF) in the primary sequence (Figure 1d). The aniline side chain (T19*p*AF) was introduced into the coat protein of the MS2 viral capsid using amber stop codon suppression (Figure 2a).[18] The MS2 capsid self-

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[**] This work was supported by a grant from the NSF (CHE 1059083). A.C.O was supported by an NSF graduate research fellowship and the UC Berkeley Chemical Biology Program (NRSA Training Grant 1 T32 GMO66698). J.B.J. was supported by a SURF Rose-Hills summer research fellowship, and C.N. was supported by an HHMI International Student Fellowship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201307386.

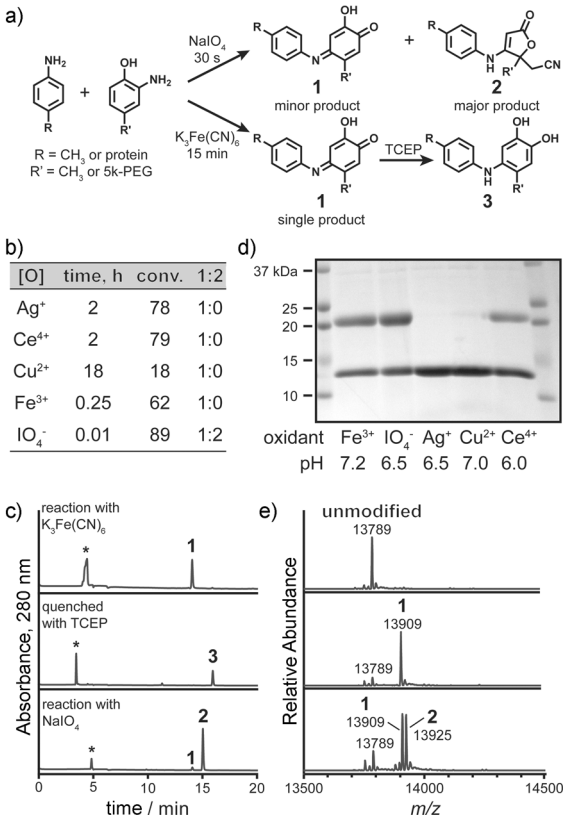


Figure 1. a) Schematic for the oxidative coupling of anilines and *o*-aminophenols. b) Oxidants were screened by HPLC to determine suitable alternatives to $NaIO_4$. c) Reverse-phase HPLC traces of the reactions shown in (a). Asterisks denote peaks that correspond to the oxidant. d) The same oxidants as in (b) were tested for their ability to mediate the coupling reaction on proteins using T19*p*AF MS2 and *o*-aminophenol 5k-PEG as model substrates. e) LC-MS analysis of MS2 monomers (top) modified with 2-amino-*p*-cresol shows that only one product is formed when ferricyanide is used as the oxidant (middle), whereas the use of periodate resulted in a mixture of two different products (bottom).

