

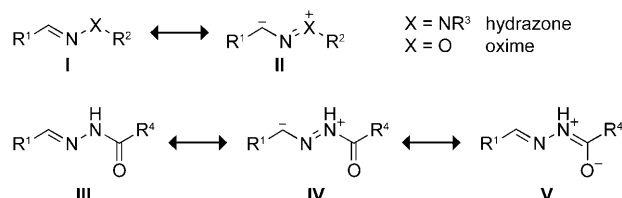
Conjugate Stability

Hydrolytic Stability of Hydrazones and Oximes**

Jeet Kalia and Ronald T. Raines*

Molecules containing carbon–nitrogen double bonds are prevalent in both chemical and biological contexts. The foundations for our current understanding of carbon–nitrogen double-bond formation and hydrolysis were laid by seminal work on hydrazone hydrolysis and formation,^[1] and by contributions from mechanistic studies on enzymes that utilize pyridoxal phosphate.^[2] In particular, the meticulous kinetic analyses of Jencks resulted in the delineation of a carbinolamine intermediate in carbon–nitrogen double-bond formation and hydrolysis, and elucidation of the general mechanism of carbonyl-group addition reactions.^[3,4] These principles were summarized in a landmark review.^[5]

Hydrazones and oximes ($C=N^1-X^2$) possess greater intrinsic hydrolytic stability than do imines. The textbook explanation for this greater stability invokes the participation of X^2 in electron delocalization (Scheme 1).^[6] The contribu-



Scheme 1. Major resonance forms of conjugates.

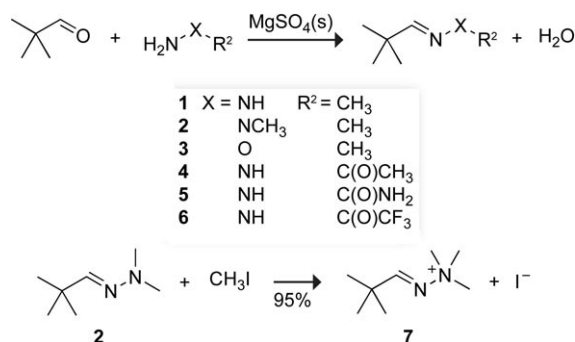
tion of resonance form **II** in alkyl hydrazones and oximes, and resonance form **IV** in acyl hydrazones increases the negative-charge density on C^1 and hence reduces its electrophilicity, thereby imparting greater hydrolytic stability to hydrazones and oximes. An alternative explanation is based on the

repulsion of the lone pairs of N^1 and X^2 being relieved in the conjugates.^[7]

Although the greater stability of hydrazones and oximes than imines is well-appreciated, a consensus on the comparative stability of hydrazones and oximes is lacking. To the best of our knowledge, the only report of a direct comparison of the rates of hydrolysis of hydrazones and oximes was from Stieglitz and Johnson in 1934.^[8] These workers assayed the hydrolysis of benzophenonehydrazone and benzophenone-oxime in extremely acidic solutions by titrating the respective hydrazine and hydroxylamine products. This rudimentary study provided little insight. More recently, other workers have discussed the stability of the hydrazones and oximes used in particular applications,^[9,10] but without direct comparisons.

Herein, we report the first detailed investigation of the hydrolysis of isostructural alkylhydrazones, acylhydrazones, and an oxime. Half-lives for the hydrolysis of these conjugates were measured with ¹H NMR spectroscopy in deuterated buffers (pD 5.0–9.0) to obtain pD rate profiles. In addition, pD titrations of the conjugates were performed with ¹H NMR spectroscopy to determine relevant pK_a values and thereby provide mechanistic insight. Our findings establish oximes as the linkage of choice for the stable conjugation of molecules via a carbon–nitrogen double bond.

Conjugates **1–6** were synthesized by condensation of the respective nitrogen bases with pivalaldehyde (*t*BuCHO) and removal of the water by-product with anhydrous MgSO₄ (Scheme 2). Pivalaldehyde was chosen because it lacks enolizable protons, thus preventing obfuscating side reactions such as aldol condensations. Methoxyamine and all the alkyl hydrazines and acyl hydrazines were available commercially except for trifluoroacetylhydrazine, which was generated in situ by the deprotection of Boc-trifluoroacetylhydrazine (compound **8**, see Supporting Information; Boc = *tert*-butyloxycarbonyl). Trimethylhydrazonium ion **7** was synthesized by treating dimethylhydrazone **2** with methyl iodide (Scheme 2). The synthesis of **7** by the condensation of



Scheme 2. Synthesis of conjugates.

