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Oximes and Hydrazones in Bioconjugation: Mechanism and Catalysis

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Abstract

The formation of oximes and hydrazones is employed in numerous scientific fields as a simple and versatile conjugation strategy. This imine-forming reaction is applied in fields as diverse as polymer chemistry, biomaterials and hydrogels, dynamic combinatorial chemistry, organic synthesis, and chemical biology. Here we outline chemical developments in this field, with special focus on the past ~10 years of developments. Recent strategies for installing reactive carbonyl groups and α -nucleophiles into biomolecules are described. The basic chemical properties of reactants and products in this reaction are then reviewed, with an eye to understanding the reaction's mechanism and how reactant structure controls rates and equilibria in the process. Recent work that has uncovered structural features and new mechanisms for speeding the reaction, sometimes by orders of magnitude, is discussed. We describe recent studies that have identified especially fast reacting aldehyde/ketone substrates and structural effects that lead to rapid-reacting α -nucleophiles as well. Among the most effective new strategies has been the development of substituents near the reactive aldehyde group that either transfer protons at the transition state or trap the initially formed tetrahedral intermediates. In addition, the recent development of efficient nucleophilic catalysts for the reaction is outlined, improving greatly upon aniline, the classical catalyst for imine formation. A number of uses of such second- and third-generation catalysts in bioconjugation and in cellular applications are highlighted. While formation of hydrazone and oxime has been traditionally regarded as being limited by slow rates, developments in the past 5 years have resulted in completely overturning this limitation; indeed, the reaction is now one of the fastest and most versatile reactions available for conjugations of biomolecules and biomaterials.

Graphical abstract

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1. Introduction

In recent years, bioconjugation reactions have become an indispensable tool for studying and manipulating biomolecules *in vitro* and *in vivo*. Reactions that allow for covalent tagging of biomolecules in their native environment with high selectivity and specificity are of particular interest, since this can yield new insights into cellular processes.^{1,2} Among many other labeling reactions that can be done under physiological conditions,^{3,4} the formation of oximes and hydrazones is nowadays commonly used to facilitate link biomolecules to various probes. Generally, oximes **4** and hydrazones **5** can be easily formed from the corresponding α -effect nucleophile (alkoxylamine **1** or hydrazine **2**, respectively) and a carbonyl compound (aldehyde or ketone **3**, Scheme 1) with water being the sole byproduct.

Notably, those two reactions are considerably older than many other bioconjugation reactions. As early as 1882, oximes and their formation have been intensively studied by Meyer and Janny.^{5–8} Shortly later, the term hydrazone was coined by Fischer in 1888.⁹ Due to the simplicity of these venerable ligation reactions, hydrazones and oximes have also had a pervasive influence on numerous other research fields ever since. They have been extensively used for ¹⁸F-labeling of peptides and proteins,¹⁰ syntheses of molecular switches, metallo-assemblies and sensors,^{11,12} treatment of organophosphorus poisoning,^{13,14} decoration of nanoparticles,^{15,16} preparation of palladacycle precatalysts,^{17,18} syntheses of alicycles and heterocycles,^{19–23} and derivatization of carbohydrates for mass spectrometric analysis.²⁴ Other specific aspects and applications of oximes and hydrazones that have previously been reviewed include their biological activities,^{13,25} their utility as valuable synthetic intermediates,^{26–30} the multifunctionalization of biological scaffolds,³¹ and the preparation of protein conjugates.³²

This review will highlight the most recent strategies to improve oxime/hydrazone bioconjugation reactions with a particular emphasis on approaches that enable rapid

conjugation under physiological conditions (see the Supporting Information for a selection of detailed, exemplary oxime/hydrazone bioconjugation protocols). After a brief account of the strengths and features of these linker moieties, synthetic methods that incorporate α -effect nucleophiles or carbonyl groups into diverse classes of biomolecules will be described. The mechanism of the formation of oximes and hydrazones will be explained in detail. The stability of those conjugates will subsequently be critically analyzed, since they can theoretically undergo hydrolysis in aqueous solution. Finally, the latest advances in the designs of efficient bifunctional catalysts as well as aldehydes and α -effect nucleophiles with intrinsically high reactivity will be examined.

2. Utility of Oxime/Hydrazone Ligation

Ideally, ligation reactions have to meet certain requirements for greatest utility in bioconjugations. The new linkage should preferably be stable across a broad range of biological environments and should be small in size to minimize its steric hindrance. Furthermore, the ligation should proceed with high selectivity and specificity, which means that the two bond-forming moieties should be rather scarce among naturally occurring molecules and not too reactive to avoid random labeling. Moreover, the reaction should be bioorthogonal;^{1,33,34} i.e., the conjugation can be conducted in living systems without interfering with biological processes or metabolites.

Oxime and hydrazone bond formation meets several of these requirements and is therefore frequently applied for bioconjugations. The oxime/hydrazone tether is very small in size, consisting only of three non-hydrogen atoms ($C=N-X$ with $X = O$ or NH), and thus constitutes a minimal perturbation of the native biomolecule. Notably, the stability of this linkage must be regarded with caution. Although Scheme 1 depicts the oxime/hydrazone formation as a straightforward condensation, the reaction is in fact reversible and the conjugates can undergo hydrolysis in aqueous media (see section 4 for the detailed mechanism). However, the hydrolytic stability can be effectively tailored by making structural changes to the linking moiety, for example, by employing oximes, which generally display much higher stability than hydrazones.³⁵ In addition, the pH of the solution also has a marked influence on the conjugates' stability. As a consequence, the half-life of oxime and hydrazone conjugates can span several orders of magnitude. Hence, the reversibility of the oxime/hydrazone conjugation can be exploited as a powerful feature for the controlled release of biologically active molecules. The diverse stability factors will be discussed in greater detail in section 5.

Oxime- and hydrazone-based conjugations are occasionally listed as bioorthogonal reactions.^{36–42} However, the bioorthogonality of these ligations is not unconditional and must be treated with caution. Although α -effect nucleophiles are rarely found in nature,⁴³ aldehydes and ketones are common. Importantly, the hemiacetal of every carbohydrate is in equilibrium with the respective carbonyl compound, which makes many of them amenable to reactions with α -nucleophiles.^{44–46} Furthermore, numerous aldehydes and ketones (e.g., ketone bodies)⁴⁷ are generated through normal and pathogenic metabolism.⁴⁸ Specific conditions, like oxidative stress,^{49–51} are also known to trigger the production of a variety of

aldehydes. Consequently, the concentration of cellular carbonyl compounds can span several orders of magnitude, from low nanomolar to high micromolar.⁵²

To gauge their bioorthogonality, the reactivity of both reaction partners must be scrutinized as well. Due to the α -effect,^{53–55} hydrazines and alkoxyamines are strongly nucleophilic and readily react with a wide range of electrophiles. Aldehydes and ketones, on the other hand, can form imines with various amines, albeit in a reversible manner, which also explains their potential cytotoxicity.⁵⁶

Although the oxime/hydrazone conjugation is not strictly bioorthogonal, it can be effectively bioorthogonal, if highly reactive electrophiles and aldehydes/ketones are only present at low concentration.¹⁵ Fortunately, this requirement is sufficiently met in many biological environments, which renders this reaction truly versatile.