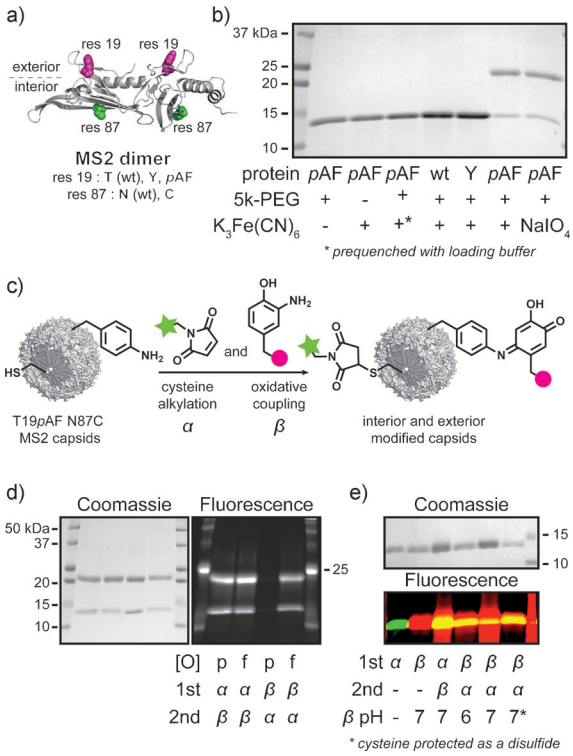


Figure 2. a) Representation of the MS2 coat protein dimer, indicating the location of residues 19 and 87 based on PDB ID: 2MS2. b) The coupling reaction was quenched by the addition of loading buffer (with DTT) and analyzed by SDS-PAGE. The lower band corresponds to unmodified MS2 monomers and the upper band corresponds to MS2 monomers modified with 5k-PEG. c) Schematic for the modification of both cysteine and *p*AF residues on the interior and exterior surface of MS2 capsids, respectively. d) MS2 capsids were first treated with either a fluorescent maleimide for cysteine alkylation (α) or *o*-aminophenol PEG for oxidative coupling (β). The modified proteins were then subjected to the other conditions (α or β) and analyzed by SDS-PAGE (periodate (p), ferricyanide (f)). e) Coomassie-stained and fluorescent images of SDS-PAGE gels of MS2 modified with a fluorescent maleimide (α) and a fluorescent *o*-aminophenol (β) under various conditions. The oxidative coupling reaction was carried out with $K_3Fe(CN)_6$ (analogous experiments with $NaIO_4$ appear in Figure S10).

assembles from 180 sequence identical monomers to form a hollow spherical structure.[19] Reactions were performed on the assembled capsid, but analysis was carried out on the monomers after disassembly. $K_3Fe(CN)_6$ and $Ce(NH_4)_2(NO_3)_6$ (CAN) maintained high reactivity on the protein substrate, whereas $AgCO_2CF_3$ and $CuSO_4$ showed poor reactivity on proteins. This was likely due to precipitation and slower reaction rates.

Ferricyanide [$K_3Fe(CN)_6$] was chosen for further development, as it was a milder oxidant than CAN and was compatible with proteins and most buffers.[20] Coupling a small molecule, 2-amino-*p*-cresol (80 μM), to the protein substrate (20 μM) with ferricyanide (1 mM) for 20 minutes showed near-complete modification to a single product by LC-MS (Figure 1e). When the reaction was run under the same reaction conditions with periodate, an equal mixture of two products was observed.

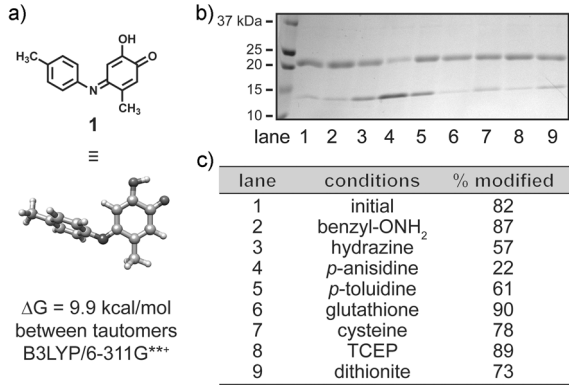
Initial observations from the HPLC reactivity screen indicated that ferricyanide formed the same iminoquinone as was the observed minor product (**1**) of the periodate reaction (see Figure S2). This product presumably arises from the 1,4-addition of the aniline to the oxidized *o*-aminophenol. Further oxidation and hydrolysis of the imine *ortho* to the alcohol gives the final product **1**. Two-dimensional NMR analyses and high-resolution mass spectrometry were used to confirm that ferricyanide formed product **1** (see Figure S3). Iminoquinone (**1**) could be reduced to corresponding hydroquinone **3** by TCEP.[13] This reduction was observed on small-molecule and protein substrates. However, over time the hydroquinone was observed to reoxidize to the iminoquinone in air.

The selectivity of the reaction was tested using several mutants of the MS2 coat protein: wild-type (wt), T19Y, and T19*p*AF (Figure 2a). Coupling was only observed when the oxidant, *o*-aminophenol, and aniline were all present (Figure 2b). In addition, the yield was comparable to that of the reaction mediated by periodate. At higher pH, some background reactivity with native amino acids was observed.

However, the coupling was selective for the *p*AF residue when the reaction pH was held between 6.0 and 6.5. This selectivity is likely because anilines are deprotonated under these conditions, thus rendering them uniquely nucleophilic. Additionally, it was found that addition of 1–10 mM imidazole could further prevent any undesired nonspecific reactivity. However this additive was not necessary for many of the protein substrates tested (see Figure S4).

