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Fast Alpha Nucleophiles: Structures that Undergo Rapid Hydrazone/Oxime Formation at Neutral pH

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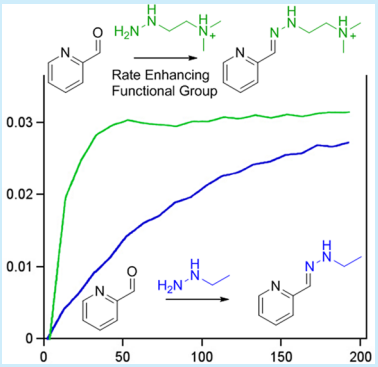
Supporting Information

ABSTRACT: Hydrazones and oximes are widely useful structures for conjugate formation in chemistry and biology, but their formation can be slow at neutral pH. Kinetics studies were performed for a range of structurally varied hydrazines, and a surprisingly large variation in reaction rate was observed. Structures that undergo especially rapid reactions were identified, enabling reaction rates that rival orthogonal cycloaddition-based conjugation chemistries.



The formation of imines by hydrazines and aminooxy compounds has been an exceedingly useful strategy for formation of conjugates in chemistry and biology (Figure 1).[1−3]

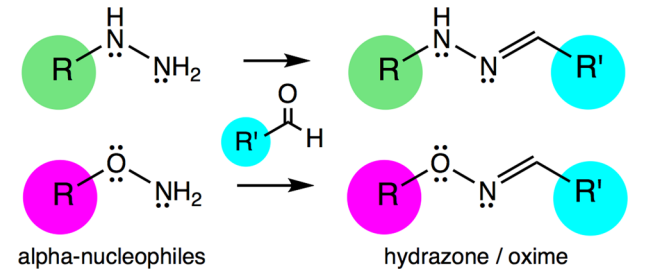


Figure 1. Formation of hydrazones and oximes by reaction of alpha-nucleophiles with carbonyl compounds.

Alpha nucleophiles such as hydrazines and aminooxy groups act as stronger nucleophiles than standard amines,[4] and their low basicity allows them to form more stable imine products as well.[5] Given these favorable attributes, hydrazone and oxime formation is of general interest and utility not only in biological chemistry[1] but also in polymer chemistry,[2] dynamic combinatorial chemistry,[3] and reaction development.[6]

Despite this widespread interest, one issue that limits the practical utility of these imine-forming reactions is their relatively slow rate, particularly at neutral pH.[1d,7] For one example, the reaction of aminooxy Peg with glyoxyl modified peptides has been reported to proceed with an observed second-order rate constant of 6×10^{-3} M^{-1} s^{-1} at pH 7.0.[7] This is much slower than ideal for reactions in, for example, biological settings where reactants occur at micromolar concentrations.[8]

Nucleophilic catalysis can speed hydrazone and oxime formation. Aniline has been traditionally used for this purpose;[1d,9] however, it exhibits relatively low efficiency and significant toxicity.[10] As a result, we and others were motivated to find water-soluble organocatalysts that are considerably more effective and less toxic than aniline,[7,9b,11,12] and we subsequently described the development of further improved third-generation catalysts as well.[13] However, catalysts add complexity to the reaction and may not be compatible with some reactant structures, or with cellular experiments.

To address these issues, we recently undertook more general studies of aldehyde and ketone structure and their effects on reaction rate in the absence of catalysts. We found a large range of reactivates, depending on structure, and identified specially reactive carbonyl compounds with acid/base groups near the reactive center.[14] These latter compounds formed products rapidly even without an added catalyst at biological pH.

Although carbonyl reactivity in hydrazone and oxime formation is now becoming better understood, the reactivity of the other partner is less well-defined. Indeed, we are aware of no general studies of the effects of structure on alpha nucleophile reaction rates in imine formation. One might expect that, since the nucleophilic amino group is generally not the site of most structural variation in hydrazines, reaction rates might be relatively insensitive to structural differences. Here we report that, on the contrary, these alpha nucleophiles vary considerably in their rates of hydrazone formation. We survey a range of structurally varied hydrazines and find a ≥100-fold range of rate constants for reaction. The studies have allowed us to identify structural features that yield surprisingly rapid

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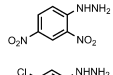
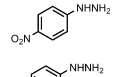
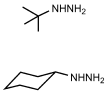
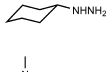
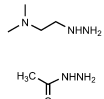
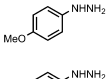
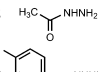
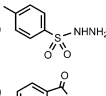
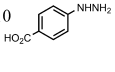
rates in hydrazone and oxime formation comparable to other existing rapid bioorthogonal reactions.

