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Rapid, Hydrolytically Stable Modification of Aldehyde-Terminated Proteins and Phage Libraries

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Supporting Information

ABSTRACT: We describe the rapid reaction of 2-amino benzamidoxime (ABAO) derivatives with aldehydes in water. The ABAO combines an aniline moiety for iminium-based activation of the aldehyde and a nucleophilic group (Nu:) ortho to the amine for intramolecular ring closure. The reaction between ABAO and aldehydes is kinetically similar to oxime formations performed under stoichiometric aniline catalysis. We characterized the reaction by both NMR and UV spectroscopy and determined that the rate-determining step of the process is formation of a Schiff base, which is followed by rapid intramolecular ring closure. The relationship between apparent rate constant and pH suggests that a protonated benzamidoxime acts as an internal general acid in Schiff-base formation. The reaction is accelerated by substituents in the aromatic ring that increase the basicity of the aromatic amine. The rate of up to 40 M^{-1} s^{-1} between an electron-rich aldehyde and 5-methoxy-ABAO (PMA), which was observed at pH 4.5, places this reaction among the fastest known bio-orthogonal reactions. Reaction between M13 phage-displayed library of peptides terminated with an aldehyde moiety and 1 mM biotin-ABAO derivative reaches completion in 1 h at pH 4.5. Finally, the product of reaction, dihydroquinazoline derivative, shows fluorescence at 490 nm suggesting a possibility of developing fluorogenic aldehyde-reactive probes based on ABAO framework.

Rapid, site-specific, and hydrolytically stable modifications of biomolecules are critical for unnatural modification of proteins, labeling of cells, synthesis of antibody-drug conjugates, and other applications. Aldehydes are versatile functional groups for protein functionalization. The popularity of aldehyde chemistry is rapidly growing due to continuous expansion of methods for incorporation of this functional group into proteins. Important examples include: periodate oxidation of the N-terminal Ser/Thr residues,[1] pyridoxal-5′-phosphate-mediated N-terminal transamination,[2] and periodate oxidation of sialic acids displayed on proteins,[3,4] oxidation of glycoproteins by galactose oxidase,[5] incorporation through protein farnesyltransferase,[6] and enzymatic oxidation of proteins by formylglycine-generating enzyme.[7] Aldehyde reactions on proteins were used in a number of therapeutic products, such as proteins PEGylated through N-terminal glyoxal.[8] Prominent new applications of aldehyde ligations developed in the past 10 years are the generation of antibody-glycan conjugates,[9] the labeling of the proteins in cells,[10] protein ligation,[11,12] and the diversification of genetically-encoded libraries.[13]

The formation of oximes and hydrazones is the most widely used chemical modification of aldehyde-displaying proteins. The relatively slow kinetics of these ligations can be accelerated by aniline catalysis.[14,11,12] We used this approach for the rapid modification of phage-displayed peptides in the presence of 100 mM aniline (Figure 1A).[13] The resulting oxime and hydrazone linkages, however, suffer from susceptibility to hydrolysis.[15] To overcome this problem, Bertozzi[16] and Rabuka[17] modified the Pictet–Spengler reaction to yield hydrolytically stable six-membered rings starting from protein-displayed aldehydes in water at pH 4–7. Here, we expand the scope of bio-orthogonal aldehyde chemistry by introducing hydrolytically stable linkages in model aldehydes, peptides, and bacteriophage-displayed peptide libraries.

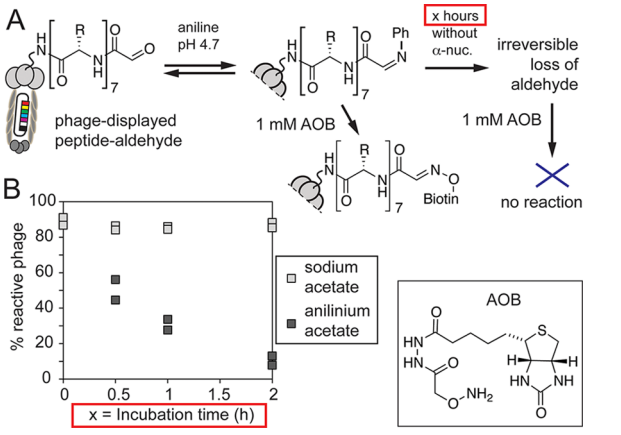

Figure 1. (A) Ligation of AOB to phage-displayed aldehydes is accelerated by aniline, but preincubation of phage with aniline in the absence of AOB leads to rapid loss of aldehyde and loss of reactivity toward AOB. (B) In anilinium acetate, pH 4.7, the aldehyde disappears with $t_{1/2}$ ~40 min; however, aldehydes alone are stable at the same pH in sodium acetate buffer.

