

## Fast Hydrazone Reactants: Electronic and Acid/Base Effects Strongly Influence Rate at Biological pH

Eric T. Kool,\* Do-Hyoung Park, and Pete Crisalli

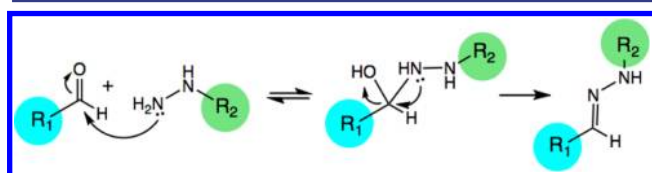
Department of Chemistry, Stanford University, Stanford, California 94305-5080, United States

## Supporting Information

**ABSTRACT:** Kinetics studies with structurally varied aldehydes and ketones in aqueous buffer at pH 7.4 reveal that carbonyl compounds with neighboring acid/base groups form hydrazones at accelerated rates. Similarly, tests of a hydrazine with a neighboring carboxylic acid group show that it also reacts at an accelerated rate. Rate constants for the fastest carbonyl/hydrazine combinations are  $2\text{--}20\text{ M}^{-1}\text{ s}^{-1}$ , which is faster than recent strain-promoted cycloaddition reactions.

Extensive efforts<sup>1</sup> to develop new bond-forming reactions having improved selectivity, lower interference and cross-reactivity with biological molecules, and enhanced rates have resulted in the development of important classes of reactions such as Cu-catalyzed azide–alkyne cycloadditions,<sup>2</sup> strain-driven cycloadditions,<sup>3,4</sup> photoclick reactions,<sup>5</sup> and Staudinger ligations.<sup>6</sup> Relative to earlier, less-selective reactions, these new bond-forming strategies greatly enhance the ability to construct conjugates of biomolecules, particularly under challenging aqueous conditions at pH 7.4, at low concentrations, and in cellular settings.

One of the earliest reactions used for bioconjugations is that of hydrazone/oxime formation (Figure 1), involving the stable



**Figure 1.** Mechanism of hydrazone formation. Breakdown of the tetrahedral intermediate is typically rate limiting at neutral pH.<sup>7a,b</sup>

imine formation of aldehydes and ketones with  $\alpha$ -nucleophiles such as hydrazines and aminoxy groups. This venerable reaction<sup>7</sup> has been widely useful in bioconjugation,<sup>8</sup> due to its biomolecular orthogonality and because carbonyl and hydrazine functional groups are readily installed into small molecules. Early mechanistic studies of the reaction were performed by Jencks in the 1960s,<sup>7a</sup> and work by Dawson<sup>8d</sup> and Tam<sup>8a</sup> has highlighted the utility of the reaction in peptide labeling. Very recent studies by our laboratory<sup>9</sup> and by Raines,<sup>10</sup> Distefano,<sup>11</sup> and Canary<sup>12</sup> are also contributing to the utility of the reaction, which is employed not only in bioconjugations but also in other fields, such as polymer chemistry<sup>13</sup> and dynamic combinatorial chemistry.<sup>8d,14</sup>

However, there is a significant limitation of hydrazone and oxime formation that hinders its broader use: the slow rate of reaction of most substrates at neutral pH. This can be inconvenient for reactions *in vitro* (sometimes requiring hours to days<sup>8b,15</sup>) and can be strongly limiting *in vivo*, where concentrations of reactants are low. Although aniline can be used as a nucleophilic catalyst,<sup>7c</sup> high concentrations are required, and the rate acceleration is moderate.<sup>8b,14a</sup> New-generation catalysts such as anthranilic acids and phosphonates have improved upon aniline catalysis,<sup>9b,c</sup> but in some applications the use of a catalyst is undesirable, adding complexity and, in some cases, toxicity.<sup>16</sup>

Thus the identification of structural features that might speed hydrazone/oxime formation without the addition of a catalyst could significantly enhance the utility of this reaction. Surprisingly, there exist few general studies of what structural features in carbonyl substrates affect reaction rates, particularly at pH 7.4.<sup>15</sup> Early mechanistic studies were typically carried out at acidic pH, where rates are much more rapid.<sup>17</sup> However, the modern expansion of biological chemistry has greatly increased the relevance of reactivity at pH values near neutral.




