Other iron(III) sources were evaluated for their ability to perform the coupling (see Figure S5). Most ferric salts tested were poorly soluble in water or rapidly formed insoluble iron oxides, and thus resulted in little to no coupling. Optimization of the equivalents, time, pH, and buffer revealed that high levels of conversion (> 75 %) could be achieved with only 2.5–5 equivalents of the aminophenol substrate in 15–20 minutes. In addition, neither the reaction pH nor the buffer salt was found to have an effect on the efficiency of the reaction (see Figure S6). However, caution should be taken when running the reaction at higher pH as the reaction may lose selectivity for the aniline side chain under more basic conditions. To reach high levels of conversion, 5 equivalents of *o*-aminophenol (relative to protein) and 10 equivalents of $K_3Fe(CN)_6$ (relative to *o*-aminophenol) should be used. Additionally, it was critical to purify the *o*-aminophenol substrate thoroughly and store the purified substrate at −20 °C before use to achieve high levels of modification.

Computational studies of **1** indicated a strong preference (ca. 10 kcal mol^{-1}) for the iminoquinone tautomer shown relative to the *o*-quinone structure (Figure 3a). This was confirmed by NMR spectroscopy, as only **1** was observed. Despite the presence of the imine moiety, **1** was found to be resistant to hydrolysis and relatively stable with respect to nucleophilic attack. The iminoquinone stability was assayed by subjecting purified, modified protein (ca. 80 % modified, 20 μM) to a range of nucleophilic and reducing additives (10 mM for 18 h at room temperature). Only in the presence of competing nucleophilic amines, such as *p*-anisidine, was appreciable loss of product observed (Figure 3b,c). Exposure



c)

lane	conditions	% modified
1	initial	82
2	benzyl-ONH_2	87
3	hydrazine	57
4	*p*-anisidine	22
5	*p*-toluidine	61
6	glutathione	90
7	cysteine	78
8	TCEP	89
9	dithionite	73

Figure 3. a) Computational studies and NMR analysis indicated that product **1** has a strong preference for the tautomer shown. b,c) The stability of the product was tested on MS2 after modification with *o*-aminophenol 5k-PEG. SDS-PAGE, followed by Coomassie staining and densitometry analysis (±5 % accuracy) was used to assess the stability. Additional data on pH and temperature stability is found in Figure S7.

to a wide range of physiologically relevant pH values (4.0–10.0), glutathione, or increased temperature (37 and 50 °C) for 18 hours did not result in product loss, thus indicating the relative stability of the product (see Figure S7). Additionally, no loss in product was observed after seven days of storage at room temperature at neutral pH.

Despite the mild nature of ferricyanide, we wanted to confirm that excess oxidant could be completely removed from the bioconjugation reaction.[20d,21] Using standard biomolecule purification techniques, such as gel filtration and ion exchange, it was possible to remove all detectable iron (< 0.1 μM,; see Figure S8).

The ferricyanide-mediated coupling was also evaluated for its compatibility with cysteine chemistry. In addition to the important biological role of cysteine, it is frequently a target for protein modification.[7] We tested the ability of the coupling to be used in conjunction with cysteine maleimide chemistry following the scheme outlined in Figure 2c. A cysteine introduced to the interior surface of MS2 capsids[5] was first modified with a fluorescent maleimide and then subjected to the oxidative coupling conditions. Up to 150 copies of PEG and DNA *o*-aminophenol-containing substrates were coupled to the fluorescently labeled viral capsid (see Figure S9).

We also confirmed that unmodified cysteines were still reactive after the oxidative coupling step. T19*p*AF N87C MS2 capsids were first reacted with *o*-aminophenol 5k-PEG using either $K_3Fe(CN)_6$ or $NaIO_4$ and were subsequently treated with a fluorescent maleimide. After exposure to periodate, the cysteine no longer reacted with the maleimide. However, after oxidative coupling with ferricyanide, the cysteine was successfully labeled with the fluorophore (Figure 2d). To rule out the possibility that this reactivity was seen because the 5k-PEG substrate was too large to diffuse into the interior of the capsids, we also verified that the cysteine maintained reactivity after oxidative coupling with a small-molecule aminophenol (Figure 2e). A fluorescent rhodamine aminophenol (compound **S5**, see Scheme S2 in the Supporting Information) was synthesized and reacted with the capsids. A spectrally separated fluorescent maleimide (Alexa Fluor 680 C_2-maleimide) was then used to assay the reactivity of the cysteine. The modified protein was analyzed by SDS-PAGE with two-color fluorescence detection. Only when ferricyanide was used as the oxidant was the thiol moiety still reactive after the oxidative coupling step (Figure 2e; see Figure S10 for reactions with periodate).

