Bioconjugation Hot Paper

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Dialdehydes Lead to Exceptionally Fast Bioconjugations at Neutral pH by Virtue of a Cyclic Intermediate**

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Abstract: *One of the open challenges in chemical biology is to identify reactions that proceed with large rate constants at neutral pH values. As shown here, dialdehydes react with O-alkylhydroxylamines at rates of 500 $M^{-1}s^{-1}$ at neutral pH values in the absence of catalysts. The key to these conjugations is an unusually stable cyclic intermediate, which ultimately undergoes dehydration to yield an oxime. The scope and limitations of the method are outlined, as well as its application in bioconjugation and a mechanistic interpretation that will facilitate further developments of reactions with alkylhydroxylamines at low substrate concentrations.*

Condensations of hydroxylamine and hydrazine derivatives with carbonyl groups are widely used methods for bioconjugation.[1] At neutral pH values, however, oxime condensations are too slow to be employed at the high dilution that is often required in biological settings. The fastest small-molecule bioconjugations are the tetrazine inverse-electron-demand Diels–Alder reactions with strained olefins ($k \approx 10^2$–$10^4\ M^{-1}s^{-1}$),[2] and a recently described amide-forming ligation of acyltrifluoroborates with *O*-carbamoylhydroxylamines ($k = 20\ M^{-1}s^{-1}$).[3] We describe here that dialdehydes react with *O*-alkylhydroxylamines with second-order rate constants in the range of 10^2–$10^3\ M^{-1}s^{-1}$ at neutral pH values, thus competing with the fastest small-molecule systems.[4] In practical terms, rate constants of this magnitude mean that even at concentrations of 5 μM for each substrate, the first half-life is less than seven minutes. A mechanistic analysis indicates that the key to these reactions is the rapid and irreversible formation of an isoindole bis(hemiaminal) (IBHA), which gradually dehydrates to the oxime (see Figure 1c).

Jencks' seminal mechanistic work showed that oxime condensations typically proceed through a rapid pre-equilibrium with a tetrahedral hemiaminal intermediate followed by a rate-limiting dehydration (Figure 1a).[5] Most attempts to optimize oxime and hydrazone condensations have therefore focused on lowering the barrier of the dehydration step: The use of low pH values,[6] nucleophilic catalysis,[7] and intramolecular proton assistance[8] are prominent examples (Figure 1b). At high dilution, however, the initial bimolecular addition step becomes rate-limiting and therefore warrants scrutiny. Herein, we show that dialdehydes provide an internal trap to stabilize hemiaminals as IBHAs, leading to an enormous shift in the equilibrium position. The K_{eq} is so large that the initial addition step becomes effectively irreversible; this simplifies hydroxylamine-based bioconjugations because the timing of the typically slow dehydration reaction is rendered immaterial: Both IBHAs and oximes represent successful conjugations. The results we present should find broad applicability in the labelling of biomolecules at high dilution and provide a framework for engineering further variants of *O*-alkylhydroxylamine addition reactions.

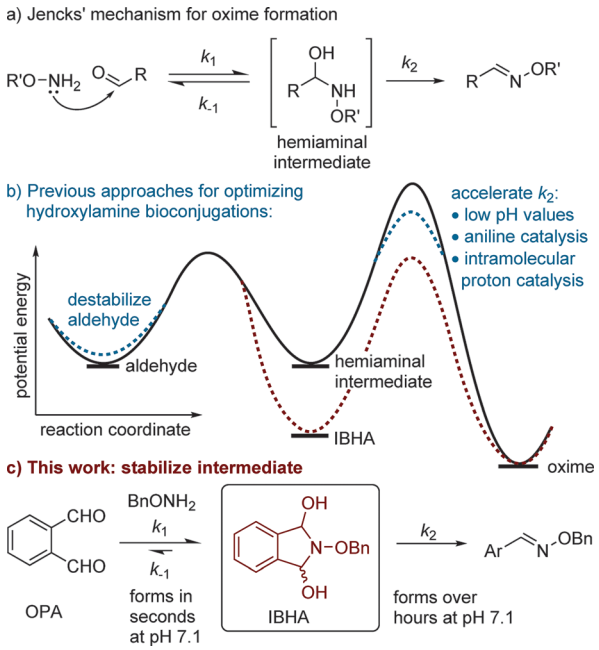

Figure 1. a) The Jencks mechanism for condensations with *O*-alkylhydroxylamines. b) Known approaches for accelerating the reaction are shown in blue on the reaction coordinate diagram. c) The approach outlined here is given in red: *O*-alkylhydroxylamines react with *ortho*-phthalaldehyde (OPA) to give stable isoindole bis(hemiaminals) (IBHAs) within seconds at neutral pH values (conditions: 10 mM phosphate buffer, pH 7.1, 20% acetonitrile). Please note that the reaction coordinate diagram is qualitative; smooth curves are drawn between kinetically relevant steps although proton transfer steps and intermediate addition products are likely to be involved.

