Synlett O. Dilek et al. Letter

Intramolecular Catalysis of Hydrazone Formation of Aryl-Aldehydes via *ortho*-Phosphate Proton Exchange

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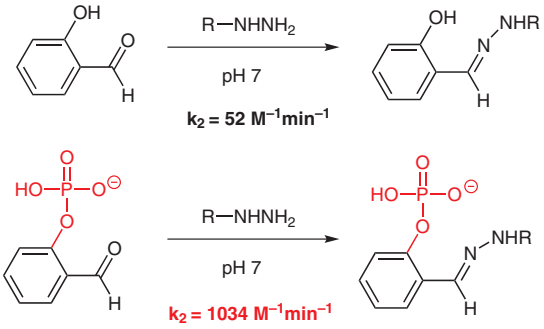

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Abstract Bioorthogonal site-specific chemical reaction to label biomolecules in vitro and in living cells is one of the most powerful and convenient tools in chemical biology. Reactive pairs frequently used for chemical conjugation are aldehydes/ketones with hydrazines/hydrazides/hydroxylamines. Although the reaction is generally specific for the two components, even in a cellular environment, the reaction is very slow under physiological conditions. Addition of a phosphate group at the *ortho* position of an aromatic aldehyde increases the reaction rate by an order of magnitude and enhances the aqueous solubility of the reagent and the product. We have synthesized phosphate-substituted aldehyde synthetic models to study kinetics of their reactions with hydrazines and hydrazides that contain a fluorophore. This rapid bioorthogonal reaction should therefore be potentially a very useful reaction for routine site-specific chemical ligations to study and image complex cellular processes in biological systems.

Key words site-specific conjugation, kinetics, bioorthogonal reaction

Site-specific conjugation of molecules in biological systems is a general method with many applications.[1] Ideally, two molecules are joined in a reaction that occurs rapidly under physiologically compatible conditions without cross-reaction with other functional groups in the molecules or milieu. Multiple reactive pairs are in use, such as azide/phosphine, alkyne/azide, tetrazine/alkyne, and new bioorthogonal reactions continue to be developed.[2] It has long been known that amino-substituted heteroatoms, amines bonded to oxygen or nitrogen, react with aldehydes and ketones in aqueous solutions to form reversible but stable covalent bonds.[3] Generally, hydrazines or hydroxylamines are used as nucleophiles, forming hydrazones or oximes, respectively, the carbonyl-containing molecule. The mechanism of this class of reactions has been very well studied.[4] In particular, it is known that the rate of product formation as a function of pH is a bell-shaped curve. The rate normally peaks around pH 4, which is well below physiological pH. The reaction rate can be increased by addition of a nucleophilic catalyst such as aniline and derivatives of aniline.[5] Although impressive rate enhancement can be achieved by substituted anilines,[6] large excess of the catalyst is required to overcome the generally low equilibrium constants for imine formation in neutral aqueous solution.

Another approach to increasing the rate of hydrazone or oxime formation is to modify the structure of the reagent. Nucleophilicity of the hydrazine amine is dependent of the substitutents on the hydrazine.[7] In very general terms, aromatic hydrazines are more reactive toward carbonyls than aliphatic hydrazines. Attaching electron-withdrawing groups to the α nitrogen, such as in a hydrazide, decreases the reactivity of the functional group, while other groups, such as a β-dialkylamine, can enhance the rate.

Less attention has been focused on enhancing the reaction by changing the structure of the electrophile. This approach is more limiting in the sense that naturally occurring carbonyls, such as those generated in proteins, lipids, and nucleic acids by reactive oxygen species, by definition have a fixed structure of the electrophile. Site-specific coupling is often performed with exogenous aldehydes or ketones and carbonyl-containing partner in the conjugation reaction, however, modification of the carbonyl-containing partner can also be considered.