Formation of aniline Schiff bases at the N-terminal glyoxal motif displayed on phage provides 100-fold acceleration in the reaction with α-nucleophiles, such as aminooxybiotin (AOB) (Figure 1A).[13] Interestingly, this Schiff base rapidly and irreversibly degrades when glyoxal is incubated in aniline buffer

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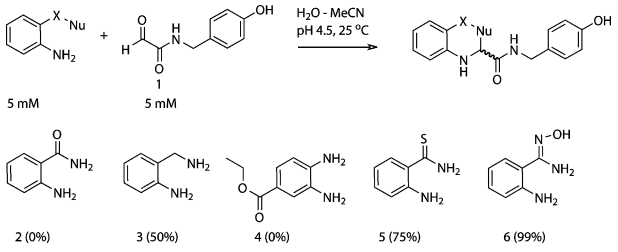
in the absence of any α-nucleophiles (Figure 1A,B). Imine-activated N-terminal aldehydes in proteins, therefore, are susceptible to attack by a variety of nucleophiles, whereas the aldehyde is stable under these conditions. We could not isolate a definitive product of this reaction, but based on the evidence from the literature, we hypothesize that the intermediate aromatic imine could be quenched either by amines (Lys) or reactive aromatic size chains (Tyr, Trp). To further explore the reactivity of glyoxal Schiff bases with nucleophiles, we prepared a model water-soluble aldehyde (**1**) that mimics the N-terminal glyoxal in a protein and tested its reaction with five commercially available anilines containing a weak nucleophilic group at the ortho position (**2**–**6**, Scheme 1). The reactions of **1** with **2**–**6** at pH 4.5 were monitored by HPLC (Figure S1).

Scheme 1. Reaction of Aldehyde 1 with Aniline-Containing Nucleophilic Substituents in the ortho Position[a]



[a]In brackets shown is % conversion after 1 h.

Anthranilamide **2** is known to react with aldehydes in highly acidic conditions to yield stable quinazolinones; however, at pH = 4.5, more than 24 h was required to achieve 50% conversion. 2-(Aminomethyl)aniline (**3**) reacted rapidly with the aldehyde, but the product was hydrolytically unstable. Diaminobenzene (**4**) reacted slower than **3** due to the suboptimal "anti-Baldwin" 5-endo-trig geometry required for ring closure. An amino-thiobenzamide (**5**) yielded a single product in 1 h, which, however, degraded into a complex mixture of products upon incubation for 24 h (Figure S1C). The reaction of 2-amino benzamidoxime (ABAO, **6**) was fastest and yielded a single stable product. A similar reaction in ethanol was previously described.[18] We isolated the product of the reaction and confirmed its structure as a 1,2-dihydroquinazoline-3-oxide (**7**, Figures 2, S2).

The product **7** formed irreversibly and did not undergo hydrolysis upon storage in acetate buffer (pH 4.5) over 13 days, as monitored by HPLC (Figure S3). This observation suggested that the ABAO–aldehyde reaction can be applied for the introduction of hydrolytically stable modifications into proteins. We investigated the kinetics by ^{1}H NMR spectroscopy (Figures 2, S4). The rate of this reaction was moderate at acidic pH. The rate constant of the reaction between ABAO and N-terminal glyoxyl derivative **1** was 0.086 M^{-1} s^{-1} at pH 4.5. The reaction was first order with respect to ABAO and the aldehyde and exhibited weak pH dependence in the range of 4–6 (Figure 2B), which is characteristic of general-acid-catalyzed formation of the Schiff base.[19] No accumulation of Schiff base was observed in NMR experiments. Consumption of the reactants was accompanied by concomitant appearance of the final product (Figure 2C). This observation suggested that the formation of Schiff base is the rate-determining step, while the attack of amidoxime is a relatively rapid process.