The mild nature of ferricyanide increases the compatibility of the oxidative coupling reaction with a broader scope of protein targets. Glycosylated proteins are attractive targets for modification, with antibody–drug conjugates serving as a prominent example.[3,22] While sodium periodate can be used to modify glycoproteins, it is also known to oxidize the 1,2-diols found in sugars.[23,24] To test the ability of ferricyanide to modify glycosylated proteins without this side reaction, an aniline moiety was first site-selectively introduced on the N-terminus of an engineered antibody fragment (Fc). The Fc was transaminated using pyrodixal-5′-phosphate (PLP), thus generating a uniquely reactive ketone at each N-terminus (Figure 4a).[24,25] This ketone was then modified with

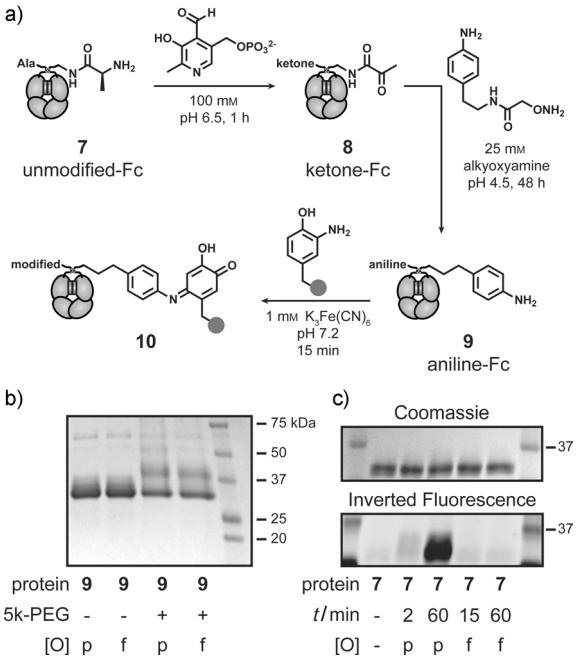


Figure 4. a) Schematic for the modification of anilines introduced by transamination of the N-terminus. The terminus was first converted into a ketone with pyridoxal-5′-phosphate (PLP). This ketone was modified with an alkoxyamine-functionalized aniline. The aniline then underwent oxidative coupling with *o*-aminophenol substrates. b) An engineered antibody fragment (Fc) was subjected to the conditions shown in (a). Analysis of the modification with *o*-aminophenol 5k-PEG by SDS-PAGE demonstrated the ability of ferricyanide to modify a complex glycoprotein substrate. c) The Fc sample was treated with either periodate (p) or ferricyanide (f) followed by a fluorescent alkoxyamine to probe for reactive, oxidized sugars (full gels from (b) and (c) appear in the Supporting Information).

an alkoxyamine-functionalized aniline. The aniline-Fc, **9**, was successfully PEGylated with a 5 kDa *o*-aminophenol PEG using ferricyanide (ca. 40% modified, Figure 4b). The lower level of modification was likely due to increased sterics as the N-termini of the two Fc chains are in close proximity of each other.

The undesired glycan oxidation was probed by treating the Fc (**7**) with either $NaIO_4$ or $K_3Fe(CN)_6$ followed by a fluorescent alkoxyamine (Figure 4c).[24] After the oxidants were quenched with TCEP, the oxidant-treated protein was incubated with an aminooxy dye to probe for reactive aldehydes formed as a byproduct of sugar oxidation. Exposure to periodate for 2 minutes resulted in a slight amount of oxidation of the glycan, with extensive oxidation observed after 1 hour of exposure. No oxidation of the Fc was observed after treatment with ferricyanide. Glycopeptide standards were also used to assess the reactivity of the oxidants toward 1,2-diols. Treatment of a glycosylated erythropoietin fragment (EPO, 117-131) with $K_3Fe(CN)_6$ resulted in no oxidation of the GalNAc residue over the course of 1 hour (see Figure S13). Incubation of $NaIO_4$ with glycosylated-EPO, however, resulted in rapid oxidation of the sugar. To investigate the compatibility of ferricyanide with 1,2-diols further, several additives were screened for their effect on reactivity. Mannose, glucose, and glycerol had little to no effect on the ferricyanide-mediated coupling, even at concentrations of 1M (see Figure S14). Periodate reactivity was significantly quenched in the presence of a vast excess of 1,2-diols. However, at lower concentrations (10 mM) these additives could be used to protect glycoproteins from periodate while still allowing the desired oxidative coupling to take place, as previously reported.[24]