3. Functionalization of Biomolecules For Oxime/Hydrazone Ligation

In order to undergo an oxime/hydrazone conjugation reaction, the respective biomolecules must contain either a carbonyl moiety or an α -nucleophile. The former can be found in some biologically relevant molecules, most notably reducing sugars, but typically methods that install either of these two reactive groups are prerequisite. The following section will give a brief overview of strategies that allow for the site-selective decoration of different biomolecules with the respective functional groups.

3.1. Functionalization with Aldehyde and Ketone Moieties

An old but still widely used method for the functionalization of biomolecules with carbonyl groups is the oxidative cleavage of vicinal diols with sodium periodate (Scheme 2).⁵⁷ This strategy can be used for facile modification of the glycoproteins of the glycocalyx by oxidizing sialic acid residues.⁵⁸

Nucleic acids that display 3'-ribonucleotides (**12**) are likewise known to undergo oxidation to yield the respective dialdehyde **13**, which exists in its hydrated 1,4-dioxane form **14** in aqueous solution (Scheme 3).^{59,60} In addition, 5-formylcytosine (5-fC) and 5-formyluracil (5-fU) can be used as building blocks for the site-specific aldehyde labeling of synthetic DNA. The latter is more reactive, which allows for selective tagging of DNA that contains both of these naturally occurring modifications.⁶¹

Similarly, 1,2-amino alcohols **7** are susceptible to oxidative cleavage as well (see Scheme 2), which can be exploited for the selective labeling of peptides with N-terminal serine or threonine.⁶² As an extension, the unnatural amino acids **15–19** and the 3'-appendix **20** have been developed, which can be incorporated into synthetic peptides or nucleic acids to enable site-selective side chain or backbone cleavage with periodate under mild conditions (Figure 1).^{63–66}

A wide variety of ketone-containing amino acid congeners have been introduced into peptides and proteins (Figure 2). They can be installed site-specifically either by classical solid-phase peptide synthesis⁶⁷ or, as an alternative, by using the biosynthetic machinery via amber-stop codon suppression.^{68–73}

Furthermore, Francis and co-workers have demonstrated that proteins can be modified postsynthetically with a ketone via azo coupling with keto-diazonium ion **29** (Scheme 4).^{74,75} Importantly, this modification targets exclusively the electron-rich aromatic ring of tyrosine residues.

Cysteine-containing peptides and proteins can be readily functionalized with the enzyme protein farnesyltransferase (PFTase) and the farnesyl pyrophosphate derivative **32** (Scheme 5).^{76,77}

In that method, the cysteine residue is required to be positioned at the C-terminus as a part of a tetrapeptide sequence termed the CAAX-box. In addition, Bertozzi and co-workers have shown that proteins can be equipped with an aldehyde moiety in living cells by recruiting the formylglycine-generating enzyme (FGE) to the 6-amino acid tag LCTPSR.⁷⁸

Concomitantly, methods for the selective functionalization of the N-terminus of peptides and proteins have been devised. A biomimetic approach enables N-terminal transamination with pyridoxal phosphate (**35**) as the carbonyl oxygen donor (Scheme 6).⁷⁹ This remarkable selectivity over lysine chains arises from the increased acidity of the N-terminal α -C-H bond due to the adjacent carbonyl moiety, which facilitates the isomerization from imine **36** to **37**.

The same result can be obtained by site-specific oxidation of N-terminal amine **34** with oxone, yielding oxime **40**, which can subsequently undergo exchange with other alkoxyamines (Scheme 7).⁸⁰

3.2. Functionalization with Alkoxyamine, Hydrazone, and Hydrazine Moieties

Due to the fact that most biomolecules can be readily functionalized with aldehyde or ketone moieties,⁸¹ fewer methods for the implementation of α -effect nucleophiles have been developed. Thus, the introduction of α -nucleophiles into biomolecules is typically limited to synthetic nucleic acids and peptides. Several phosphoramidite derivatives have been applied for the 5'-modification of synthetic nucleic acids (Figure 3).⁸²⁻⁸⁶

At the same time, the building blocks **49-53** have been designed for internal modification of nucleic acid strands (Figure 4). The α -nucleophile can be tethered to the 1'-position either as a replacement (**49**)⁸⁷ or as a modification of the nucleobase (**50** and **51**).^{88,89}

Furthermore, ribonucleotide building blocks **52** and **53** can be obtained by functionalization of the 2'-hydroxy function.^{89,90}

Synthetic peptides can be easily equipped with a C-terminal hydrazide moiety by cleaving the respective solid-supported peptide **54** from the Wang resin with hydrazine (Scheme 8).⁹¹ Alternatively, the peptidyl hydrazide **55** can be directly synthesized on the corresponding supports **56** or **57**.^{92,93} The final cleavage can be achieved either photolytically or under acidic conditions, respectively.

N-Terminally decorated peptides can be generated by using N,N,N' -tris(Boc)hydrazinoacetic acid for the last coupling step of their solid-supported assembly.⁹⁴

Additionally, unnatural amino acids **58–64** have been created to generate synthetic peptides with internal α -nucleophiles (Figure 5).^{64,95–98}

4. Mechanism of Oxime/Hydrazone Formation

In order to develop and apply oxime/hydrazone formation to its full potential, it is crucial to understand the underlying mechanism. As shown in sections below, this knowledge helps users understand the equilibria and rates involved and is empowering for future catalyst development and for designing fast-reacting substrates.

In the 1930s, seminal works on the formation and hydrolysis of semicarbazones brought first insights into the mechanism of this reaction.^{99,100} This was followed by important mechanistic studies by Jencks in the 1960s.¹⁰¹ An important finding was that this conjugation is fully reversible. The following mechanistic reflections will outline the formation of oximes **4** and hydrazones **5**; the hydrolysis reaction is obtained by reversing the order of the reaction steps.

The reaction commences by a proton-catalyzed attack of the α -effect nucleophile **1** or **2** on the carbonyl carbon atom of electrophile **3** (Scheme 9). Upon proton transfer, the hemiaminal **67** or **68**—the tetrahedral intermediate—is obtained. This hemiaminal can undergo dehydration via protonation of the hydroxyl function and subsequent elimination of water. The resulting protonated intermediate is represented by two resonance forms (**71/72**). The final deprotonation yields oxime **4** or hydrazone **5**.

Typically, this reaction is under general acid catalysis.^{99,100,102} Jencks and co-worker proposed the transition state **73** for the acid-catalyzed nucleophilic attack on the carbonyl carbon atom, which leads to the protonated intermediate **65** or **66** (Scheme 10).¹⁰³ According to their detailed studies on semicarbazone formation, the proton transfer from a general acid to the carbonyl oxygen atom occurs concurrently with the attack of the nucleophilic reagent. Thereby, the termolecular complex **73/74** would likely arise from two consecutive bimolecular reactions, rather than a ternary collision. Facilitated by hydrogen bonding, the carbonyl compound **3** can exist in a pre-equilibrium complex with the general acid, which would be followed by the C–N bond-forming attack of the α -nucleophile **1** or **2**.