We began by performing kinetics studies of hydrazone formation with 20 structurally varied commercial hydrazines. The reactions were carried out at pH 7.4 in phosphate-buffered saline at room temperature. 10% DMF was added to ensure solubility; the cosolvent was not needed for many substrates but was included in all cases for consistency, and does not greatly affect rates (see Supporting Information (SI), Table S2). As a reaction partner for this first survey we chose 2-formylpyridine, which provides a useful chromophore during hydrazone formation for measuring reaction progress by UV−vis spectroscopy (Table S1). All reactions were performed in triplicate under standard pseudo-first-order conditions, with hydrazine in excess ([$RNHNH_2$] = 500 μM; [RCHO] = 10 μM). Linear first-order fits were quite good; UV−vis scans, reaction progress curves, and line fits are provided in the SI (Figures S1−S3).

Table 1 displays observed first-order rate constants and apparent second-order rate constants as a function of hydrazine

Table 1. Reactivity of Varied Hydrazines with 2-Formylpyridine[a]

	substrate	$k_{1(obs)}$ (min^{-1})	$k_{2(app)}$ ($M^{-1}sec^{-1}$)	k_{rel}		substrate	$k_{1(obs)}$ (min^{-1})	$k_{2(app)}$ ($M^{-1}sec^{-1}$)	k_{rel}
1		0.0037 (0.0006)	0.12 (0.02)	1	11		0.047 (0.006)	1.6 (0.2)	13
2		0.0043 (0.0006)	0.14 (0.02)	1.2	12		0.040 (0.001)	1.3 (0.1)	11
3		0.0053 (0.0006)	0.18 (0.02)	1.4	13		0.012 (0.001)	0.40 (0.03)	3.2
4		0.0063 (0.0006)	0.21 (0.02)	1.7	14		0.0093 (0.0006)	0.31 (0.02)	2.5
5		0.007 (0.001)	0.23 (0.03)	1.9	15		0.0073 (0.0006)	0.24 (0.02)	2.0
6		0.017 (0.001)	0.57 (0.03)	4.6	16		0.011 (0.001)	0.37 (0.03)	3.0
7		0.014 (0.002)	0.47 (0.06)	3.8	17		0.084 (0.006)	2.8 (0.2)	23
8		0.0073 (0.0032)	0.24 (0.10)	2.0	18		0.0077 (0.0015)	0.26 (0.05)	2.1
9		0.0073 (0.0006)	0.24 (0.02)	2.0	19		0.0050 (0.0010)	0.17 (0.03)	1.4
10		0.018 (0.001)	0.60 (0.03)	4.9	20		0.005 (0.002)	0.17 (0.07)	1.4

[a]Conditions: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, 10% DMF, 25 °C. Values measured 3 times and averaged (std. dev. in parentheses). Pseudo-first-order $k_{(abs)}$ normalized to standard 500 μM [hydrazine].

structure. Note that overall second-order behavior is expected for hydrazone formation at the low concentrations employed.[15] In the current study, second-order behavior was documented for two cases (Figure S4). Analyzing the data, we find that the hydrazines vary by over 20-fold in their rate of reaction with 2-formylpyridine (see Figure 2 for two examples). The slowest reactions were observed with the electron-deficient pentafluorophenylhydrazine and diphenylhydrazine, while methoxy- and methyl-substituted arylhydrazines were substantially faster. Some general trends were noted: first, electron-poor arylhydrazines react more slowly than electron-rich ones (see