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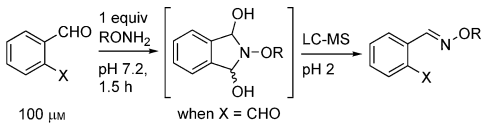
While testing the propensity of various *ortho*-substituted aldehydes to accelerate oxime condensations, we discovered that a stable intermediate formed within seconds of mixing *ortho*-phthalaldehyde (OPA) with *O*-benzylhydroxylamine in a 1:1 ratio at 100 μM at neutral pH (Figure 1c). A complete NMR characterization of the intermediate suggested a 9:1 ratio of diastereomers of the IBHA structure that is shown in red in Figure 1.[9] The IBHA further dehydrated to give the expected oxime over a time scale of hours. The rate of intermediate formation and its stability under neutral conditions immediately suggested its potential in bioconjugation. Indeed morpholino-type bis(hemiaminals) have been observed during the 3′-end labelling of periodate-cleaved RNAs;[10] surprisingly, however, RNA 3′-labelling has never been subjected to a detailed kinetic characterization, nor has it inspired a general bioconjugation approach based on dialdehydes.

Two reports have described accelerating effects of neighboring substituents on oxime or hydrazone condensations: Jencks and Wolfenden observed up to a tenfold rate enhancement with certain *ortho*-substituted benzaldehydes,[11] an effect that they attributed to a change in the addition equilibrium owing to destabilization of the starting aldehyde (indicated in blue in Figure 1). Kool et al. have recently shown that neighboring protons facilitate hydrazone condensations, and they proposed that intramolecular proton assistance helped the dehydration (blue annotation in Figure 1).[8a] The stabilization of adducts prior to dehydration, such as with IBHAs, would offer a new way of controlling alkylhydroxylamine conjugations.

Table 1: Comparison of oxime ligation reactions with different aldehydes and hydroxylamines.[a]



Entry	Aldehyde	$RONH_2$	Product	Conv. [%]
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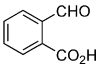
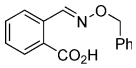
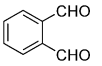
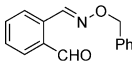
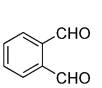
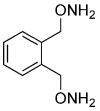
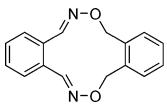
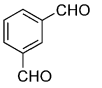
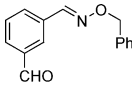
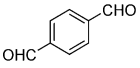
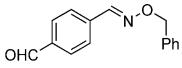
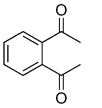
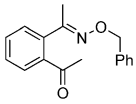
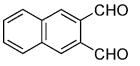
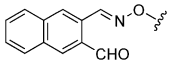
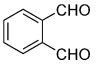
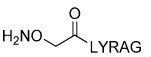
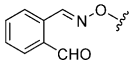
2[b]				<10
3[c]				>98
4[c]				>98
5[b]				<10
6[b]				<10
7[b]				<1
8[b]				98
9[b]				79

[a] Note that the oxime products are reported as acidic LC-MS detection was employed; with OPA, the adducts present prior to LC-MS injection are mixtures of the IBHAs and oximes. Each reaction in Table 1 that employs *ortho*-dialdehydes leads to complete conversion, we show the 90 min time point for convenient comparison. [b] Substrate concentrations: 100 μM; phosphate buffer (100 mM), pH 7.2. [c] Substrate concentrations: 10 μM.