Attack of strong α -nucleophiles on aldehydes and ketones is a fast reaction and is not rate-limiting in the large majority of cases. In the pH range from ca. 3 to 7, the acid-catalyzed dehydration of the tetrahedral intermediate **67/68** is typically the rate-determining step.¹⁰³ This is supported by the observation that the formation of oximes and hydrazones becomes concentration-independent if the excess of α -nucleophile is sufficiently high.¹⁰⁴ More supporting evidence is provided by density functional theory (DFT) calculations.¹⁰⁵

Although the reaction rate for hydrazone/oxime formation can be greatly accelerated under general acid catalysis, the reaction slows down again if the pH of the reaction medium is too low. This is due to the fact that the α -nucleophile **1** or **2** is in equilibrium with up to two different protonation states (Scheme 11). Hydrazines **2** can undergo protonation at either of their nitrogen atoms, forming hydrazinium ion **76** or **78**, respectively.¹⁰⁶ For alkoxyamines **1**, the protonation occurs exclusively at the terminal nitrogen atom to yield

alkoxyammonium ion **77**. Owing to the low basicity of oxygen, the formation of oxonium ion **75** is of no importance. Since these protonated species do not lead to product formation, due to their low nucleophilicity, the reaction slows at more acidic pH (typically under pH 3).¹⁰⁷ Under such conditions, the attack of the α -effect nucleophile **1** or **2** will become rate-determining.¹⁰³ Consequently, this reaction is fastest when the acidity of the solution strikes a balance between fast acid-catalyzed dehydration of hemiaminal **67** or **68** and negligible formation of unreactive protonated α -effect nucleophiles **76–78**.¹⁰⁸ For the formation of oximes and hydrazones, a pH of ca. 4.5 is typically advantageous.^{109,110} However, many biological applications require this ligation to proceed under physiological conditions, which is challenging due to the slow reaction rate at neutral pH and the low concentrations of the reaction partners.¹¹¹ Thus, the development of efficient catalysts and fast-reacting substrates, which could lead to more rapid bond formation under biological conditions, has been a strong focus of recent work (see sections 6 and 7).

5. Stability Factors

Among commonly used bioconjugation reactions, a unique feature of hydrazones and oximes is that the stability of the linkage can be fine-tuned by virtue of the general reversibility of this ligation. Since the hydrolysis of oximes and hydrazones can be obtained by reversing the order of the reaction steps (Scheme 9), it can be easily seen that the back-reaction is initiated by protonation of the imine nitrogen.¹⁰⁷ Mechanistic studies by Kalia and Raines showed that the negative inductive effect of the group X attached to the imine-forming nitrogen directly influences the stability of the respective conjugate.³⁵ If X is an electronegative heteroatom, the basicity of the imine nitrogen is diminished. This explains a commonly observed trend: imines (X = CH₂) hydrolyze readily under aqueous conditions, whereas oximes and hydrazones are much more stable due to the negative inductive effect of the additional heteroatom (X = O or NH), with the former being the most stable conjugate in this series due to the high electronegativity of oxygen [$\chi_p(\text{O}) = 3.5$ vs $\chi_p(\text{N}) = 3.0$]. This reversibility can be an appealing feature of the oxime/hydrazone conjugation, which enables valuable applications, like the controlled release of small molecules from suitable drug delivery platforms.^{112–114} Due to their inherently greater stability, oximes are typically preferred in bioconjugation reactions if a more stable linkage is required, while the more labile hydrazones are employed for the controlled release of biologically active molecules. Moreover, in principle one can use a catalyst to “switch on” this reversal in an otherwise relatively stable linkage, thus allowing the controlled breaking of the bond.

The following two subsections will discuss intrinsic and extrinsic factors that govern this bond stability.

5.1. Intrinsic Stability Factors

As outlined above, the intrinsic stability of oxime and hydrazone conjugates is directly influenced by steric and, more importantly, electronic factors in the vicinity of the linkage. In general, conjugates obtained from ketones have a higher stability than the respective aldehyde derivatives.¹¹⁵ The equilibrium constants of hydrazones are typically in the range

of 10^4 – 10^6 and $>10^8$ M⁻¹ for oximes.¹¹⁶ Figure 6 depicts the equilibrium constants of some prototypical oximes, hydrazones, and semicarbazones for comparison.^{117–119}

The thermodynamic stability of oximes formed from different aldehydes and ketones increases in the following order: acetone < cyclohexanone ~ furfural ~ benzaldehyde < pyruvic acid (Figure 7).¹⁰⁸ Aromatic aldehydes and derivatives of α -oxo acids are thus frequently used for bioconjugations.^{31,200}

The substituents at the α -nucleophile moiety can also have a distinct influence on the stability. In a series of isostructural conjugates, the first-order rate constant for the hydrolysis of oxime **95** was 160-fold lower than semicarbazone **94**, 300-fold lower than acetylhydrazone **93**, and 600-fold lower than methylhydrazone **92** (Figure 8).³⁵ Hydrazones bearing two electron-withdrawing groups were found to be especially labile and hydrolyzed rapidly even at neutral pH.¹¹⁸

If required, the stability of oxime and hydrazone conjugates can be enhanced by reducing their C=N double bond, e.g. with sodium cyanoborohydride.^{121,122} Kalia and Raines made the interesting observation that trimethylhydrazonium ions **96** are exceptionally stable and even exceed the hydrolytic stability of oximes.³⁵ In this case, the intrinsically charged trimethylammonium group inductively disfavors the protonation of the imine nitrogen (Scheme 12). However, those conjugates are not readily accessible in a biological environment, because the quaternization involves the strong and toxic electrophile methyl iodide.

Simanek and co-workers have investigated the hydrolytic stability of a novel series of hydrazones **99**, which were formed from *s*-triazinylhydrazines and various aldehydes/ketones (Figure 9).¹²³ At pH >5, those conjugates had a lower stability compared to structurally analogous acetylhydrazones **100**.

Interestingly, this stability trend reversed around pH 5. The authors noted that this observation can be explained by the protonation of the *s*-triazinyl moiety ($pK_a \sim 5$), which subsequently disfavors the required protonation of the imine nitrogen due to the presence of a delocalized positive charge (Scheme 13).