To design ABAO derivatives with enhanced reaction rates, we explored the rate of reaction of the model aldehyde **1** and

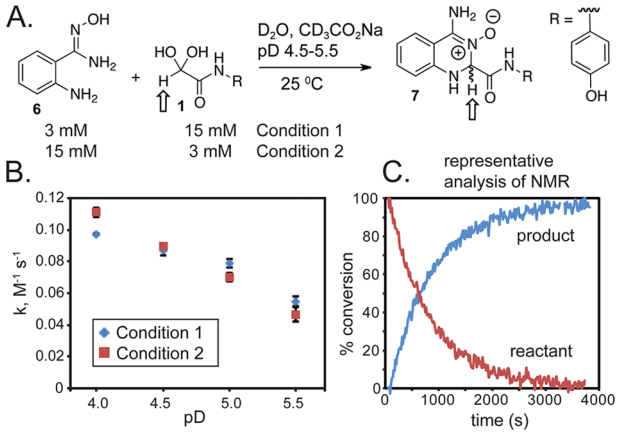


Figure 2. Kinetics of the reaction between **6** and **1**. (A) Reaction scheme and conditions. Block arrows indicate protons used for monitoring reaction progress by ^{1}H NMR spectroscopy. (B) Rate constants measured by ^{1}H NMR spectroscopy. Reactions were conducted in CD_3COONa buffer (100 mM) at pD 4.5 at ~10 fold excess of either **6** or **1**, and the resulting data were fitted to first-order kinetics. We used first-order expression $A(t) = A_0(1 - \exp(-k_1 t))$ to fit the data, and the second-order rate constant was derived as $k_2 = k_1/C_{ex}$, where C_{ex} is concentration of the excess reagent (here ABAO). (C) Disappearance of the reactants and concurrent appearance of the product as determined by ^{1}H NMR spectroscopy. Spectra were acquired every 17 s (~230 data points are in this graph). Ratio of product resonance and the sum of product and starting material resonance were used to determine % conversion.

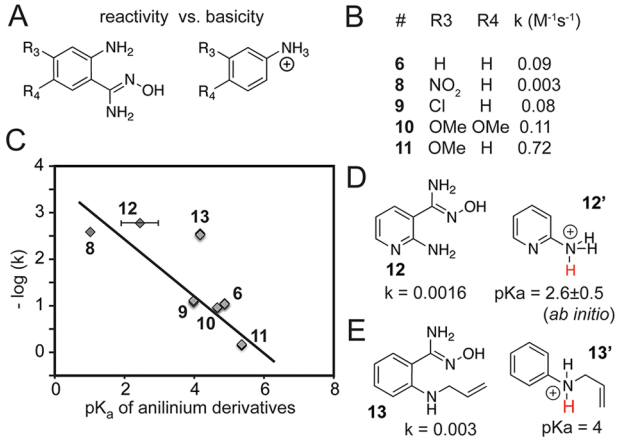


Figure 3. Relationship between the measured reaction rates and pK_a of the corresponding protonated anilines for seven ABAO analogues. (A) Structures of ABAOs and corresponding anilines. (B) Reaction rates measured by NMR in CD_3COONa buffer (100 mM) at pD 4.5. (C) Scatter plot showing relationship between log k and pK_a. (D–E) Structures and reaction rates for **12** and **13**. Basicity of aniline nitrogen in **12**′ was estimated by an ab initio calculation (see Figure S8).

derivatives of ABAO (Figure 3). The reactivity of derivatives **6**–**12** exhibited an apparent linear free-energy relationship (LFER) with the pK_a of the corresponding anilinium cations and suggested that a high basicity of the nitrogen is necessary to accelerate the Schiff base formation. As a result, we identified *para*-methoxy ABAO (PMA) (**11**) as the most reactive derivative of the tested analogues. Deviation from the LFER was observed for the analogue with alkyl substitution at the aromatic amine (**13**, Figure 3E). It provided a mechanistic insight: reaction of N-alkylated ABAO proceeds through an obligatory positively charged Schiff base, whereas nonalkylated ABAO could avoid

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significant positive charge buildup in the corresponding transition state by engaging reaction pathways catalyzed by general base and acid (Figure S5).

To identify the nature of general acid and base catalysts, we measured the reaction at different pH and buffer compositions. The formation of the 1,2-dihydroquinazoline-3-oxide derivatives from PMA or ABAO was accompanied by a visible change in color, which permitted characterization of the reaction by UV spectroscopy. Reaction rates in deuterated buffer were similar when measured by UV and NMR spectroscopy. Upon replacement of the deuterated buffer with protic buffer we observed a significant inverse solvent kinetic isotope effect (sKIE, Figure S6); the rate constant of the reaction was up to 2-fold higher in deuterated acetate buffer. An inverse sKIE was previously observed for other nucleophilic additions to aldehydes in aqueous solutions.[20] The expanded pH profile measured by UV (Figure 4) suggested that reaction is catalyzed by general acid with pK_a ~ 4–5.