To explore the nature of the acceleration and to establish the reaction scope, we first varied the substrates (Table 1). A survey of carbonyl derivatives confirmed the primacy of OPA: The reactions of benzaldehyde (entry 1), 2-formylbenzoic acid (entry 2), diacetylbenzene (entry 7), and dialdehydes with other substitution patterns (entries 5 and 6) were significantly slower. As shown in entry 4, bis(oximes) can also be formed, an approach that may be important when the presence of a residual aldehyde is undesirable. The special reactivity of dialdehydes is not limited to OPA; for example naphthalene dialdehyde was also a highly effective substrate, converting completely into the corresponding oxime within minutes (entry 8). The acceleration with OPA is maintained with complex substrates since a pentapeptide (LYRAG) bearing an N-terminal hydroxylamine gave 79% conversion after 1.5 hours at concentrations of 100 μM in each reaction partner (entry 9).

Time course NMR analysis provided a value for k_2 and a lower bound for K_{eq} (see Figure 2). As the dehydration is effectively irreversible over the time scales we monitored,[12] the appearance of oxime could be taken as a direct measure of k_2 (see the H_c protons in Figure 2). The value obtained, $1.2 \pm 0.2 \times 10^{-5}\ s^{-1}$, is two to three orders of magnitude smaller than those of typical oxime condensations at pH 7,[7a] consistent with the intermediate IBHA sitting in a deep energy minimum. No equilibrium for the initial addition was measurable over the observed time scale (20 h), a point independently confirmed in a separate experiment: When the IBHA[9] was dissolved in phosphate buffer, and the reaction mixture monitored by NMR spectroscopy, the formation of OPA was not observed; instead, the substrate was gradually converted into the oxime. Although an accurate K_{eq} value could not be determined, a lower bound of approximately $10^7\ M^{-1}$ may be assumed since low micromolar concentrations of the aldehyde would be well above the detection limit of the 700 MHz NMR spectrometer. In terms of the reaction coordinate diagram (see Figure 1), the fact that the IBHA led to oxime formation but not to formation of OPA suggests that dehydration is faster than retro-IBHA formation (i.e., $k_2 > k_{-1}$).[13] Overall, the mechanism of oxime formation that we observe with dialdehydes parallels Jencks' proposal for simple oximes (see Figure 1), with the key distinction that the equilibrium constant for the formation of the intermediate overwhelmingly favors addition.

The formation of the IBHA was so rapid that even at a concentration of 100 μM, NMR analysis lacked the time

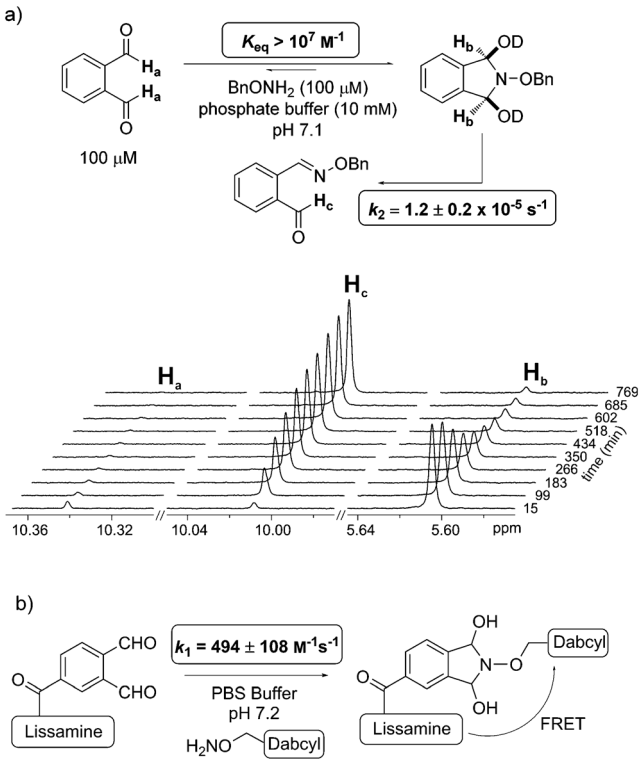
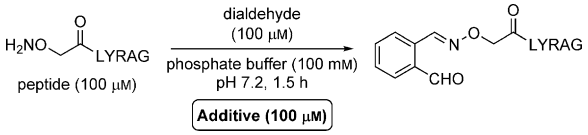


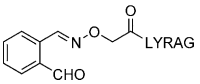
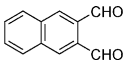
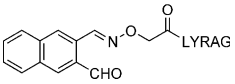
Figure 2. Kinetic analysis. a) NMR analysis indicating the peaks that were monitored with time to determine the value of k_2. NMR spectra covering the entire ppm range are included in the Supporting Information, Figure S8. Kinetic parameters are an average of two experiments. b) A fluorescence quenching assay was used to establish the rate of the initial bimolecular addition. k_1 is the combined average of six repeats at two different concentrations (see Figure S13 for the kinetic plots).