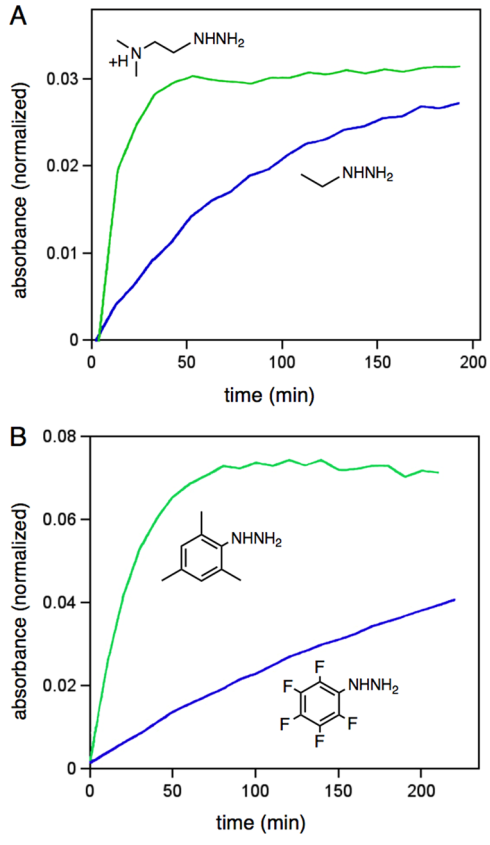


Figure 2. Examples of strongly varied reaction rates with changes in hydrazine structure, as shown by curves of reaction progress. (A) Alkylhydrazines with and without a basic amino group; (B) electron-rich vs electron-poor arylhydrazines. Conditions same as those in Table 1.

trimethylphenylhydrazine, entry 12 and Figure 2). A fit of sigma values in the aryl cases afforded a roughly linear correlation with ρ = −1.3 (Figure S5), consistent with a nonconjugated inductive effect lowering the nucleophilicity of the reacting amino group. Similarly, acylhydrazides and sulfonylhydrazides were also sluggish reactants, consistent with this explanation. Second, simple alkylhydrazines react at similar rates as phenylhydrazine and show little variation in rate. Finally, two hydrazines containing acid/base groups escape these trends by reacting significantly more rapidly: *ortho*-carboxyphenylhydrazine (OCPH; 13-fold more reactive than the slowest hydrazine) and 2-(dimethylamino)ethylhydrazine (DMAEH; 23-fold more reactive; see Figure 2). A similar survey (excluding alkylhydrazines due to the lack of a chromophore) was carried out with 2-butanone, and again the electron-poor hydrazines reacted more slowly than the electron-rich ones (Table S3). A correlation plot of reaction rates for these two carbonyl substrates shows a general correlation of reactivity of most aryl hydrazines, although the *o*-carboxy compound and (to a lesser extent) trimethylphenylhydrazine fall well off the line due to their substantially higher reactivity with the aldehyde (Figure S6).

Having identified two exceptionally reactive hydrazines (fast alpha nucleophiles, FANs) for the aldehyde substrate, we then explored the scope of their reactivity by reacting them with a range of aldehydes and ketones. The data are presented in Table 2. The *o*-carboxy compound OCPH reacts more rapidly with all new aldehyde and ketone substrates than it does with 2-

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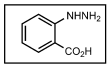
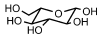
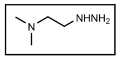
Table 2. Scope of Reactivity of Two Fast Hydrazines (Boxed)[a]

	substrate	$k_{1(obs)}$ (min^{-1})	$k_{2(app)}$ ($M^{-1}sec^{-1}$)	k_{rel}
				
1		0.027 (0.001)	0.90 (0.03)	7.3
2		0.039 (0.002)	1.3 (0.1)	11
3		0.038 (0.006)	1.3 (0.2)	10
4		0.037 (0.002)	1.2 (0.06)	10
5		0.034 (0.006)	1.1 (0.2)	9.2
6		0.031 (0.004)	1.0 (0.1)	8.4
7		0.13 (0.03)	4.3 (1.0)	35
8		0.051 (0.06)	1.7 (0.2)	14
9		0.37 (0.06)	12.3 (2.0)	100
10		0.090 (0.032)	3.0 (1.0)	24
				
11		0.045 (0.015)	1.5 (0.05)	12
12		0.062 (0.011)	2.1 (0.4)	17
13		0.078 (0.006)	2.6 (0.2)	21
14		0.21 (0.04)	7.0 (1.3)	57

[a]Conditions: 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, 10% DMF, 25 °C. Values measured 3 times and averaged (std. dev. in parentheses). Pseudo-first-order $k_{(abs)}$ normalized to standard 500 μM [carbonyl].

butanone. It forms hydrazones rapidly with aryl and alkyl aldhehydes and with aryl and alkyl ketones as well. Among aryl substrates, the fastest reactions occur with carbonyl compounds having proximal imino groups[14] (e.g., quinoline-8-carboxaldehyde, entry 8; 2-acetylpyridine, entry 10). The latter case proceeds with a rate 24-fold faster than the slowest reaction in the study. Alkyl aldehydes are yet faster, yielding rates up to 100-fold greater (butyraldehyde, entry 9) than the reference slow reaction. Intriguingly, dimethylaminoethylhydrazine (DMAEH, entries 11−14) is found to react even more rapidly than OCPH. Although it could be measured with only a few aryl aldehydes due to the lack of a chromophore in the hydrazone product, in each case the reaction rate was 2−4 times that of OCPH with the same carbonyl compound. We speculate that reactions of DMAEH hydrazine with alkyl aldehydes would yield the highest rates of all, but this possibility could not be measured with current methods.