[*] J. Kalia, Prof. R. T. Raines

Department of Biochemistry, University of Wisconsin-Madison
433 Babcock Drive, Madison, WI 53706-1544 (USA)

Fax: (+1) 608-262-3453

E-mail: rtraines@wisc.edu

Homepage: <http://www2.biochem.wisc.edu/raines>

Prof. R. T. Raines

Department of Chemistry, University of Wisconsin-Madison
1101 University Avenue, Madison, WI 53706-1322 (USA)

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trimethylhydrazinium ion and pivalaldehyde was unsuccessful, consistent with reports by others,^[11] nor was this condensation reaction facilitated to a detectable extent by aniline^[3d,10] at pD 5.0–9.0. (As trimethylhydrazinium ion did not even condense with the unhindered carbonyl group of formaldehyde, the likely problem is that nucleophilic attack by trimethylhydrazinium ion ($\text{H}_2\text{N}^1\text{N}^2(\text{CH}_3)_3^+$ generates a positive charge on N^1 when N^2 already bears a positive charge.) ^1H NMR spectroscopy in deuterated phosphate buffers (pD 5.0–9.0) was used to monitor the appearance of the aldehydic proton of pivalaldehyde ($\delta = 9.4$ ppm), a signal for conjugate hydrolysis.

The hydrolytic cleavage of carbon–nitrogen double bonds is reversible. An excess of a deuterated aldehyde or ketone can be used to trap the liberated nitrogen base and thereby push the hydrolysis reaction to completion, allowing the forward (hydrolysis) reaction to be monitored without interference from the reverse (condensation) reaction. Various aldehydes and ketones were tested as potential chemical traps. Deuterated acetone was an inefficient trap—a 100-fold excess drove the hydrolysis of a methylhydrazone to only 62% completion at pD 7.0 (data not shown). Another dialkyl ketone, levulinic acid, has been used for a similar purpose,^[12] but would have added a muddling carboxy group to the reaction mixture. Hexachloroacetone, tribromoacetaldehyde, and calcium mesoxylate could not be used because of their low aqueous solubility. Alloxan, an electrophilic ketone, was unstable in water. Finally, a 10-fold excess of deuterated formaldehyde (CD_2O) was identified as an effective trap, driving the hydrolysis reactions of all the conjugates (except that of trimethylhydrazonium ion **7**) to completion at pD 5.0–9.0. A typical kinetic trace is shown in Figure 1.

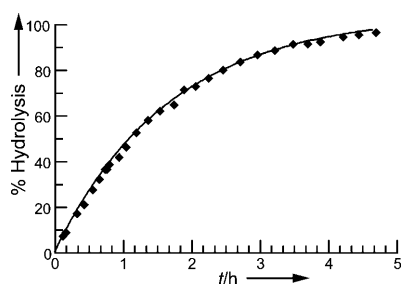


Figure 1. Kinetic trace for the hydrolysis of methylhydrazone **1** at pD 7.0 in the presence of a 10-fold molar excess of D_2CO . Each data point was obtained by integration of a ^1H NMR spectrum. Similar kinetic traces were obtained for other hydrolysis reactions.

At pD 5.0–9.0, the half-life of oxime **3** was much larger than those of each hydrazone, except for trimethylhydrazonium ion **7** (Table S1 in the Supporting Information). At pD 7.0, the first-order rate constant for the hydrolysis of oxime **3** was approximately 600-fold lower than for methylhydrazone **1**, 300-fold lower than for acetylhydrazone **4**, and 160-fold lower than for semicarbazone **5**. Although the linkage in a trialkylhydrazonium ion (such as conjugate **7**) is highly stable, it is not suitable for bioconjugation because its synthesis involves treatment with methyl iodide—a reagent that is not chemoselective in a biological system—subsequent

to condensation. Thus, oximes are the most preferable linkages for carbon–nitrogen double-bond-mediated bioconjugation.

The hydrolysis of the conjugates is catalyzed by acid (Figure 2). This finding is consistent with conjugate hydrolysis being accelerated by protonation. The hydrolysis of oxime **3** at pD > 7.0 and that of trimethylhydrazonium ion **7** at pD > 5.0 were too slow to yield a complete kinetic trace within a reasonable time frame.

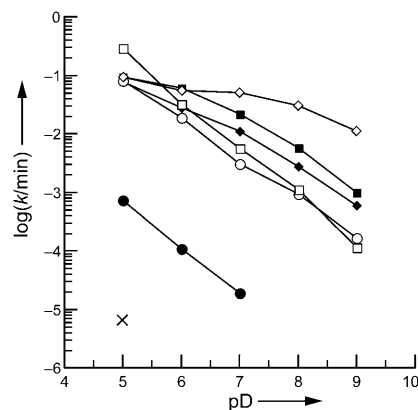


Figure 2. pD-rate profiles for the hydrolysis of conjugates **1** (◆), **2** (■), **3** (●), **4** (□), **5** (○), **6** (◇), and **7** (×). First-order rate constants (k) were calculated from kinetic traces (see Figure 1 for example).

pD-Titration experiments monitored with ^1H NMR spectroscopy revealed that some (but not all) of the conjugates experience a substantial change in protonation state between pD 0.7 and 13.4 (Figure 3). The δ value of C^1H for methyl-

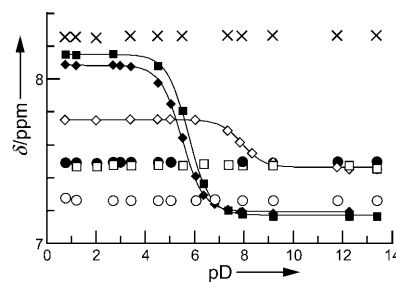


Figure 3. pD-Titration of the chemical shift of C^1H of conjugates **1** (◆), **2** (■), **3** (●), **4** (□), **5** (○), **6** (◇), and **7** (×).