Here we have performed kinetics studies on a range of aldehyde and ketone substrates, to examine how structure affects reactivity at pH 7.4 in aqueous buffer. We find that substrate structures can have marked effects on reaction rate, and we have identified specific structural features that yield reactions with rates that rival many modern cycloaddition reactions.

Reaction rates were measured for a range of commercially available carbonyl reactants (Tables 1, 2) dissolved in phosphate-buffered saline (137 mM Na<sup>+</sup>, 2.7 mM K<sup>+</sup>, 12 mM phosphate, pH 7.4, 25 °C). A small amount of organic cosolvent (10% DMF) was added to ensure the solubility of both reactants. To evaluate structural effects, we tested a range of aldehydes and ketones, both aryl- and alkyl-substituted. Electron-rich and -poor cases were included, and steric substitution near the carbonyl was varied. Finally, carbonyl compounds with proximal acid/base groups were examined as well. A standard hydrazine (phenylhydrazine) was chosen to react with these; in limited cases we tested other hydrazines as well. Rates were measured under pseudo-first-order conditions by following changes in absorption, with aldehydes at 125  $\mu\text{M}$ –1 mM and hydrazines at 2.5–20  $\mu\text{M}$ . In general, fits were very good (see Supporting Information), although a few examples

Received: July 18, 2013

Published: November 12, 2013

Table 1. Rates of Reaction of Varied Carbonyl Compounds with Phenylhydrazine in Aqueous Buffer at pH 7.4<sup>a</sup>

Substrate	$k_{1(\text{obs})}$ (min <sup>-1</sup> )	$k_{2(\text{app})}$ (M <sup>-1</sup> sec <sup>-1</sup> )	$k_{\text{rel}}$	Substrate	$k_{1(\text{obs})}$ (min <sup>-1</sup> )	$k_{2(\text{app})}$ (M <sup>-1</sup> sec <sup>-1</sup> )	$k_{\text{rel}}$
Aryl aldehydes							
1 	0.0020 (0.0005)	0.033 (0.008)	1	11 	0.20 (0.06)	3.4 (1.0)	100
2 	0.009 (0.004)	0.15 (0.06)	4.5	12 	0.076 (0.032)	1.3 (0.6)	39
3 	0.007 (0.002)	0.12 (0.03)	3.6	13 	0.030 (0.002)	0.50 (0.03)	15
4 	0.009 (0.004)	0.15 (0.07)	4.5	14 	0.026 (0.002)	0.43 (0.03)	13
5 	0.009 (0.002)	0.15 (0.04)	4.3	Ketones			
6 	0.005 (0.003)	0.09 (0.04)	2.8	15 	0.013 (0.004)	0.22 (0.07)	6.7
7 	0.013 (0.008)	0.22 (0.13)	6.7	16 	0.017 (0.006)	0.28 (0.10)	8.6
8 	0.008 (0.001)	0.13 (0.02)	3.9	17 	0.034 (0.002)	0.57 (0.03)	17
9 	0.006 (0.002)	0.10 (0.03)	3.0	18 	0.046 (0.006)	0.77 (0.10)	23
Alkyl aldehydes				19 	0.022 (0.010)	0.37 (0.17)	11
10 	0.58 (0.14)	9.7 (2.3)	290				

























<sup>a</sup>Conditions: 137 mM NaCl, 2.7 mM KCl, 12 mM phosphate, 25 °C. Values measured 3–6 times and averaged (std. dev. in parentheses). Pseudo-first-order  $k_{1(\text{obs})}$  normalized to standard 1 mM aldehyde concentration.

showed some curvature at later time points, indicating a deviation from strictly second-order behavior.