Oxime bonds are in some applications more useful than hydrazones because of their greater hydrolytic stability.[5,9b] Inspired by this fastest FAN reactant, we synthesized the aminooxy analogue of DMAEH in order to test its reactivity in oxime formation. The new compound, dimethylaminoethyloxyamine (see SI for synthetic details), was reacted with 2-formylpyridine, and its rate was compared to that of the simplest alkyl aminooxy compound, methoxyamine. The results show (Figure 3) that the dimethylamino-substituted compound reacts with a rate 3-fold higher than that of the control compound. Thus, the data suggest that the favorable effect of the dimethylaminoethyl group on reaction rates may be general. More detailed studies and synthesis of analogs will be needed to test this possibility, but strategies for accelerating reaction rates may be especially useful for oxime ligations, which are slower than corresponding hydrazone formation reactions at biological pH.[5,9b]

The rate-limiting step for hydrazone formation at neutral pH is generally the breakdown of the tetrahedral intermediate

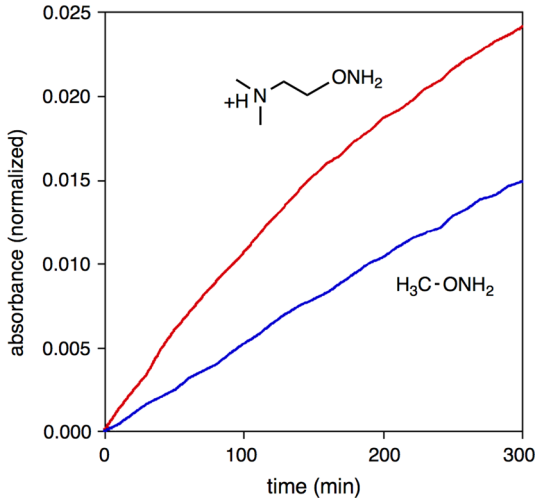


Figure 3. Favorable effect of a dimethylamino group on oxime formation rate, as shown by reaction progress curves for aminooxy compounds reacting with 2-formylpyridine at pH 7.4.

formed when the alpha nucleophile attacks the carbonyl carbon.[9a,15] We speculate that the origin of the high reactivity of the two fast-reacting hydrazines in this study (OCPH and DMAEH) is their ability to donate a proton intramolecularly at the transition state of the reaction. Although detailed mechanistic studies will be needed to test this, such proton donation could assist in the elimination of water from this tetrahedral intermediate, accelerating formation of the imine product.[15] The current FAN substrates have nonideal pK_a's for such a proton transfer, so we speculate that analogs with pK_a values closer to solution pH would be yet faster in hydrazone or oxime formation. This has indeed been found to be the case with nucleophilic catalysts for the reaction,[13] which adopt a closely analogous transition state. Future studies of this possibility for new, designed alpha nucleophiles are planned.

Significantly, the apparent second-order rate constants for reaction of the current two FAN hydrazines with a range of aldehydes are greater than 1 M^{-1} s^{-1} and, with the fastest substrate, over 10 M^{-1} s^{-1} (Table 2). This is more rapid than many bioorthogonal conjugation reactions, including most azide/strained-alkyne cycloadditions,[16] and is competitive even with recently described strain-driven Diels−Alder reactions.[17] Although we have recently reported structural features in aldehydes and ketones that yield rapid hydrazone formation,[14] the identification of rapid-reacting hydrazines and aminooxy compounds has special significance, because aldehyde reactive groups can be generated on a wide range of native biomolecules. For example, sugars and oligosaccharides can react directly with hydrazines[18] (see Table 2 for an example with a sugar). RNAs can be readily modified to generate reactive aldehydes in one step at the 3′ end via periodate oxidation,[19] and peptides and proteins can similarly be oxidized at N-terminal serine residues to generate a reactive aldehyde.[20] Thus one can envision the design of FAN hydrazine and aminooxy reagents carrying useful labels for rapid conjugation to these biomolecules. Future studies will be directed toward this possibility.

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■ ASSOCIATED CONTENT

Supporting Information

Synthesis and kinetics procedures, kinetic fit data, and supporting figures are provided. 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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