Bertozzi and co-workers have recently presented a variation of the classical oxime formation, termed Pictet–Spengler ligation, which yields hydrolytically stable conjugates (Scheme 14).¹²⁴ In analogy to the Pictet–Spengler reaction, this conjugation involves the indolyl-substituted nucleophile **102**, which reacts with aldehyde **11** to form oxyiminium ion **103**. Ensuing intramolecular C–C bond formation results in the tricyclic heterocycle **104**. This Pictet–Spengler ligation was also applied for the conjugation of isostructural hydrazine nucleophiles.¹²⁵

Recently, Gillingham and co-workers found that aromatic aldehydes/ketones **105**, which feature a boronyl moiety in the ortho position, will yield 4,3-borazaroisoquinolines (BIQs) **107** upon reaction with hydrazines **106** (Scheme 15).¹²⁶ Most notably, those BIQs proved to form irreversibly and thus they were found to be indefinitely stable in aqueous solution. This

approach could be further developed into a fluorogenic reaction by using 2-hydrazinylphenol as a substrate, which results in the formation of highly emissive tetracyclic BIQ **108**.

Another irreversible condensation takes place between aldehydes **11** and α -aminoxy acetohydrazides **109** (Scheme 16).¹²⁷

5.2. Extrinsic Stability Factors

The stability of the oxime/hydrazone linkage is also influenced by some external factors. As expected, the hydrolysis and transimination of hydrazones and oximes are significantly faster under acidic conditions and at elevated temperature.^{48,128–131} Senter and co-workers studied the release of the antimetabolic agent auristatin E from a monoclonal antibody conjugate and found that the half-life time $t_{1/2}$ of the respective hydrazone linkage dropped from $t_{1/2} = 183$ h at pH 7.2 to $t_{1/2} = 4.4$ h at pH 5.¹³² This can be a very useful feature for targeted drug delivery. Hydrazone linkers have been shown to release their covalently bound payload primarily in the acidic environment of specific organelles, like the endosomes (pH 5.5–6.2) or lysosomes (pH 4.5–5.0).¹³³ For in vivo applications of oximes and hydrazones, it is also important to consider the composition of the biological medium they are exposed to. It was shown that various arylhydrazones rapidly decomposed in plasma, whereas they were relatively stable in phosphate-buffered saline (PBS).¹³⁴ Low molecular weight compounds (<30 kDa) and, to a lesser extent, plasma proteins (e.g., albumin) were identified as the origin for this marked stability difference. This is in line with previous reports that proteinogenic amino acids can catalyze hydrazone hydrolysis in cell culture medium and serum.¹³⁵

6. Catalysts For Oxime/Hydrazone Formation

Since reactants for oxime and hydrazone formation may exist at low concentrations in some applications, the rates of these reactions, and finding mechanisms to enhance these rates, are important from a practical standpoint. For example, it has been pointed out³ that biomolecular conjugation and/or labeling is often carried out under conditions in which the biomolecule quantity and concentration are limiting. Indeed, it is common to work with biomolecules at low micromolar concentrations or below. In such situations, reactions must proceed at an appreciably rapid inherent rate in order to produce high reaction yields.

Many hydrazone and oxime formation reactions proceed with second-order rate constants of $0.01 \text{ M}^{-1} \text{ s}^{-1}$ or below at neutral pH, which is slower than many of the faster cycloaddition-based bioconjugation reactions.^{136–139} This is particularly true of oxime and acylhydrazone formation, which (in the absence of catalysts or specialized reactants) typically proceeds more slowly than hydrazone formation. This was recently illustrated by Bode and co-workers, who compared the second-order rate constants of several widely used chemo-selective ligation reactions (Figure 10).¹⁴⁰ The kinetic studies were carried out with functionalized heptapeptides, which were reacted with azo dyes featuring the complementary reactive group. The two reactants of each conjugation were used in a 1:1 ratio. As expected, at neutral pH the formation of oxime **111** was significantly slower than other bioconjugation reactions. Notably, the analogous Pictet–Spengler conjugate **112** forms 10 times faster than oxime **111**.

To overcome those shortcomings, there has been considerable interest of late in developing catalysts to speed hydrazone and oxime formation reactions and in developing fast-reacting substrates. Both of those strategies lead to greatly improved reaction rates (Figure 11).^{141,142} The former of these topics is discussed here and the latter in section 7.

It is important to note that catalysts for the bond-forming reaction also by definition catalyze the back-reaction as well. This allows them to be useful in promoting equilibration among different hydrazones in exchange reactions, some of which are mentioned below.

Hydrazone-based dynamic combinatorial libraries can thus greatly benefit from the application of appropriate catalysts to achieve thermodynamic control under mild conditions.^{143,144} Second, catalysts for imine formation can be useful not just for hydrazone formation, but also in related imine hydrolysis reactions, such as removal of formaldehyde adducts from biomolecules of formalin-fixed tissue specimens, also described below.

In the search for improved catalysts and new catalytic frameworks, it is useful to have in hand simple assays of hydrazone/oxime formation with easily measured signals. To that end, multiple color-changing hydrazine substrates **119–121** have been described,^{142,145,146} and the FRET-based fluorescence probe **122** for evaluating catalysts was recently reported as well (Figure 12).¹⁴⁷

6.1. Monofunctional Catalysts

Jencks was a pioneer in the study of the catalysis of hydrazone (semicarbazone) formation.¹⁰¹ He documented the rate-determining step of the reaction, namely, breakdown of the tetrahedral intermediate (see Scheme 9), and reported on mechanisms of catalysis as well. In 1962, he described the general acid catalysis of semicarbazone formation using simple organic acids of varying pK_a .¹⁰³ Although this established that proton donation can speed the reaction, the studies were carried out at acidic pH (in the range 2–4) and so may not be highly relevant to current interests in biological application.

Also importantly, Jencks early on documented the fact that aniline acts as a nucleophilic catalyst for semicarbazone formation at pH 2–4.¹⁴⁸ At 4 mM (pH 2.6) aniline enhanced the rate by 3.5-fold. Substituted anilines were also tested. Jencks was the first to establish that the mechanism occurred via nucleophilic catalysis (Scheme 17). In this mechanism, aniline (**123**) adds first to the carbonyl, producing the first tetrahedral intermediate, **124**, which breaks down to form the catalyst imine intermediate **125**. This then is subject to attack by the α -effect nucleophile **1/2**, resulting in the second tetrahedral intermediate, **126/127**, which then breaks down by eliminating the catalyst **123** to form the ultimate product **4/5**. Although this pathway requires more steps and involves more intermediates than the uncatalyzed reaction, the energy of the highest transition state barrier is lower than that of the uncatalyzed reaction, thus speeding the reaction overall. Aniline (**123**) acts as a successful catalyst because it has a nucleophilic amine, adding rapidly to the carbonyl. In addition, because the lone pair electrons of the amine are delocalized into its aromatic ring, it behaves as an effective leaving group. The rate-determining step of the catalyzed reaction is still a breakdown of a tetrahedral intermediate, but this proceeds more favorably in the catalyzed reaction.

In the same study, Jencks reported that glycine also acts as a nucleophilic catalyst for the reaction, albeit less effectively. More recently, Cid reported another simple amine, pyrrolidine, as a catalyst for oxime and hydrazone formation. The amine was employed at relatively high concentrations, and no reaction rates were reported.¹⁴⁹ Subsequent studies (described below) have consistently found aromatic amines (aniline derivatives) to have more favorable catalytic properties than aliphatic examples.