Structures and rate constants are given in Tables 1 and 2. Examination of the whole set shows some broad trends and identifies specific features of interest that affect reaction rates very significantly. First, among all substrates, simple alkyl aldehydes are the fastest reactants. Indeed, the three fastest-reacting carbonyl compounds in the study are butyraldehyde, 2-methylbutyraldehyde, and pivaldehyde (entries 10–12, Table 1). Butyraldehyde, the fastest carbonyl substrate overall, formed a hydrazone 65-fold more rapidly than did benzaldehyde (entry 2). It seems likely that the faster reaction of alkyl carbonyls compared to aryl substrates is due to the conjugation in the aryl cases, which is disrupted upon formation of the tetrahedral intermediate. This is also consistent with the observation that cinnamaldehyde reacts 6-fold more slowly than phenylacetaldehyde. A second general observation is that aldehydes are in most cases faster than comparable ketones (e.g., butyraldehyde is faster than 2-butanone by 44-fold and pivaldehyde is 2.3-fold faster than di-*tert*-butylketone (entries 17, 19). One exception to this general rule is acetophenone (entry 20), which reacted 2.4-fold more rapidly than benzaldehyde. Overall, the two fastest-reacting ketones in the study were 2-acetylpyridine and 1,1,1-trifluoroacetone (entries 18, 31), which may be due in part to their more electron-deficient character.

On this topic, it has long been known that electron-withdrawing groups can increase the reactivity of aldehydes in hydrazone formation.<sup>7d,17</sup> However, the overall effect has been found to be small (Jencks observed a Hammett  $\rho$  value of 0.91 at pH 1.75).<sup>17</sup> In our experiments at pH 7.4 we observed a

Table 2. Rates of Reaction of Aldehydes and Ketones with Phenylhydrazine and Other Hydrazines in Aqueous Buffer at pH 7.4<sup>a</sup>

Substrate	$k_{1(\text{obs})}$ (min <sup>-1</sup> )	$k_{2(\text{app})}$ (M <sup>-1</sup> sec <sup>-1</sup> )	$k_{\text{rel}}$	Substrate	$k_{1(\text{obs})}$ (min <sup>-1</sup> )	$k_{2(\text{app})}$ (M <sup>-1</sup> sec <sup>-1</sup> )	$k_{\text{rel}}$
Carbohydrates				Bulky hydrazine ( $\phi_2\text{NNH}_2$ )			
20 	0.013 (0.003)	0.22 (0.02)	6.7	32 	0.051 (0.024)	0.85 (0.13)	25
21 	0.011 (0.001)	0.18 (0.02)	5.2	33 	0.024 (0.012)	0.40 (0.27)	12
22 	0.032 (0.002)	0.53 (0.03)	16	34 	0.009 (0.003)	0.15 (0.05)	4.5
Acid/Base substituents				35 	0.010 (0.005)	0.17 (0.08)	5.2
23 	0.045 (0.025)	0.75 (0.42)	23	36 	0.007 (0.001)	0.12 (0.01)	3.6
24 	0.010 (0.002)	0.16 (0.02)	4.8	2-Carboxyphenylhydrazine			
25 	0.014 (0.001)	0.24 (0.02)	7.1	37 	1.5 (0.2)	24 (3)	710
26 	0.022 (0.006)	0.37 (0.10)	11	38 	0.021 (0.004)	0.35 (0.07)	10
27 	0.018 (0.006)	0.30 (0.10)	9.0	39 	0.077 (0.006)	1.3 (0.1)	39
28 	0.036 (0.012)	0.58 (0.25)	17	40 	0.11 (0.02)	1.9 (0.4)	57
29 	0.025 (0.001)	0.42 (0.02)	12	41 	0.16 (0.03)	2.7 (0.5)	81
30 	0.050 (0.004)	0.83 (0.07)	25	42 	0.14 (0.03)	2.4 (0.4)	73
31 	0.036 (0.010)	0.60 (0.17)	18	43 	0.16 (0.02)	2.7 (0.4)	81

<sup>a</sup>Conditions are the same as those in Table 1. Values measured 3–6 times and averaged (standard deviations in parentheses). Pseudo-first-order  $k_{1(\text{obs})}$  normalized to the standard 1 mM aldehyde concentration.

general trend favoring electron-deficient aldehydes and ketones over electron-rich ones. For example, the slowest-reacting compound in the study was 4-methoxybenzaldehyde (entry 1), while 4-nitrobenzaldehyde reacted 4.5-fold more rapidly. Similarly, trifluoroacetone formed a hydrazone 3.4-fold more rapidly than 2-butanone, which could also be attributed to the electron deficiency of the former. However, this effect did not always hold; for example, dimethoxyacetaldehyde reacted 8-fold more slowly than 2-methylbutyraldehyde (compare entries 11,13).