resolution to measure the initial bimolecular rate constant k_1. However, a value for k_1 is important in the planning of experiments because it forecasts the required concentrations and stoichiometry. We therefore developed a fluorescence quenching assay to determine k_1. A lissamine-tagged version of OPA and a dabcyl derivative bearing a hydroxylamine were synthesized, and kinetic parameters were determined at concentrations of 5 μM and 2 μM for each substrate (100 mM phosphate buffer, pH 7.2). The resulting data fit best to a second-order rate equation and gave an average over the two concentrations of $k_1 = 494 \pm 108\,\text{M}^{-1}\text{s}^{-1}$ (see Figure 2b). Although the fluorescence quenching assay was run with different substrates (the tags necessary for the readout), the magnitude of k_1 is qualitatively consistent with the rapid formation of the intermediate that was observed by NMR spectroscopy. Using these numbers we can now annotate the major elementary steps for oxime formation with dialdehydes (bold-faced values in Figure 2). The most rapid extant small-molecule bioconjugation is the tetrazine-strained olefin Diels–Alder reaction, which typically has second-order rate constants between 1 and $10^3\,\text{M}^{-1}\text{s}^{-1}$,[2a,b,14] and in the best cases up to $10^6\,\text{M}^{-1}\text{s}^{-1}$.[4,14–15] Despite these impressive rate constants, the widespread adoption of the tetrazine Diels–Alder reaction has been hindered by challenging substrate syntheses and the instability of the tetrazines. In comparison, IBHA formation has rate constants that compete with the fastest cycloaddition reactions,[4] but employs far simpler substrates.

For the labelling of complex samples, the dialdehyde must tolerate other biological nucleophiles. With simple amines, dialdehydes can form a great variety of products depending on the reaction conditions. Reactions with amines, however, are slower than what we observe with alkylhydroxylamines, and, although postulated as intermediates, IBHAs have never been observed with amines under aqueous conditions.[16] Under dilute conditions in cold DMSO, an ammonia-derived IBHA has been observed by NMR spectroscopy, but it rapidly eliminated water to deliver the Schiff base.[17] In studies using dialdehydes to capture substrate–kinase pairs, the groups of Shokat[18] and others[19] have shown that reactions with amines[20] can be mitigated by employing less reactive dialdehydes. In our case, the IBHA conjugation was equally effective in the presence of equimolar tryptophan. In the presence of lysozyme, however, the conversions were lower (see entries 3 and 6, Table 2), likely as a result of nucleophilic side-chains in the protein trapping some of the dialdehyde.

Table 2: Effect of amino acids and proteins on the IBHA reaction.[a]



Entry	Dialdehyde	Additive	Product	Conv. [%]
1		–		79
2		tryptophan		67
3		lysozyme		37
4		–		77
5		tryptophan		76
6		lysozyme		57

[a] Please note that we report the oxime products since acidic HPLC analysis was employed; with OPA, the adducts present prior to HPLC injection are mixtures of the IBHAs and oximes.

These results suggest that alkylhydroxylamine additions may be conducted with complex samples, but that a slight excess of the dialdehyde may be necessary for complete conversion.

To further demonstrate the practical potential of the method we examined a bioconjugation with a complex DNA substrate. We synthesized a 5′-*O*-alkylhydroxylamine terminated DNA 41-mer and tested it with various concentrations of an OPA derivative bearing the lissamine fluorophore (Figure 3, top). As the gel data illustrate, under neutral conditions, even down to 5 μM of DNA, almost complete conversion into the labelled product was observed after one hour (compare lanes 3–7), whereas the control lanes using either a monoaldehyde (lane 8) or a native DNA (lane 9) showed no labelling. Given the proclivity of nucleic acids to depurinate at the low pH values needed for most oxime condensations, the neutral conditions employed here provide a mild alternative for DNA and RNA bioconjugations.[21]