Subsequent to Jencks' early study, many laboratories have investigated arylamines (anilines) as catalysts, but with a trend toward biological applications and to pH values closer to neutral. An important study by Dawson and co-workers documented the use of aniline (**123**) both at pH 4.5 and 7.0 to speed oxime ligations involving modified peptides.¹⁵⁰ The catalyst was employed at 10 or 100 mM. At the high aniline concentration, reaction rates were increased by a factor of up to 40 at neutral pH and even more (400-fold) at pH 4.5. Neutral-pH reactions with lower catalyst concentration were not described, possibly because of the slow reaction rate.

Subsequent to Dawson's initial study, several additional reports have appeared further documenting catalysis by the parent compound aniline and its use in a number of applications. Dawson's own laboratory used aniline (10 mM, pH 5.7) to catalyze hydrazone formation in dynamic covalent chemistry of peptides.¹⁵¹ In a more recent study, Varghese and co-workers used a combination of aniline and acetate catalysis to enhance coupling of aminooxy compounds to ketones.¹⁵² Meijler and co-workers described aminooxy-substituted BOD-IPY dye labeling of LasR receptor protein in bacteria with aniline (1 mM) catalysis.¹⁵³ Moving to eukaryotic cells, Dawson, Paulson, and their co-workers employed 10 mM aniline in the cell-surface labeling of sialylated glycoproteins, with little toxicity observed after 90 min incubation with K88 or CHO cells (Figure 13).⁵⁸ Finally, Domaille and Cha described the tethering of aniline on DNA and the use of DNA duplex formation to increase its local concentration relative to an acylhydrazide group during its reaction with nitrobenzaldehyde.¹⁵⁴

It is now clear that substitutions on the aryl ring of aniline can enhance its catalytic efficiency. Dawson and co-workers' early study¹⁵⁰ tested *p*-methoxyaniline (**128**, 100 mM) at neutral pH due to its higher pK_a (5.3 vs 4.6 of aniline). As pointed out, enhanced basicity should promote protonation of the aniline Schiff base intermediate. Dawson did not directly compare aniline (**123**) to its *p*-methoxy analogue **128**, however. Jensen and co-workers later confirmed its superiority at neutral pH in catalyzing oxime formation with carbohydrates, although no rate comparisons were given.¹⁵⁵ A recent study of hydrazone exchange reactions in the detection of biological aldehydes showed that *p*-methoxyaniline (**128**, 10 mM) enhanced the exchange rate by a factor of 2.6-fold relative to aniline at pH 7.⁴⁸ Because of its enhanced performance, *p*-methoxyaniline (**128**, 100 mM) was employed in the pH 7 spin labeling of a human sulfite oxidase enzyme.¹⁵⁶

Recent studies have explored numerous multisubstituted aniline derivatives in catalysis of oxime and hydrazone formation (Figure 14), and some of these have improved performance over both aniline (**123**) and *p*-methoxyaniline (**128**). 4-Aminophenylalanine (**129**) was shown to catalyze the reaction between formyl amino acids and hydrazine-substituted

fluorophores, albeit at a rate slower than that of aniline.¹⁵⁷ More recently, Yuen et al. tested 16 different catalysts for hydrazone exchange reactions (5 mM, pH 7) and found that both 5-aminoindole (**130**) and 2,4-dimethoxyaniline (**131**, 2,4-DMA) were superior to 4-methoxyaniline (**128**), besting the parent aniline by nearly a factor of 3 in rate.⁴⁸ Interestingly, 2,4-dimethoxyaniline (**131**) was far superior to the 3,5-dimethoxy isomer **132**, by a factor of over 50, suggesting the importance of ortho and para electron donation enhancing the basicity of the amine. Notably, 2,4-DMA (**131**, 10 mM) showed little toxicity to human cells and was employed by Yuen et al. to perform intracellular hydrazone exchange reactions, using a dark hydrazone (“DarkZone”) dye to label and image cellular aldehydes (Figure 15).⁴⁸

A number of research groups have observed good catalytic activity in phenylenediamines, albeit with some drawbacks in stability and toxicity. Distefano and co-workers found that *m*-phenylenediamine (**133**) was approximately 2-fold faster than aniline (**123**) as a catalyst of oxime formation and offered the advantage of greater solubility, allowing the application in bioconjugations at up to 900 mM catalyst concentration.¹⁵⁸ The same group went on to describe protocols for protein labeling using phenylenediamines.¹⁵⁹ Baca and co-workers extended this finding by testing 12 different aniline and phenylenediamine derivatives and found *p*-phenylenediamine (**134**) to be superior to aniline (**123**) over a range of pH values, with a 19-fold faster rate than aniline at oxime formation measured at neutral pH.¹⁶⁰ Yuen et al. found *p*-phenylenediamine (**134**) to be nearly 5-fold faster than aniline (**123**) at hydrazone exchange reactions.⁴⁸ A drawback of *p*-phenylenediamine (**134**) and similar compounds is their oxidative instability, which results in their conversion to *p*-benzoquinones in air. Indeed, commercial samples are often brown in color, and reactions containing them turn brown over time,⁴⁸ likely because of this reaction. Moreover, *p*-phenylenediamine (**134**) was shown to cause ~60% cellular toxicity at 10 mM after only 1 h exposure.⁴⁸ Meta-substituted isomers are less likely to suffer the oxidative instability; an example of such a catalyst is 3,5-diaminobenzoic acid (**135**, 3,5-DABA), which was found by Crisalli and Kool to be an effective catalyst of hydrazone and oxime formation, yielding reaction rates ~3-fold greater than that of aniline (**123**) at pH 7.4.¹⁶¹

6.2. Bifunctional Catalysts

Developing catalysts with higher activity remains attractive not only for enhancing reaction rates further, but also for their utility at low concentrations, suppressing potential toxicity and background reaction effects that might occur at high concentrations. In this light, Crisalli and Kool in 2013 described the discovery of bifunctional catalysis in hydrazone and oxime formation.¹⁶¹ This bifunctionality was defined as two nearby functional groups playing a direct role in catalysis. Over 20 potential catalysts containing a nucleophilic amine were studied, and anthranilic acids were found to have the highest activity of those tested with an aryl aldehyde substrate. Controls replacing the carboxy group with a nitrile or amide group showed no activity, suggesting a mechanism involving general acid catalysis by the *o*-carboxy group at the transition state **136** (Scheme 18). Testing of varied anthranilic acids showed that substitution by electron-donating groups on the aryl ring enhanced catalysis further at neutral pH, consistent with the need for a more basic and nucleophilic amine

group. 5-Methoxyanthranilic acid (**137**, 5MA) was shown to have activity 6-fold greater than aniline (**123**) in hydrazone formation at pH 7.4 using only 1 mM catalyst (Figure 16).¹⁶¹

Subsequent work in multiple laboratories has confirmed the superior catalysis of anthranilates over anilines. McKay and Finn described the testing of polyvalent and dendrimer-based anthranilic acid catalysts, confirming superior activity over anilines in dendrimer labeling.¹⁶³ Langenhan and co-workers used mono- and bifunctional aniline derivatives for fluorescent conjugation of aldose sugars, and confirmed 5MA as the most effective catalyst among 10 tested.¹⁶⁴

Crisalli and Kool followed up on their earlier work to investigate the mechanism of bifunctional catalysis in hydrazone and oxime formation, resulting in the design of further improved catalysts.¹⁴² To test their hypothesis that proton donors ortho to the nucleophilic amine group of aniline provide general acid/base catalysis during the reaction, they synthesized a series of catalysts having ortho groups of varied pK_a (Figure 17). They found that the two acidic groups (tetrazole **138** and phosphonate **139**) having a pK_a closest to neutral (i.e., the solution pH) were the most effective catalysts. This is strong evidence of the proposed general acid/base mechanism of catalysis (Scheme 18).