The reactivity of an aromatic aldehyde varies sharply with the structure of the molecule. This property has been recently exploited by Wang and Canary, who take advantage of the naturally occurring pyridoxal phosphate (PLP) as the source of the aldehyde for hydrazone ligation reactions.[8] Both the phosphate and the phenol are known to participate in imine formation in PLP reactions in solution. When PLP is used as a ligation ligand, though, the phosphate is converted into a phosphoamidate, which is less likely to participate in the hydrazone-forming reaction.

Recently, Cristalli and Kool showed that aniline derivatives with certain proton-donating substituents *ortho* to the amine display enhanced catalytic activity.[9] The best catalyst possessed a phosphonate substituent, which has a pK_a close to the pH of the reaction solution. They also examined a small series of substituted aromatic aldehydes with the new catalysts and noted that certain *ortho* substituents on the aldehyde can produce a synergistic effect on the reaction rate.

We have been interested in developing hydrazine-containing fluorophores that undergo a spectroscopic change upon hydrazone formation for use as probes in biological systems.[10] Hydrazine-containing fluorophores were first synthesized and characterized later.[11] These fluorophores do react with aromatic aldehydes at neutral pH, but the reaction is sluggish. In this work we show that the reaction can be accelerated by an order of magnitude by a phosphate substituent.

Model compounds (Figure 1) were used to assess the effect of an *ortho* phosphate on the kinetics of hydrazone formation between an aromatic aldehyde and an aromatic hydrazine. The aldehyde components were salicylaldehyde (**1**, SA) or 3-formyltyrosine (**2**, fY), and 2-formylphenyldihydrogen phosphate (**3**, SA-P); fY is of interest because it can be incorporated into proteins. Furthermore, it is much more water soluble than SA. Since formation of hydrazone makes a more hydrophobic product enhanced water solubility of the components of the model reaction is desirable.

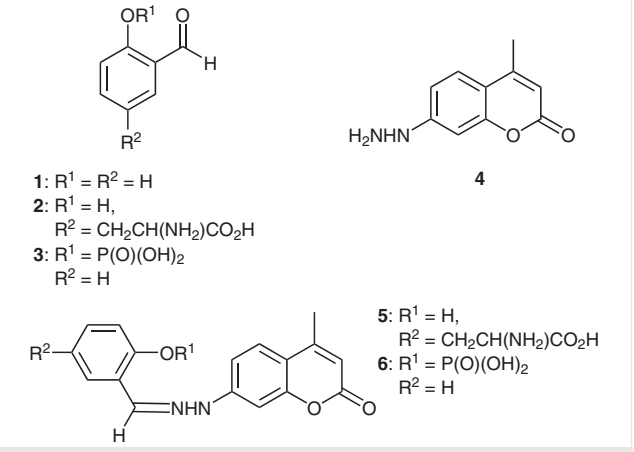


Figure 1 Structures of model aldehydes, hydrazine, and hydrazones

The hydrazine, 7-hydrazino-4-methyl coumarin (**4**, CH), was selected as a model aromatic hydrazine because its absorption maximum at longer wavelength is more than the commonly used 2-hydrazinopyridine. It also has a larger extinction coefficient so it can be used as the limiting reagent while still providing a visible signal. In addition, we have shown previously that hydrazone formation affects the absorption and emission properties of the fluorophore and were thus assured of a signal to monitor kinetics.[10]

Kinetics of hydrazone formation was assessed at 25 °C using absorption difference spectroscopy as described in Supplementary Material. Figure 2 shows a kinetic trace at a single wavelength for the reaction of fY or SA-P with the same concentration of CH, forming hydrazones **5** or **6**. Data were fit to a single pseudo-first order reaction to obtain peudo-first order rate constant. The kinetics for SA-P hydrazone formation at pH 4 and pH 5 was too fast to be measured on a standard spectrophotometer. Therefore these data were collected under pseudo-first order conditions using twofold lower concentrations of reagents. In order to compare all of the reactions, apparent second-order rate constants were calculated (Table 1). A plot of these data is shown in Figure 3.