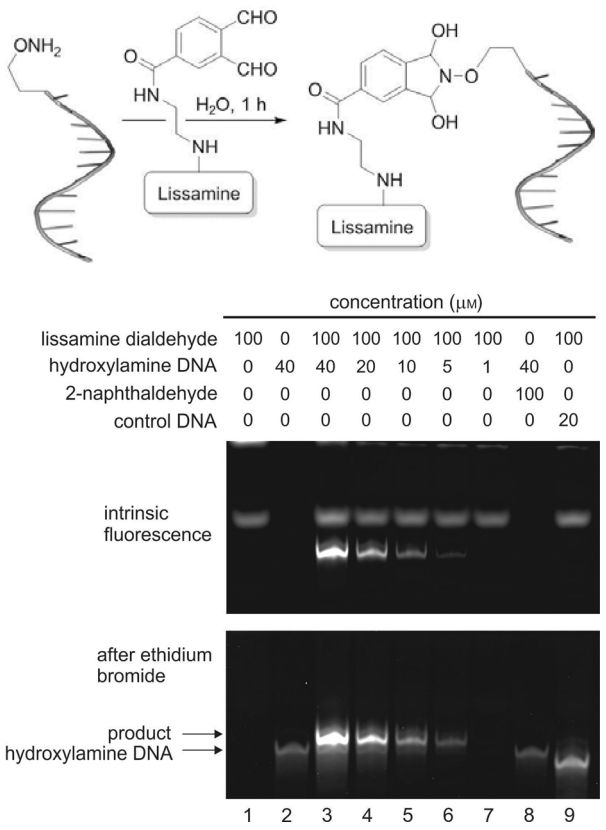


Figure 3. Top: DNA was efficiently labelled under mild conditions (pH 7). Please note that for simplicity, the IBHA is drawn, but the conjugation products were likely to be a mixture of IBHA and oxime. Gel: Lane 1: lissamine dialdehyde. Lane 2: 41-mer 5′-*O*-alkylhydroxylamine DNA. Lanes 3–7: IBHA conjugations at various DNA concentrations. Lane 8: control reaction of alkylhydroxylamine DNA with the fluorescent monoaldehyde 2-naphthaldehyde. Lane 9: control reaction confirming that the lissamine dialdehyde does not react with a native DNA oligomer (39-mer). Note that the reactions were run in unbuffered water; the pH value measured at the end of the reaction was 7.

Most effort in optimizing oxime and hydrazone condensations has been devoted to accelerating the final dehydration step as it has the highest activation barrier. However, at sufficiently low concentrations, bimolecular elementary steps will begin to dominate the rate equation of any reaction irrespective of barrier heights. Further development of alkylhydroxylamine bioconjugations at low concentrations will therefore require an increased focus on the initial bimolecular addition step. The propensity of OPA to form a stable IBHA is one such example, but additional possibilities could be imagined: Other dicarbonyl compounds that lead to stable IBHAs or alkylhydroxylamines that also deliver stable intermediates present new targets for future studies.

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Keywords: aldehydes · bioconjugation · hydroxylamines · kinetics · oximes