This mechanistic study resulted in new phosphonate and tetrazole catalysts having high activity with aryl and alkyl aldehyde substrates. For example, the methyl-substituted phosphanilate catalyst **139** (4-methyl-2-phosphonoaniline, 4MPA) gave an 83-fold rate enhancement over the uncatalyzed reaction at only 1 mM catalyst concentration (pH 7.4).¹⁴² One notable exception to the effective catalysis was a bulky aryl ketone substrate, for which aniline was the superior catalyst. It was speculated that the added bulk of the bifunctional catalysts hinders the transition state for the reaction. Hydrazone and oxime formation with aryl ketones (an indeed, ketones in general) remains a challenge for improved catalyst design.

In an effort to identify new structural motifs for bifunctional catalysts of hydrazone and oxime formation, a recent study by Larsen et al. measured 28 different bifunctional catalyst candidates having potential proton donors near a nucleophilic amino group. The study resulted in the identification of two effective new catalyst scaffolds (Figure 18): 2-aminophenols and 2-(aminomethyl)-benzimidazoles.¹⁶² Tuning the pK_a in these systems resulted in improved catalysts with considerably greater activity than aniline for a range of substrates. To evaluate the scope of catalysis, the study compared rates of hydrazone formation for 10 varied carbonyl substrates. Notably, at 1 mM, aniline (**123**) showed only modest activity for aldehyde substrates and no activity at all with four different ketone substrates. Some of the new bifunctional amino-methylbenzimidazole catalysts showed measurable and significant activity (up to 10-fold rate enhancement) at 1 mM, even with ketone substrates. With aldehyde substrates, the phosphanilate 4MPA (**139**) described above was superior to all catalysts. This latter compound appears to be the most effective existing catalyst to date for common aldehyde substrates in hydrazone and oxime formation.

Bifunctional catalysts were used recently in a related transamination reaction, namely, reversal of formaldehyde adducts on RNA preserved with formalin. 4MPA (**139**) was used

successfully to enhance the removal of hemiaminal adducts and aminor cross-links from RNA in vitro and enabled the recovery of considerably greater quantities of RNA amplification signals from preserved tissue.¹⁶⁵

7. Discovery and Design of Fast-Reacting Substrates

Since hydrazone formation (and particularly oxime formation) has historically been regarded as slower than ideal for some applications, it is of interest to study how structural variations in the reacting substrates affect their reaction rates. Knowledge gained from such studies can result in the design of improved substrates that react at much greater rates.

7.1. Fast-Reacting Aldehydes and Ketones

In early work, Wolfenden and Jencks noted that aryl aldehydes react somewhat more rapidly in semicarbazone formation when ortho groups (e.g., methoxy, chloro) were present.¹⁶⁶ At the time, a mechanistic reason for this was not proffered. Nevertheless, Wang and Li recently took advantage of this reactivity in documenting acylhydrazone formation of peptides with *o*-halo-substituted benzaldehydes (Figure 19).¹⁶⁷ The *o*-chloro aldehydes **146** gave the highest reaction yields among the halogens, and a similarly derivatized version of a fluorescent label was shown to react favorably. Rates were not reported in the study. In another application of the ortho-effect, Wang and Canary studied pyridoxal phosphoramidate aldehyde substrates **147** (Figure 19) and showed that they react particularly rapidly,⁴² likely due both to the electron deficiency of the pyridine (see below) and to the *o*-hydroxyl group. A rate constant of $10 \text{ M}^{-1} \text{ s}^{-1}$ was documented at pH 7.4, and applications to protein conjugation were reported.

A 2016 report by Bane and co-workers used an *o*-phosphate group to strongly enhance reaction rates for the conjugation of aryl aldehyde **148** with a hydrazine-substituted coumarin dye.⁴⁰ A second-order reaction rate constant of up to $17 \text{ M}^{-1} \text{ s}^{-1}$ was documented at pH 7, giving a reaction rate approximately 20-fold faster than with an *o*-hydroxy group. The authors cited intramolecular general acid catalysis by the phosphate group, as proposed earlier,¹⁴² as the mechanism of rate enhancement.

In a general effort to explore the effects of structure on reactivity, Kool et al. carried out kinetics studies of 50 different structurally varied aldehydes and ketones reacting with arylhydrazines.¹¹¹ Surveying the structural effects on rates, one general conclusion was that electron-deficient carbonyl groups react somewhat more rapidly on average; for example, 4-nitrobenzaldehyde (**150**) reacted 4.5-fold more rapidly than the 4-methoxy derivative **149**, and trifluoroacetone (**156**) was an especially fast-reacting ketone (Figure 20). Steric effects were moderate to small for hydrazone formation, and while some bulky aldehydes (pivaldehyde, **157**) showed some apparent steric inhibition, highly bulky ketones (di-*tert*-butyl ketone, **155**) reacted as fast as nonbulky ones (e.g., 2-butanone, **154**). A major effect was found in comparing alkyl systems to aryl examples; alkyl substrates were found to react much more rapidly. For example, butyraldehyde (**158**) reacted 65-fold faster than benzaldehyde (**151**). Another clear and strong effect was the greater reactivity of aldehydes over ketones: for example, butyraldehyde (**158**) reacted 44-fold faster than 2-butanone (**154**).

Finally, the authors noted that aldehydes with nearby basic groups (2-formylpyridine **152**, 8-formylquinoline **153**) reacted up to 8-fold faster than control compounds lacking the basic groups. This was hypothesized to be due to internal acid–base catalysis at the reaction transition state. A similar effect was seen in hydrazines as well (see section 7.2).

Continuing the exploration of the importance of ortho-substitution in enhanced reaction rates, Gillingham and co-workers in 2014 documented that phthalaldehyde (**159**) is an exceptionally fast reactant in oxime formation.⁸³ Mechanistic studies showed that the mechanism is changed from standard oxime formation, as the first-formed tetrahedral intermediate is trapped intramolecularly to form an isoindoline(bis)-hemi-aminal heterocycle **160** (Scheme 19). This heterocyclic intermediate then slowly rearranges to oxime **161** (Figure 21). Because the rapid first step joins the reactants it can be considered the “ligation” step, and the rate constant for this step is high, on the order of $500 \text{ M}^{-1} \text{ s}^{-1}$, which is faster than the large majority of bioorthogonal “click”-type reactions used in bioconjugations.