In this study, we used a small-molecule-based screen to identify alternative oxidants for the coupling of *o*-aminophenols and anilines. Ferricyanide efficiently performed the coupling on both small molecules and proteins. The updated coupling reaction formed a single product with excellent conversion using only a few equivalents of the *o*-aminophenol coupling partner. The biomolecule compatibility of this reaction was demonstrated with several protein and aminophenol substrates. Importantly, the ferricyanide-mediated coupling of *o*-aminophenols and anilines was completely orthogonal to both thiol and 1,2-diol moieties, thus facilitating its use with cysteine chemistry and glycosylated substrates. These attributes make the reaction particularly appropriate for applications that require installation of two synthetic components onto a protein. As an example, we demonstrated the dual modification of a viral capsid using both cysteine-maleimide chemistry as well as the ferricyanide-mediated coupling. The oxidative coupling reaction can reliably be used on glycoproteins, such as antibodies or antibody fragments, without any undesired oxidation of the glycan. While the periodate-mediated coupling is both unfailing and useful, the ferricyanide-mediated reaction can successfully be used in situations when $NaIO_4$ is not compatible with the substrate. Taken together, both oxidative coupling strategies offer valuable new ways to create nearly any complex bioconjugation target.

Received: August 22, 2013
Published online: December 5, 2013

Keywords: amines · bioconjugation · iron · oxidation · proteins

[1] H. M. O'Hare, K. Johnsson, A. Gautier, *Curr. Opin. Struct. Biol.* **2007**, *17*, 488–494.
[2] S. Zalipsky, *Bioconjugate Chem.* **1995**, *6*, 150–165.
[3] S. C. Alley, N. M. Okeley, P. D. Senter, *Curr. Opin. Chem. Biol.* **2010**, *14*, 529–537.
[4] a) W. Wu, S. C. Hsiao, Z. M. Carrico, M. B. Francis, *Angew. Chem.* **2009**, *121*, 9657–9661; *Angew. Chem. Int. Ed.* **2009**, *48*, 9493–9497; b) N. Stephanopoulos, G. J. Tong, S. C. Hsiao, M. B. Francis, *ACS Nano* **2010**, *4*, 6014–6020.
[5] a) J. J. Day, B. V. Marquez, H. E. Beck, T. A. Aweda, P. D. Gawande, C. F. Meares, *Curr. Opin. Chem. Biol.* **2010**, *14*, 803–809; b) G. J. Tong, S. C. Hsiao, Z. M. Carrico, M. B. Francis, *J. Am. Chem. Soc.* **2009**, *131*, 11174–11178.
[6] a) E. Baslé, N. Joubert, M. Pucheault, *Chem. Biol.* **2010**, *17*, 213–227; b) E. M. Sletten, C. R. Bertozzi, *Angew. Chem.* **2009**, *121*, 7108–7133; *Angew. Chem. Int. Ed.* **2009**, *48*, 6974–6998; c) C. H. Kim, J. Y. Axup, P. G. Schultz, *Curr. Opin. Chem. Biol.* **2013**, *17*, 412–419; d) M. F. Debets, J. C. M. van Hest, F. P. J. T. Rutjes, *Org. Biomol. Chem.* **2013**, *11*, 6439–6455; e) Y. Takaoka, A. Ojida, I. Hamachi, *Angew. Chem.* **2013**, *125*, 4182–4200; *Angew. Chem. Int. Ed.* **2013**, *52*, 4088–4106.