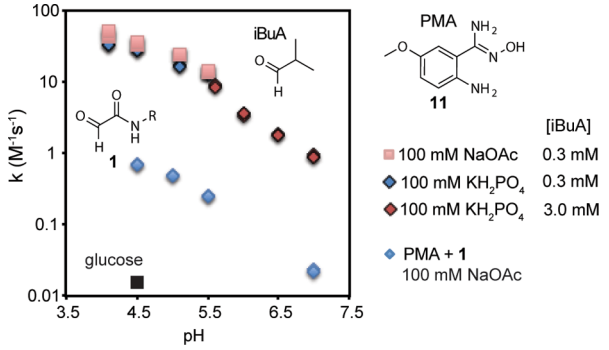

Figure 4. The pH dependence on rates of the reaction between **11** and aldehydes. Reaction rates were measured by monitoring the change in UV absorbance at 400 nm. For the representative raw traces see Figure S16.

We identified acetate (pK_a = 4.7) as a weak catalyst: at pH = 4.5, the rate increased by a factor of <2 with an increase in buffer concentration from 50 to 400 mM (Figure S7). We hypothesized that the main general acid catalysis is performed in an intramolecular fashion by the benzamidoxime (BAO) functionality (calculated pK_a = 4.2, Figure S8). Catalysis of imine formation by intramolecular general acids is well-known from original mechanistic investigations by Hine[21] and recent reports by Dawson[22] and Kool.[23] Based on the above observations, we proposed a three-step reaction mechanism, which satisfies the observed order in reagents and pH profile (see Figure S9 and kinetic equations on page S20–S21). It starts from formation of a carbinolamine catalyzed by protonated BAO functionality (general-acid), followed by a general acid and general-base-catalyzed elimination of the carbinolamine to form a neutral Schiff base, and ends with a rapid intramolecular ring closure.

PMA reacted remarkably fast with electron-rich aldehydes (Figures 4, S10–S11). At pH 4.5, the rate constant was 30–40 M^{-1} s^{-1} for isobutyraldehyde (iBuA), which was used by Bertozzi et al. as a model of a formyl-glycine residue.[16] The reaction was four times faster than Pictet–Spengler cyclization with iBuA at pH = 4.5; PMA ligation was ~2-fold faster than Pictet–Spengler cyclization in near neutral buffer (pH 6–7).[16] Reaction with another biologically relevant aldehyde, electron-deficient N-terminal glyoxal **1**, proceeded with the rate of ~1 M^{-1} s^{-1}. This rate is comparable to Pictet–Spengler reactions of the same aldehyde in the same conditions (see Figure 3 in ref 16). Most importantly, glucose reacts with PMA very slowly with the rate ≪0.1 M^{-1} s^{-1} (Figure 4, black square), thus suggesting that this reaction can be conducted in biological medium in the presence of large excess of reducing carbohydrates.

To confirm that ABAO derivatives are useful for protein bioconjugation, we modified functionality-rich peptide WY-DANHSKPL displaying an N-terminal glyoxyl. Reaction yielded two products with identical mass (diastereomers resulting from a new chiral center at the aldehyde carbon, Figure S12). It did not modify any of the reactive chains as confirmed by MS/MS fragmentation analysis of the products (Figure S13). UV monitoring confirmed that kinetics of the reaction of peptide and iso-electronic model aldehyde (**1**) are nearly identical (Figure S12). To monitor conjugation on intact proteins, we oxidized glycans on horseradish peroxidase (HRP) with $NaIO_4$ and added biotin-displayed ABAO derivative (BABAO, Figure 5A) to yield biotinylated HRP. ELISA assay (Figure S14) and