Gillingham and co-workers subsequently modified this intramolecular trapping mechanism by replacing the *o*-aldehyde trap with a boronic acid group.¹²⁶ In this design, reactants are again rapidly trapped at the tetrahedral intermediate stage **162/163**, with the oxyanion coordinated to boron, and then more slowly rearrange to oximes **164** (Scheme 19). This strategy is an important new reaction for bioconjugation development. Exceptional rate constants of $10^4 \text{ M}^{-1} \text{ s}^{-1}$ have been measured, rendering this among the fastest of “click”-type conjugation reactions. Subsequent to Gillingham's report, studies from the Gao and Bane laboratories also described boron-substituted aldehyde and ketone variants and applications.^{126,168–172}

7.2. Fast-Reacting Alkoxyamines and Hydrazines

It has long been known that, among α -nucleophiles, rates of reaction vary strongly with the electron deficiency of the non-nucleophilic atom. Thus, alkoxyamines react to form imines (i.e., oximes) more slowly than acylhydrazines (forming acylhydrazones), which in turn react more slowly than hydrazines. For example, relative rates of similar alkoxyamines and hydrazines have been measured to differ by a factor of ca. 20.¹⁴¹ The reason for this slow reactivity of aminooxy compounds is that the rate of the rate-limiting step, breakdown of the tetrahedral intermediate, relies on the position of pre-equilibrium leading to it. Lower nucleophilicity pushes the equilibrium further toward the side of starting materials, lowering the concentration of tetrahedral intermediate and thus the rate of imine formation.

Beyond this general observation, little work had been done prior to 2014 on structural influences on α -effect nucleophile activity with carbonyl substrates. To address this, Kool et al. measured kinetics of hydrazone formation for 20 varied hydrazines in the reaction with 2-formylpyridine (**152**) at pH 7.4.¹⁴¹ Notably, rates varied over a factor of 23-fold across the range of structures tested (see examples in Figure 22). Relatively slow were electron-deficient hydrazines such as pentafluorophenylhydrazine (**166**) and acylhydrazines **167**. 2-Hydrazinopyridine (**169**), a very common α -effect nucleophile generally thought to be efficient, was only 1.2-fold faster than phenylhydrazine (**168**). However, two hydrazines

with acid/ base groups near the nucleophilic site were found to be much more reactive: namely, *o*-carboxyphenylhydrazine (**170**, OCPH) and dimethylaminoethylhydrazine (**171**, DMAEH). These fast alpha nucleophiles (FAN) showed 3- and 6-fold faster rates (respectively) than phenylhydrazine (**168**) at pH 7.¹⁴¹ Combining the DMAE-substituted hydrazine with a fast-reacting aldehyde produced rapid reactions (rate constant of $10 \text{ M}^{-1} \text{ s}^{-1}$) that exceed most cycloaddition “click”-type reactions in rate (Figure 23). The same DMAE substitution on an alkoxyamine also produced enhanced reaction rates as compared with a control alkoxyamine.¹⁴¹

A special class of reactive hydrazines is found in indolylmethylhydrazines that undergo Pictet–Spengler ligation (see section 5.1).¹²⁴ Rates for ligation are fastest at lower pH values, but are as high as $4.2 \text{ M}^{-1} \text{ s}^{-1}$ at pH 6.0, as reported by the groups of Bertozzi and Rabuka.^{124,125} However, the rates for the reaction drop as neutral pH is approached.

8. Conclusions

The mechanistic studies carried out by Jencks in the 1960s established the basic mechanism of imine formation by α -effect nucleophiles. That early work, carried out chiefly at low pH in organic and partially organic solvents, was important for laying the groundwork for modern research in the field. However, modern research in the field has been focused largely on the reaction performed in water, and at pH values closer to neutral, with biological relevance in mind. Indeed, while Jencks worked only with small molecules, it is now common to work with peptides, proteins, and nucleic acids as reacting substrates and to carry out reactions not only in solution but also in living cells. It seems highly likely that this trend to biological and biomedical relevance will continue.

Before the last 5 years, the rates of hydrazone and oxime formation were widely regarded as slow as compared with modern biofunctionalization chemistries, including strain-enhanced cycloaddition reactions. However, due to recent studies, new mechanistic insights into substrate reactivity and catalysis have led to the identification of reactant features that considerably enhance these rates. This work has led to the identification of fast-reacting aldehydes with *o*-proton donors and highly reactive hydrazines with proton donors that aid in the breakdown of the tetrahedral intermediate. The use of such reactive, self-catalyzing substrates has resulted in reaction rates (rate constants on the order of $10 \text{ M}^{-1} \text{ s}^{-1}$ at neutral pH) that exceed those of many modern cycloaddition reactions based on strained alkynes. Thus, slow reaction rates should no longer be considered a limitation of hydrazone and oxime bond formation.

Another important advance in the past 5 years has been the development of specialized aldehydes with trapping groups nearby, resulting in stable conjugation and, in some cases, very rapid rates. Important examples include the dialdehydes, the *o*-boron-substituted aldehydes, and the indole methyl “Pictet–Spengler” hydrazines. Rate constants in some of the orthotrapping examples are $500 \text{ M}^{-1} \text{ s}^{-1}$, rivaling the fastest bioconjugation reactions known.

Such fast-reacting aldehydes and α -effect nucleophiles can show great utility in bioconjugation reactions and are rapid enough to proceed conveniently without the use of a catalyst. However, catalysis can also achieve high reaction rates and offers other advantages as well. Unlike fast-reacting substrates, catalysts offer temporal control: a reaction can be sped up at a desired time, using a catalyst as a switch. Moreover, catalysts speed both forward and reverse reactions, thus allowing them to be useful in reversal of conjugation (bond-breaking reactions) and in speeding equilibration in dynamic combinatorial chemistry applications. Moreover, catalysts can be highly effective for substrates where specialized fast-reacting carbonyl and α -nucleophile structures are unavailable or impractical.

Following up on Jencks' early observation of nucleophilic catalysis by aniline, work in the past decade has produced dozens of new catalysts for hydrazone and oxime formation. Although all of the most effective catalysts to date make use of a nucleophilic amine group, added acid/base functional groups and varied chemical scaffolds have improved catalyst efficiency and versatility over earlier anilines. Some of the best new catalysts are more efficient than aniline by 1–2 orders of magnitude and are at the same time less toxic and more soluble. Important examples of the most highly effective newer catalysts include the anthranilates (such as 5MA, **137**) and the phosphanilates (including 4MPA, **139**). More work is needed to develop efficient catalysts for ketone substrates, but the new aminomethylbenzimidazole catalyst scaffolds **143–145** show promise for their advantages in promoting reactions with ketone substrates.