[1] G. T. Hermanson, *Bioconjugate techniques*, Elsevier/Academic Press, Amsterdam, **2008**.
[2] a) N. Devaraj, R. Weissleder, *Acc. Chem. Res.* **2011**, *44*, 816 – 827; b) M. L. Blackman, M. Royzen, J. M. Fox, *J. Am. Chem. Soc.* **2008**, *130*, 13518 – 13519; c) M. Taylor, M. Blackman, O. Dmitrenko, J. Fox, *J. Am. Chem. Soc.* **2011**, *133*, 9646 – 9649.
[3] H. Noda, G. Erős, J. W. Bode, *J. Am. Chem. Soc.* **2014**, *136*, 5611 – 5614.
[4] D. M. Patterson, L. A. Nazarova, J. A. Prescher, *ACS Chem. Biol.* **2014**, *9*, 592 – 605.
[5] W. P. Jencks, *Progress in Physical Organic Chemistry*, Wiley, Hoboken, **1964**, pp. 63 – 128.
[6] E. H. Cordes, W. P. Jencks, *J. Am. Chem. Soc.* **1962**, *84*, 4319 – 4328.
[7] a) A. Dirksen, T. M. Hackeng, P. E. Dawson, *Angew. Chem. Int. Ed.* **2006**, *45*, 7581 – 7584; *Angew. Chem.* **2006**, *118*, 7743 – 7746; b) M. Rashidian, M. M. Mahmoodi, R. Shah, J. K. Dozier, C. R. Wagner, M. D. Distefano, *Bioconjugate Chem.* **2013**, *24*, 333 – 342; c) P. Crisalli, E. T. Kool, *J. Org. Chem.* **2013**, *78*, 1184 – 1189.
[8] a) E. T. Kool, D.-H. Park, P. Crisalli, *J. Am. Chem. Soc.* **2013**, *135*, 17663 – 17666; b) P. Crisalli, E. T. Kool, *Org. Lett.* **2013**, *15*, 1646 – 1649.
[9] The IBHA could be preparatively isolated as a mixture with the oxime product by performing the reaction in acetonitrile. See the Supporting Information, Section 4.4, for full details of the procedure and a full NMR characterization of the IBHA.
[10] a) J. A. Hunt, *Biochem. J.* **1965**, *95*, 541 – 551; b) F. Hansske, F. Cramer, *Methods Enzymol.* **1979**, *59*, 172 – 181; c) F. Hansske, M. Sprinzl, F. Cramer, *Bioorg. Chem.* **1974**, *3*, 367 – 376; d) D. K. Willkomm, R. K. Hartmann in *Handbook of RNA Biochemistry*, Wiley-VCH, Weinheim, **2008**, pp. 86 – 94; e) J. Summerton, D. Weller, *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 187 – 195.
[11] R. Wolfenden, W. P. Jencks, *J. Am. Chem. Soc.* **1961**, *83*, 2763 – 2768.
[12] J. Kalia, R. T. Raines, *Angew. Chem. Int. Ed.* **2008**, *47*, 7523 – 7526; *Angew. Chem.* **2008**, *120*, 7633 – 7636.
[13] A separate NMR experiment also supports that $k_2 > k_{-1}$, and that the first step is not an equilibrium: When a sample containing the IBHA of napthalene dialdehyde was treated with a large excess of OPA, no scrambling to the OPA-derived IBHA was observed. This suggests that once the IBHA is formed, the alkylhydroxylamine is never released again. See the Supporting Information, Section 4.3, for the NMR experiments supporting this conclusion.
[14] See Ref. [2c].
[15] R. Selvaraj, J. M. Fox, *Curr. Opin. Chem. Biol.* **2013**, *17*, 753 – 760.
[16] a) E. Kulla, P. Zuman, *Org. Biomol. Chem.* **2008**, *6*, 3771 – 3780; b) J. Klíma, M. Polášek, J. Ludvík, J. Urban, *J. Heterocycl. Chem.* **2012**, *49*, 1202 – 1209.
[17] T. Do Minh, A. L. Johnson, J. E. Jones, P. P. Senise, *J. Org. Chem.* **1977**, *42*, 4217 – 4221.
[18] A. V. Statsuk, D. J. Maly, M. A. Seeliger, M. A. Fabian, W. H. Biggs, D. J. Lockhart, P. P. Zarrinkar, J. Kuriyan, K. M. Shokat, *J. Am. Chem. Soc.* **2008**, *130*, 17568 – 17574.
[19] K. Liu, K. A. Kalesh, L. Bing Ong, S. Q. Yao, *ChemBioChem* **2008**, *9*, 1883 – 1888.
[20] P. Zuman, *Chem. Rev.* **2004**, *104*, 3217 – 3238.
[21] An additional example of DNA labelling with a bis(alkylhydroxylamine) is also provided in the Supporting Information, Section 2.3.

Communications

Bioconjugation

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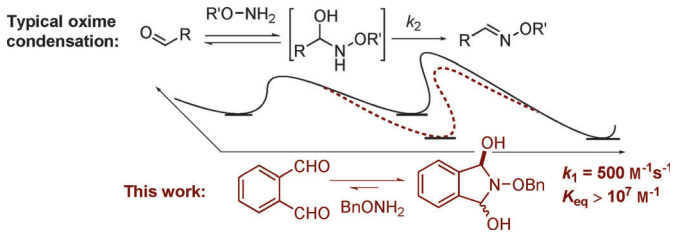
Dialdehydes Lead to Exceptionally Fast Bioconjugations at Neutral pH by Virtue of a Cyclic Intermediate



For aldehydes, 1 + 1 = 500: Dialdehydes react with *O*-alkylhydroxylamines at rates of 500 $M^{-1}s^{-1}$ at neutral pH values in the absence of catalysts. The key to these conjugations is an unusually stable cyclic intermediate, which ultimately undergoes dehydration to yield an oxime. The application of this method in bioconjugations and a mechanistic interpretation are outlined.