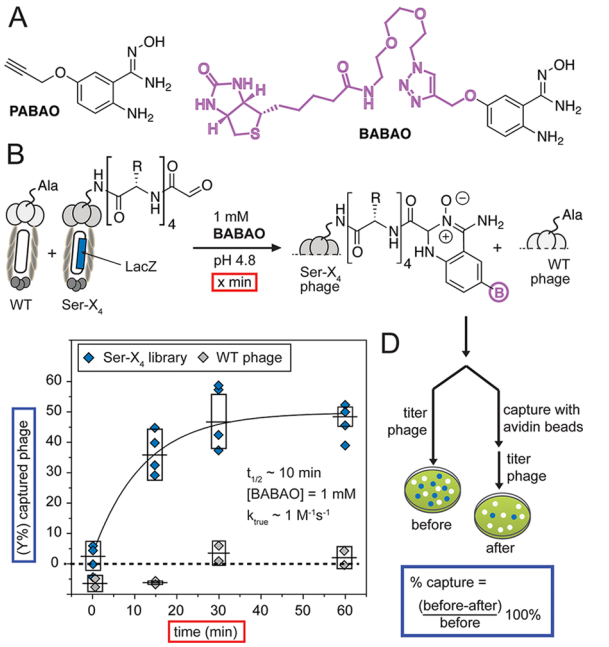

Figure 5. (A) Structures of ABAO derivatives that contain propargyloxy group and biotin. (B) Quantification of the reaction of BABAO with the M13 phage displaying a library of peptides with N-terminal glyoxal and wt phage displaying no aldehyde (10^{11} PFU/mL each). (C) Phage mixture was exposed to BABAO for the specified time. (D) To measure the reaction rate, we exposed the mixture of phage to 1 mM BABAO and measured biotinylation as a function of time by avidin capture. Error bars represent standard deviations from four independent kinetics experiments.

MALDI mass spectrometry confirmed modification albeit heterogeneity of glycosylation precluded identification of exact stoichiometry. Bovine serum albumin, which was present in the same solution, lacks glycosylation and was not modified under these conditions (Figure S14). Modification with BABAO had no effect on enzymatic activity of HRP, and it turned over its substrate in ELISA assay (Figure S14).

We then confirmed reaction of ABAO with glyoxal at the N-terminus of phage-displayed peptide library. An indirect biotin capture[13] confirmed that the rate of disappearance of the aldehyde on phage in 1 mM PMA in sodium acetate buffer (pH 4.8, Figure S15) was similar to the reactions observed on purified

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substrates (Figure 4). To rule out the degradation of aldehyde observed in aniline buffers (Figure 1), we measured the rate of direct biotinylation (Figure 5). Again, we observed that phage was biotinylated by BABAO at a rate similar to aforementioned rates (Figures S15, 4). Phage retained its ability to replicate, confirming that reaction conditions are mild enough to maintain the integrity of the M13 virion. Wild-type M13 phage particles displaying no aldehyde, which were present in the same solution, were not biotinylated, confirming the specificity of the reaction.

The change in π-conjugation upon reaction and high rigidity of the product opens an opportunity to design fluorogenic ABAO derivatives. Indeed the PMA derivative exhibited a dramatic increase in fluorescence upon reaction with aldehydes (Figure 6, Movie S1). The rate of emergence of fluorescence at 490 nm was similar to the rate of the reaction as monitored by UV spectrometry (Figure 6). To confirm fluorescence in complex substrate, we labeled the peptide WYDANHSKPL displaying an N-terminal glyoxyl and measured its UV signature and fluorescence spectrum before and after reaction (Figure S12). Fluorescence resulting from the reaction with ABAO exceeds the intrinsic fluorescence of Trp present in the peptide structure by at least 2 orders of magnitude.

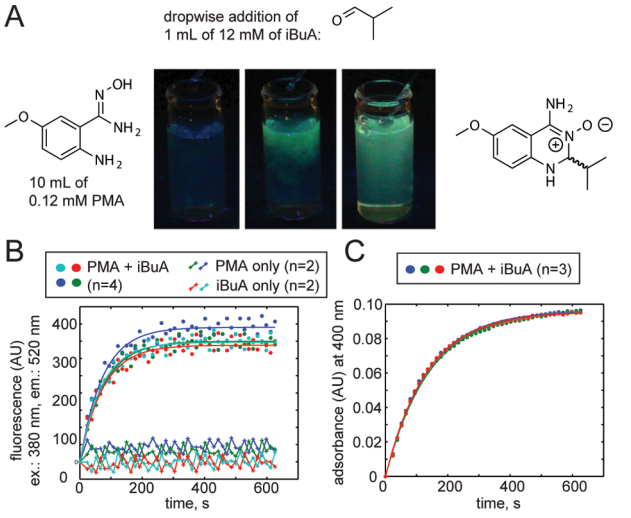

Figure 6. Emergence of fluorescence upon reaction of **11** and iBuA. (A) Images of the reaction mixture under UV lamp. (B) Reaction progress monitored by fluorescence. (C) Reaction progress monitored by UV absorbance ("*n*" is the number or independent experiments).