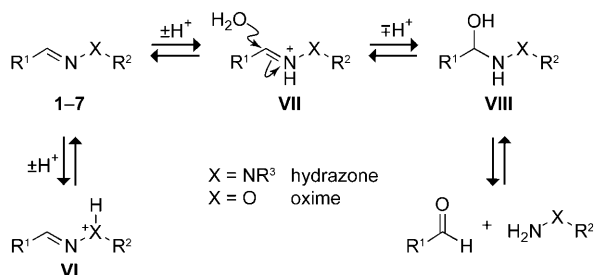
hydrazone **1** ($\text{p}K_a = 5.5$), dimethylhydrazone **2** (5.8), and trifluoroacetylhydrazone **6** (7.9) exhibited a sigmoidal dependence on pD. The δ value of C^1H in conjugates **3–5** and **7** was not a function of pD, indicating that an insignificant fraction of these conjugates is protonated at pD 0.7–13.4.

What is the site of protonation in the conjugates? The titration curves for methylhydrazone **1** and dimethylhydrazone **2** presumably result from protonation of either N^1 or N^2 . The similarity of δ values for the protonated forms of **1** and **2** to the δ value for the trimethylhydrazonium ion **7** (Figure 3), in which N^2 bears a positive charge, suggests that the site of

protonation of methylhydrazone **1** and dimethylhydrazone **2** is N² (**VI**; see Scheme 3). This interpretation is also supported by N² of dimethylhydrazone **2** being more nucleophilic than N¹ toward methyl iodide (Scheme 2). The only other report of attempts to determine the site of hydrazone protonation reached the same conclusion.^[13] The observed titration of trifluoroacetylhydrazone **6** is due to the loss of its N² proton, which is made acidic by the proximal trifluoromethyl group.

The value of δ does not correlate with conjugate stability. A high δ value of C¹H is indicative of low electron density on C¹, which portends a high susceptibility to attack by nucleophiles. Surprisingly, despite having the largest δ value (Figure 3), trimethylhydrazonium ion **7** is the most stable conjugate (Figure 2 and Table S1 in the Supporting Information). Moreover, oxime **3** and acetylhydrazone **4** have similar δ values, but at pD 7.0 the half-life of oxime **3** is 25 days whereas that of acetylhydrazone **4** is 2 h (Table S1 in the Supporting Information).

The data are consistent with a mechanism of C¹=N¹-X² hydrolysis that entails protonation of N¹ (Scheme 3). The resultant protonated species (**VII**) would be highly suscep-



Scheme 3. Putative mechanism for the hydrolysis of hydrazones and oximes.

tible to hydrolysis because of the enhanced electrophilicity of C¹. None of the conjugates is protonated to a significant extent on N¹ at pD 0.7–13.4 (Figure 3), indicating that the pK_a value of species **VII** is less than 0.7 in each conjugate, which is consistent with estimates of pK_a values for protonated oximes.^[14] The protonation of N¹ of trimethylhydrazonium ion **7** is discouraged by the adjacent quaternary ammonium group. Consequently, trimethylhydrazonium ion **7** is highly stable (Figure 2), even without the ability to access resonance form **II** or the presence of a repulsive lone pair on X². This finding belies the textbook^[6] and alternative^[7] explanation for the stability of hydrazones and oximes being greater than that of imines. Rather, these conjugates are more stable than imines because of the inductive effect of X²=N or O. This explanation is analogous to one for the origin of the α -effect.^[15]

The protonation of N¹ of oxime **3** is more favorable than that of trimethylhydrazonium ion **7**, accounting for the lower stability of oxime **3**. Still, the protonation of the oxime is less favorable than is the protonation of alkyl hydrazones **1** and **2** and acyl hydrazones **4–6**, because of the higher electronegativity of X² in the oxime ($\chi_{\text{O}}=3.5$ ^[16]) versus the hydrazones ($\chi_{\text{N}}=3.0$). Hence, oxime **3** is more resistant to

hydrolysis than are alkyl hydrazones **1** and **2** and acyl hydrazones **4–6**.

Finally, we note that the NMR spectra revealed no evidence of a carbinolamine intermediate (**VIII**). This observation, along with the high acidity of species **VII** (pK_a < 0.7), indicates that the rate-limiting transition state is that for the attack of water on species **VII**. The decomposition of a carbinolamine intermediate limits the rate of hydrolysis only under extremely acidic conditions.^[4,14]

In summary, we have evaluated the hydrolytic stability of a series of isostructural hydrazones and an oxime. We found the oxime to be much more stable than the simple hydrazones. pD-Rate profiles and pD-titrations suggest that the anomalous stabilities of the oxime (as well as a trialkylhydrazonium ion) is due to its resistance to protonation. These data can inform the proper use of compounds containing carbon–nitrogen double bonds.^[9,10]

Experimental Section

See the Supporting Information for experimental details.

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