Carbohydrates are of special interest for hydrazone formation because they naturally contain reactive ketone or aldehyde functional groups, which can be useful in labeling and bioconjugation.<sup>18</sup> Interestingly, the three aldoses tested here—ribose, deoxyribose, and glucose—reacted at reasonably good rates despite the fact that they exist largely in the hemiacetal state in solution. Ribose and deoxyribose displayed rates similar to that of benzaldehyde, while glucose reacted 3.5-fold more rapidly and was similar in reactivity to an electron-deficient alkyl aldehyde (entry 13). Reactions of anomeric carbons in deoxyribose are of interest in the development of reagents for detecting abasic damage in DNA,<sup>18a</sup> and reactions of hexoses are useful in making bioconjugates;<sup>18b</sup> the current results show that such reactions can proceed with moderate to good rates.

Steric effects could potentially have significant effects on the rate of hydrazone formation, by blocking the approach of the hydrazine nucleophile to the carbonyl carbon. However, the rate of breakdown of the tetrahedral intermediate might well be enhanced by the relief of steric crowding. We are unaware of any prior literature studies of steric effects on hydrazone or oxime formation. In the current study, steric effects with the phenylhydrazine standard reactant are found to be small and inconsistent. For example, we observed a 7.5-fold decrease in rate for the reaction of pivaldehyde relative to butyraldehyde (Table 1). Interestingly, however, in the ketone series, di-*tert*-butylketone reacts *faster* than diisopropyl ketone, which in turn reacts faster than 2-butanone (entries 15–17), showing a significant enhancement by alkyl substitution. Use of the more bulky diphenylhydrazine (entries 32–36) did not result in magnified effects on rate. Overall we conclude that steric effects are generally moderate for this reaction and that increased substitution is not always detrimental to the rate, especially for ketones.

Finally, we explored the effects of substituting acid/base groups near the reactive center on the rate of reaction. It was observed several decades ago that *ortho*-substitution in benzaldehydes could speed hydrazone formation, although a satisfactory explanation for the effect was elusive.<sup>19</sup> Pyridoxal (which has an *ortho* hydroxy group) has long been known as an efficient reactant for imine and hydrazone formation,<sup>20</sup> and a recent report employed rapidly reacting pyridoxal phosphoramidate derivatives in bioconjugations.<sup>12</sup> In our recent study, we observed that 2-carboxybenzaldehyde reacted more rapidly than the 4-isomer;<sup>9c</sup> we proposed that the acid group might donate a proton at the transition state, acting as an intramolecular catalyst in the reaction.

To address this hypothesized self-catalytic effect, we tested several compounds with imino, hydroxy, or carboxy groups substituted near the reactive carbonyl (Table 2, entries 23–31 and Figure S4). Significantly, all cases were found to react more rapidly at pH 7.4 than do control compounds that either lack the group or have it substituted remotely from the carbonyl. Imino groups (as pyridine derivatives) and *ortho* carboxy groups had similar effects. *Ortho* hydroxy groups accelerate the reaction by 2–4-fold (see entries 26, 27 relative to benzaldehyde). Finally, the greatest effect occurred with the imino group of quinoline-8-carboxaldehyde (entry 30), which accelerates the rate by a sizable factor of 8.3 relative to 1-naphthaldehyde (entry 9). Notably, while pyridoxal is a moderately fast reactant in the aromatic carbonyl series, both this new quinoline aldehyde substrate and 2-acetylpyridine are considerably faster, and thus these latter two are prime candidates for further development in bioconjugation reactions.

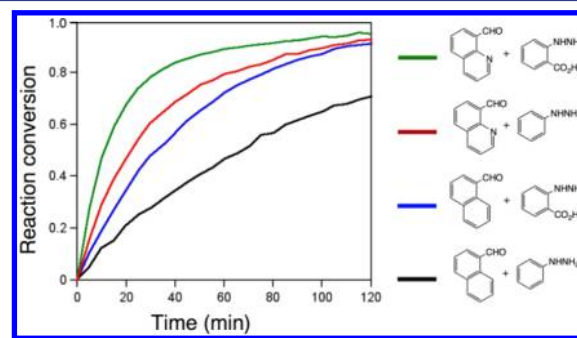
The rate-limiting step for hydrazone formation at neutral pH is the breakdown of the tetrahedral intermediate to eliminate water.<sup>7b</sup> Thus, catalysis of the reaction could occur by intramolecular transfer of a proton to the leaving hydroxy group.<sup>9c</sup> We tentatively propose that an arylhydroxy group ( $pK_a \sim 9$ –10 in the substrates tested here), a pyridine-type imino group ( $pK_a \sim 5.2$ ), or a carboxy group ( $pK_a \sim 4.2$ ) could achieve proton transfer via 5-, 6-, or 7-membered ring transition states (Figure 2). More studies will be needed to test this mechanistic hypothesis, but the results suggest that careful tuning of  $pK_a$  and geometry in the future could result in new and yet faster carbonyl substrates for this reaction.