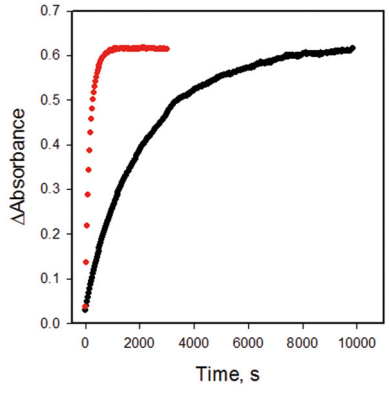


Figure 2 Formation of hydrazone **5** (black circles) or **6** (red circles) at 25 °C. The concentration of CH was 325 μM and the concentration of fY or SA-P was 32.5 μM.

Table 1 Apparent Second-Order Rate Constants for Hydrazone Formation at 25 °C

	Apparent k_2, M^{-1} min^{-1}		Relative rate
	CH + fY	CH + SA-P	
pH 4	804 ± 14	3950 ± 166	1.8
pH 5	548 ± 2.5	3270 ± 260	5
pH 6	196 ± 1.0	2790 ± 56	14
pH 7	52 ± 0.4	1034 ± 73	20
pH 8	50 ± 0.9	186 ± 1.2	3.8
pH 9	41 ± 1.4	86 ± 0.5	2.1
pH 10	40 ± 0.4	64 ± 0.2	1.6

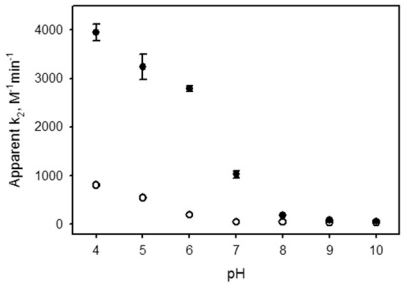
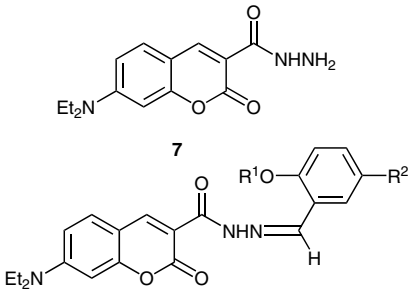


Figure 3 Apparent second-order rate constants for hydrazone formation as a function of pH. Open circles: fY plus CH. Filled circles: SA-P plus CH.

As expected, the rate of both the fY-CH and SA-P-CH reactions increases with decreasing pH.[12] The rate of the fY-CH reaction increases gradually with decreasing pH, but the rate if the SA-P-CH reaction increases suddenly at pH 7 and then more gradually as the pH decreases. Since the pK_a of the phosphate is near 7,[12] the 20-fold faster rate can be attributed to intramolecular acid catalysis by the phosphate. The difference in the apparent second order rate constants decreases with decreasing pH, which reflects the increasing influence of the protons in the buffer.

Hydrazides are much less reactive with aldehydes and ketones than aromatic hydrazines. Biological probes with hydrazides are frequently commercially available, so it was of interests to assess whether an *ortho*-phosphate would affect the rate of hydrazone formation. A commercially available coumarin hydrazide (7-diethylaminocoumarin-3-carbohydrazide **7**, Figure 4) was allowed to react with fY or SA-P at pH 7 using the same concentrations employed for the CH reactions. A typical kinetic trace is shown in Figure 5.



8: R^1 = H,
R^2 = $CH_2CH(NH_2)CO_2H$
9: R^1 = $P(O)(OH)_2$
R^2 = H

Figure 4 Structures of coumarin hydrazide (**7**) and hydrazones formed with fY (**8**) and SA-P (**9**).