In conclusion, the amino benzamidoxime framework combines fast reactivity (up to 40 M^{-1} s^{-1}), long-term stability, and intriguing changes in π-conjugation upon reaction. Ligation has maximum performance at mildly acidic pH and moderate performance at neutral pH. The nature of Schiff base intermediates complicates the design of rapid aldehyde ligation at neutral pH. For the best to-date example, hydrazino-Pictet–Spengler reaction, the reported rate constant is 4 M^{-1} s^{-1} at pH 6.0;[17] in comparison, the rates of ABAO-ligation range from 0.2 to 3 M^{-1} s^{-1} under this condition (Figure 4). The products of both ABAO and Pictet–Spengler-like reactions exhibit long-term stability, providing advantage over classical oxime/hydrazone bonds. The unexpected value-added benefit of ABAO is the change in both absorbance and fluorescence spectra during the course of the reaction. This feature facilitates rapid and accurate kinetic measurements on complex substrates such as peptides (Figure S12). We anticipate that this reaction will serve as a platform for development of new bioconjugation strategies, fluorogenic probes, and post-translational diversification of genetically-encoded libraries. We envision that it will aid applications where long-term stability and built-in quality control by UV spectrometry are desired (e.g., long-term protein labeling and synthesis of antibody-drug conjugates).

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Sklarz, B. *Q. Rev., Chem. Soc.* **1967**, *21*, 3.
(2) Scheck, R. A.; Dedeo, M. T.; Lavarone, A. T.; Francis, M. B. *J. Am. Chem. Soc.* **2008**, *130*, 11762.
(3) Reuter, G.; Schauer, R.; Szeiki, C.; Kamerling, J. P.; Vliegenthart, J. F. G. *Glycoconjugate J.* **1989**, *6*, 35.
(4) Hage, D. S.; Wolfe, C. A. C.; Oates, M. R. *Bioconjugate Chem.* **1997**, *8*, 914.
(5) Gahmberg, C. G.; Hakomori, S. *J. Biol. Chem.* **1973**, *248*, 4311.
(6) Rashidian, M.; Dozier, J. K.; Lenevich, S.; Distefano, M. D. *Chem. Commun.* **2010**, *46*, 8998.
(7) Carrico, I. S.; Carlson, B. L.; Bertozzi, C. R. *Nat. Chem. Biol.* **2007**, *3*, 321.
(8) Gaertner, H. F.; Offord, R. E. *Bioconjugate Chem.* **1996**, *7*, 38.
(9) Hudak, J. E.; Yu, H. H.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2011**, *133*, 16127.
(10) Wu, P.; Shui, W. Q.; Carlson, B. L.; Hu, N.; Rabuka, D.; Lee, J.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 3000.
(11) Dirksen, A.; Hackeng, T. M.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581.
(12) Metanis, N.; Keinan, E.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2010**, *49*, 7049.
(13) Ng, S.; Jafari, M. R.; Matochko, W. L.; Derda, R. *ACS Chem. Biol.* **2012**, *7*, 1482.
(14) Cordes, E. H.; Jencks, W. P. *J. Am. Chem. Soc.* **1962**, *84*, 832.
(15) Kalia, J.; Raines, R. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 7523.
(16) Agarwal, P.; van der Weijden, J.; Sletten, E. M.; Rabuka, D.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 46.
(17) Agarwal, P.; Kudirka, R.; Albers, A. E.; Barfield, R. M.; de Hart, G. W.; Drake, P. M.; Jones, L. C.; Rabuka, D. *Bioconjugate Chem.* **2013**, *24*, 846.
(18) Korbonits, D.; Kolonits, P. *J. Chem. Soc., Perkin Trans. 1* **1986**, 2163.
(19) Sayer, J. M.; Pinsky, B.; Schonbrunn, A.; Washtien, W. *J. Am. Chem. Soc.* **1974**, *96*, 7998.
(20) Lienhard, G. E.; Jencks, W. P. *J. Am. Chem. Soc.* **1966**, *88*, 3982.
(21) Hine, J.; Zeigler, J. P.; Johnston, M. *J. Org. Chem.* **1979**, *44*, 3540.
(22) Dirksen, A.; Hackeng, T. M.; Dawson, P. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 7581.
(23) Crisalli, P.; Kool, E. T. *J. Org. Chem.* **2013**, *78*, 1184.