Spurred by this finding, we carried out preliminary studies to investigate whether a similar self-catalytic effect might be



**Figure 2.** Proposed mechanism for rate enhancement by acid/base groups near the carbonyl center. Intramolecular protonation of the leaving group speeds breakdown of the tetrahedral intermediate.

observed in a hydrazine reactant. 2-Carboxyphenylhydrazine was reacted with selected aldehydes and ketones (Table 2 entries 37–43), and rates were compared to those with phenylhydrazine. In every case the reaction was significantly faster with the 2-carboxy-substituted hydrazine (Figure S5). The rate enhancement by the carboxy group varied from a factor of 1.6-fold (in the reaction with 2-butanone) to 8.7-fold (benzaldehyde). With the quinoline substrate, already enhanced by the nearby imino group, we observed a further 2.3-fold rate acceleration in reaction with the 2-carboxyphenylhydrazine derivative (Figure 3). Importantly, the rate



**Figure 3.** Time course plots for four related hydrazone-forming reactions, showing favorable effects of the imino group in the quinoline electrophile and of a carboxy group in the arylhydrazine nucleophile. Conditions are same as those in Table 1, with 500  $\mu\text{M}$  aldehyde and 10  $\mu\text{M}$  hydrazine.

constant for reaction of this pair of enhanced substrates was  $1.9 \text{ M}^{-1} \text{ s}^{-1}$ , which is faster than many recently developed bioorthogonal bond-forming reactions. Similarly high rates and enhancements were also seen for ketone substrates (entries 38, 41, 43). Remarkably, the rate constant for the fastest substrate in this study, butyraldehyde, with this enhanced hydrazine was  $24 \text{ M}^{-1} \text{ s}^{-1}$ , which is an order of magnitude more rapid than the rates of strain-promoted azide–alkyne cycloadditions and a tetrazine–norbornene addition/rearrangement.<sup>3,4b</sup> Thus although hydrazone formation has been generally characterized as slow at neutral pH, it is clear that specialized hydrazine and carbonyl substrates can undergo very rapid reactions.

We conclude that with the careful choice of carbonyl and hydrazine substrate structure, hydrazone formation can be rapid at biological pH even in the absence of a catalyst. Future studies will test whether additional structural tuning can yield yet faster hydrazone-forming reactants. With the advent of such rapid substrates, there are multiple reasons why this reaction may become more broadly useful, including ease of synthesis, water solubility of reactants, and facile introduction into biomolecules.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Experimental details, kinetic traces and plots. This information is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

kool@stanford.edu

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank the National Institutes of Health (GM068122) for support.