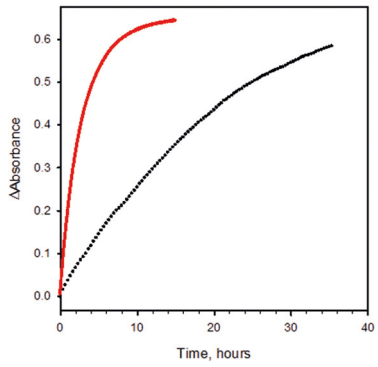


Figure 5 Formation of hydrazone **8** (black circles) or **9** (red circles) at 25 °C. The concentration of coumarin hydrazide (**7**) was 325 μM, and the concentration of fY or SA-P was 32.5 μM. Note the unit of the X-axis is hours.

The hydrazone forming reactions are much slower. The reaction between SA-P and coumarin hydrazide is essentially complete in about 12 hours. The reaction between fY and coumarin hydrazide, however, is not complete after 36 hours. Therefore, an *ortho*-phosphate increases the speed of the reaction with hydrazides as well as hydrazines at neutral pH. These data also serve to illustrate the large difference in reactivity between an aromatic hydrazine and a hydrazide. Under the same conditions, the hydrazine reaction is complete in less than 30 minutes, while the hydrazide reaction requires at least 12 hours to finish.

In conclusion, we show that directly appending a phosphate to an aromatic ring and positioning it adjacent to the aldehyde gives advantages in a hydrazone ligation reaction. First, the phosphate serves as an intramolecular general acid catalyst, which increases the rate of reaction over an order of magnitude compared to a phenol. Second, the phosphate group increases the aqueous solubility of the aldehyde and the hydrazone product. This should be particularly useful when relatively hydrophobic probes such as fluorophores are part of the ligation product.

Acknowledgment

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Supporting Information

Supporting information for this article is available online at http://dx.doi.org/10.1055/s-0035-1561387.

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(11) **Synthesis of Compound 5**

Coumarin hydrazine[2] (100 mg, 0.44 mmol) was added to dibenzyl-SA-P (0.162 g, 0.44 mmol) in MeOH. The reaction was stirred at r.t. for 2 h. Completion of the reaction was monitored by TLC. The precipitate was filtered and washed with excess MeOH and dried. Yellow product was obtained (130 mg, yield 55%). ^{1}H NMR (DMSO-d_6): δ = 2.36 (s, 3 H), 5.17 (s, 2 H), 7.26 (s, 2 H), 5.20 (s, 2 H), 6.07 (s, 1 H), 6.99–7.05 (m, 2 H), 7.25–7.39 (m, 14 H), 7.62 (d, 1 H), 8.23 (s, 1 H), 11.09 (s, 1 H) ppm. ^{13}C NMR (DMSO-d_6): δ = 18.0, 69.6, 97.9, 109.3, 109.5, 111.8, 120.6, 125.6, 125.9, 126.5, 126.6, 127.9, 128.5, 128.6, 129.7, 133.7, 135.5, 135.6, 147.7, 147.8, 148.3, 158.4, 155.1, 160.4 ppm.

Synthesis of Compound 6 (Deprotection)

Compound **5** was added to TFA–CH_2Cl_2 (1:1) and stirred at r.t. for overnight. Solvent was removed to yield actual product (60 mg, yield 100%). ^{1}H NMR (DMSO-d_6): δ = 2.35 (s, 3 H), 6.05 (s, 1 H), 6.95–7.06 (m, 2 H), 7.14–7.39 (m, 3 H), 7.60 (d, 1 H), 8.01 (d, 1 H), 8.26 (s, 1 H), 11.09 (s, 1 H) ppm. ^{13}C NMR (DMSO-d_6): δ = 18.0, 97.8, 109.3, 111.6, 120.9, 124.4, 125.4, 126.5, 126.7, 129.6, 135.1, 148.6, 149.4, 149.5, 153.6, 155.2, 160.5 ppm. ESI-MS [M^+]: 373.08; found: 374.

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