[7] a) M. W. Crankshaw, G. A. Grant in *Current Protocols in Protein Science* (Eds.: J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher, P. T. Wingfield), Wiley, New York, **2001**, pp. 15.1.1 – 15.1.18; b) J. M. Chalker, G. J. L. Bernardes, Y. A. Lin, B. G. Davis, *Chem. Asian J.* **2009**, *4*, 630 – 640.
[8] H. C. Hang, C. Yu, D. L. Kato, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14846 – 14851.
[9] a) V. W. Cornish, K. M. Hahn, P. G. Schultz, *J. Am. Chem. Soc.* **1996**, *118*, 8150 – 8151; b) A. Dirksen, P. E. Dawson, *Bioconjugate Chem.* **2008**, *19*, 2543 – 2548.
[10] a) K. L. Kiick, E. Saxon, D. A. Tirrell, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 19 – 24; b) J. C. Jewett, C. R. Bertozzi, *Chem. Soc. Rev.* **2010**, *39*, 1272 – 1279.
[11] a) M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518 – 13519; b) N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, *Bioconjugate Chem.* **2008**, *19*, 2297 – 2299.
[12] N. Li, R. K. V. Lim, S. Edwardraja, Q. Lin, *J. Am. Chem. Soc.* **2011**, *133*, 15316 – 15319.
[13] C. R. Behrens, J. M. Hooker, A. C. Obermeyer, D. W. Romanini, E. M. Katz, M. B. Francis, *J. Am. Chem. Soc.* **2011**, *133*, 16398 – 16401.
[14] L. S. Witus, M. B. Francis, *Acc. Chem. Res.* **2011**, *44*, 774 – 783.
[15] a) R. A. Miller, A. D. Presley, M. B. Francis, *J. Am. Chem. Soc.* **2007**, *129*, 3104 – 3109; b) M. T. Dedeo, K. E. Duderstadt, J. M. Berger, M. B. Francis, *Nano Lett.* **2010**, *10*, 181 – 186.
[16] A. P. Esser-Kahn, A. T. Iavarone, M. B. Francis, *J. Am. Chem. Soc.* **2008**, *130*, 15820 – 15822.
[17] a) J. M. Hooker, E. W. Kovacs, M. B. Francis, *J. Am. Chem. Soc.* **2004**, *126*, 3718 – 3719; b) J. M. Hooker, A. P. Esser-Kahn, M. B. Francis, *J. Am. Chem. Soc.* **2006**, *128*, 15558 – 15559.
[18] a) L. Wang, A. Brock, B. Herberich, P. G. Schultz, *Science* **2001**, *292*, 498 – 500; b) R. A. Mehl, J. C. Anderson, S. W. Santoro, L. Wang, A. B. Martin, D. S. King, D. M. Horn, P. G. Schultz, *J. Am. Chem. Soc.* **2003**, *125*, 935 – 939; c) Z. M. Carrico, D. W. Romanini, R. A. Mehl, M. B. Francis, *Chem. Commun.* **2008**, 1205.
[19] a) J. H. Strauss, Jr., R. L. Sinsheimer, *J. Mol. Biol.* **1963**, *7*, 43 – 54; b) K. Valegård, L. Liljas, K. Fridborg, T. Unge, *Nature* **1990**, *345*, 36 – 41.
[20] a) E. Antonini, M. Brunori, J. Wyman, *Biochemistry* **1965**, *4*, 545 – 551; b) J. F. Corbett, *J. Chem. Soc. B* **1969**, 207 – 212; c) S. Gardlik, K. V. Rajagopalan, *J. Biol. Chem.* **1991**, *266*, 4889 – 4895; d) Y. Yamagishi, H. Ashigai, Y. Goto, H. Murakami, H. Suga, *ChemBioChem* **2009**, *10*, 1469 – 1472.
[21] a) L. P. Hadjipetrou, T. Gray-Young, M. D. Lilly, *J. Gen. Microbiol.* **1966**, *45*, 479 – 488; b) C. Liu, T. Sun, Y. Zhai, S. Dong, *Talanta* **2009**, *78*, 613 – 617.
[22] a) S. C. Alley, K. E. Anderson, *Curr. Opin. Chem. Biol.* **2013**, *17*, 406 – 411; b) J. Caravella, A. Lugovskoy, *Curr. Opin. Chem. Biol.* **2010**, *14*, 520 – 528.
[23] J. K. Hamilton, F. Smith, *J. Am. Chem. Soc.* **1956**, *78*, 5910 – 5912.
[24] C. Netirojjanakul, L. S. Witus, C. R. Behrens, C.-H. Weng, A. T. Iavarone, M. B. Francis, *Chem. Sci.* **2013**, *4*, 266 – 272.
[25] a) J. M. Gilmore, R. A. Scheck, A. P. Esser-Kahn, N. S. Joshi, M. B. Francis, *Angew. Chem.* **2006**, *118*, 5433 – 5437; *Angew. Chem. Int. Ed.* **2006**, *45*, 5307 – 5311; b) L. S. Witus, T. Moore, B. W. Thuronyi, A. P. Esser-Kahn, R. A. Scheck, A. T. Iavarone, M. B. Francis, *J. Am. Chem. Soc.* **2010**, *132*, 16812 – 16817.