## ■ REFERENCES

- (1) (a) Kalia, J.; Raines, R. T. *Curr. Org. Chem.* **2010**, *14*, 138. (b) Lim, R. K.; Lin, Q. *Chem. Commun.* **2010**, 46, 1589. (c) Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272.
- (2) (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004. (b) Moses, J. E.; Moorhouse, A. D. *Chem. Soc. Rev.* **2007**, *36*, 1249. (c) Mamidyala, S. K.; Finn, M. G. *Chem. Soc. Rev.* **2010**, *39*, 1252. (d) El-Sagheer, A. H.; Brown, T. *Chem. Soc. Rev.* **2010**, *39*, 1388. (e) Lallana, E.; Riguera, R.; Fernandez-Megia, E. *Angew. Chem., Int. Ed.* **2011**, *50*, 8794.
- (3) (a) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793. (b) Ning, X. H.; Guo, J.; Wolfert, M. A.; Boons, G.-J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253. (c) Jewett, J. C.; Sletten, E. M.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 3688.
- (4) (a) Blackman, M. L.; Royzen, M.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 13518. (b) Devaraj, N. K.; Upadhyay, R.; Haun, J. B.; Hilderbrand, S. A.; Weissleder, R. *Angew. Chem., Int. Ed.* **2009**, *48*, 7013.
- (5) Gress, A.; Völkel, A.; Schlaad, H. *Macromolecules* **2007**, *40*, 7928.
- (6) (a) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007. (b) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141.
- (7) (a) Jencks, W. P. *Prog. Phys. Org. Chem.* **1964**, *2*, 63. (b) Jencks, W. P. *J. Am. Chem. Soc.* **1959**, *81*, 475. (c) Cordes, E. H.; Jencks, W. P. *J. Am. Chem. Soc.* **1962**, *84*, 826. (d) Cordes, E. H.; Jencks, W. P. *J. Am. Chem. Soc.* **1962**, *84*, 4319.
- (8) (a) Tam, J. P.; Xu, J.; Eom, K. D. *Biopolymers* **2001**, *60*, 194. (b) Cornish, V. W.; Hahn, K. M.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 8150. (c) Zeng, Y.; Ramya, T. N. C.; Dirksen, A.; Dawson, P. E.; Paulson, J. C. *Nat. Methods* **2009**, *6*, 207. (d) Dirksen, A.; Dirksen, S.; Hackeng, T. M.; Dawson, P. E. *J. Am. Chem. Soc.* **2006**, *128*, 15602.
- (9) (a) Crisalli, P.; Hernández, A. R.; Kool, E. T. *Bioconjugate Chem.* **2012**, *23*, 1969. (b) Crisalli, P.; Kool, E. T. *J. Org. Chem.* **2013**, *78*, 1184. (c) Crisalli, P.; Kool, E. T. *Org. Lett.* **2013**, *15*, 1646.
- (10) Kalia, J.; Raines, R. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 7523.
- (11) Rashidian, M.; Mahmoodi, M. M.; Shah, R.; Dozier, J. K.; Wagner, C. R.; Distefano, M. D. *Bioconjugate Chem.* **2013**, *24*, 333.
- (12) Wang, X.; Canary, J. W. *Bioconjugate Chem.* **2012**, *23*, 2329.
- (13) (a) Brinkhuis, R. P.; de Graaf, F.; Hansen, M. B.; Visser, T. R.; Rutjes, F. P. J. T.; van Hest, J. C. M. *Polym. Chem.* **2013**, *4*, 1345. (b) Lazny, R.; Nodzevska, A.; Sienkiewicz, M.; Wolosewicz, K. *J. Comb. Chem.* **2005**, *7*, 109.
- (14) (a) Sanders, J. K. *Philos. Trans. A* **2004**, *362*, 1239. (b) Gromova, A. V.; Ciszewski, J. M.; Miller, B. L. *Chem. Commun.* **2012**, 48, 2131.
- (15) (a) Dirksen, A.; Dawson, P. E. *Bioconjugate Chem.* **2008**, *19*, 2543. (b) Wu, B.; Wang, Z.; Huang, Y.; Liu, W. R. *ChemBioChem* **2012**, *13*, 1405. (c) Rayo, J.; Amara, N.; Krief, P.; Meijler, M. M. *J. Am. Chem. Soc.* **2011**, *133*, 7469.

(16) (a) Kennedy, D. C.; McKay, C. S.; Legault, M. C.; Danielson, D. C.; Blake, J. A.; Pegoraro, A. F.; Stolow, A.; Mester, Z.; Pezacki, J. P. *J. Am. Chem. Soc.* **2011**, *133*, 17993.

(17) Anderson, B. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1960**, *82*, 1773.

(18) (a) Kubo, K.; Ide, H.; Wallace, S. S.; Kow, Y. W. *Biochemistry* **1992**, *31*, 3703. (b) Beaudette, T. T.; Cohen, J. A.; Bachelder, E. M.; Broaders, K. E.; Cohen, J. L.; Engleman, E. G.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2009**, *131*, 10360.

(19) Wolfenden, R.; Jencks, W. P. *J. Am. Chem. Soc.* **1961**, *83*, 2763.

(20) French, T. C.; Auld, D. S.; Bruice, T. C. *Biochemistry* **1965**, *4*, 77.