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Supplementary Material

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Abbreviations

2,4-DMA	2,4-dimethoxyaniline
3,5-DABA	3,5-diaminobenzoic acid
5-fC	5-formylcytosine
5-fU	5-formyluracil
5MA	5-methoxyanthranilic acid
4MPA	4-methyl-2-phosphonoaniline
Ac	acetyl
Alk	alkyl residue
Ar	aromatic residue
BIQ	4,3-borazaroisoquinoline
Boc	<i>tert</i> -butyloxycarbonyl
Bu	butyl
Bz	benzoyl
c	concentration
CHO	Chinese hamster ovary
DAPI	4',6-diamidino-2-phenylindole
DFT	density functional theory
DMAEH	dimethylaminoethylhydrazine
Dmt	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acid
FAN	fast alpha nucleophiles
FGE	formylglycine-generating enzyme
HA	generic acid
$k_{2(\text{app})}$	apparent second-order rate constant
K_{eq}	equilibrium constant
k_{rel}	relative rate constant

Me	methyl
Mmt	4-methoxytrityl
NMR	nuclear magnetic resonance
NPhth	phthalimidyl
OCPh	o-carboxyphenylhydrazine
PFTase	protein farnesyltransferase
PBS	phosphate-buffered saline
Ph	phenyl
RNA	ribonucleic acid
rt	room temperature
TBTA	tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
<i>t</i>-Bu	<i>tert</i> -butyl
Trt	trityl
χ_p	electronegativity (Pauling scale)

Figure 1.
Generic structures of 1,2-amino alcohol-containing peptides **15–19** and nucleic acid **20**, which can be cleaved with periodate.

Figure 2. Selection of ketone-containing unnatural amino acids **21–27**, which have been incorporated into proteins.

Figure 3.

Selection of α -effect nucleophile-containing phosphoramidites **42–48** for 5'-modification of nucleic acids. Trt = trityl; Mmt = 4-methoxytrityl; Dmt = 4,4'-dimethoxytrityl; NPhth = phthalimidyl.

Figure 4. Examples of alkoxyamine-containing phosphoramidites **49-53** for nucleic acid synthesis. Dmt = 4,4'-dimethoxytrityl; NPhth = phthalimidyl; Bz = benzoyl.

Figure 5.

A selection of α -effect nucleophile-containing unnatural amino acids (**58–64**) that have been incorporated into proteins.^{64,95–98}

Figure 6.

Equilibrium constants of some prototypical oximes, hydrazones, and semicarbazones. $K_{\text{eq}} = \alpha(\text{conjugate})/[\alpha(\text{aldehyde}) \times \alpha(\text{a-nucleophile})]$.

Figure 7.
Stability trend in a series of oximes **87–91**.¹⁰⁸

Figure 8.

Comparison between the kinetic stabilities in a series of isostructural conjugates **92–95**.³⁵

The relative first-order rate constants k_{rel} for the hydrolysis are given [$k_{\text{rel}}(\text{oxime } 95) = 1$].

Figure 9. Comparison of hydrolytic stability between *s*-triazinylhydrazones **99** and acetylhydrazones **100** at different pH values.¹²³

Reaction	Product	Condition	Rate constant (M ⁻¹ s ⁻¹)
Oxime Coupling	111	buffer (pH 7.0)/MeOH (1:1) 10 mM, rt	0.0013
Pictet-Spengler Coupling	112	buffer (pH 4.0)/MeOH (1:1) 2.5 mM, 60 °C	0.015
Strain-promoted Alkyne-Azide Cycloaddition	113	H ₂ O/MeCN (1:1) 50 μM, rt	0.90
Cu(I)-catalyzed Alkyne-Azide Cycloaddition	114	CuSO ₄ , TBTA, sodium ascorbate, buffer (pH 7.4)/MeOH (1:1) 48 μM, rt	3.4
Thiol-Maleimide Coupling	115	buffer (pH 7.4)/MeCN (1:1) 15 μM, rt	734

Figure 10.

Comparison of reaction conditions and second-order rate constants of several bioconjugation reactions. The kinetic studies were carried out with a 1:1 stoichiometry of the respective reactants. TBTA = tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine.¹⁴⁰

Figure 11.

Apparent second-order rate constants for the formation of hydrazones **116–118**. The apparent second-order rate constants were derived from pseudo-first-order kinetic measurements, where one of the reaction partners was present in 50-fold excess. Conditions: buffer (pH 7.4)/DMF (10:1), room temperature.^{141,142}

119 **120** **121** **122****Figure 12.**

Molecular structures of α -nucleophiles that either undergo color change (**119** and **120**) or interact with a fluorescently labeled substrate (**121** and **122**) upon hydrazone/oxime formation.^{142,145–147}

Figure 13. Confocal microscopy images of untreated and periodate-treated (1 mM at 4 °C for 5 min) CHO cells subjected to ligation with alkoxyamine-functionalized biotin (100 μ M) in the absence or presence of aniline (10 mM) at 4 °C for 90 min. Cells were permeabilized and stained with dye-streptavidin conjugate (green) and DAPI (blue, nuclei). Scale bars are 15 μ m. Reprinted in part with permission from ref 58. Copyright 2009 Nature Publishing Group.

Figure 14. Selection of substituted anilines **128–135**, which have been shown to be efficient catalysts for the formation of oximes/hydrazones.

Figure 15.

Intracellular imaging of acetaldehyde by hydrazone exchange catalyzed by the cell-permeable catalyst 2,4-DMA. HeLa cells were incubated with 20 μM of a fluorogenic hydrazone dye, which undergoes hydrazone exchange with acetaldehyde (varying concentrations, 0.1–2 mM), and 10 mM of 2,4-DMA (**131**) for 1 h. Scale bars are 20 μm . Reprinted in part with permission from ref 48. Copyright 2016 American Chemical Society.

Figure 16.

Conversion of color-changing hydrazine **119** with 4-nitrobenzaldehyde (**150**) to the corresponding hydrazone in the presence of selected catalysts. Reprinted with permission from ref 161. Copyright 2013 American Chemical Society.

Figure 17.

Comparison of relative reaction rates in a series of bifunctional catalysts **137–139** and aniline (**123**).^{142,161} The relative reaction rates refer to the hydrazone formation between the chromogenic, aromatic hydrazine **119** and 4-nitrobenzaldehyde (**150**) (uncatalyzed reaction $k_{\text{rel}} = 1$).

Figure 18.

New bifunctional 2-aminophenol **140–142** and 2-(aminomethyl)benzimidazole **143–145** catalyst scaffolds discovered by Larsen et al.¹⁶² The relative reaction rates refer to the hydrazone formation between phenylhydrazine (**168**) and 4-chlorobenzaldehyde (uncatalyzed reaction $k_{\text{rel}} = 1$).

Figure 19.

Examples of aromatic aldehydes **146–148**, which exhibit greater reactivity to α -effect nucleophiles than benzaldehyde (**151**) due to the presence of an ortho substituent.

Figure 20.

Relative hydrazone formation rates for a selected set of aldehydes and ketones **149–158** at pH 7.¹¹¹ The relative reaction rates refer to the hydrazone formation between the corresponding carbonyl compound and phenylhydrazine (**168**) [$k_{\text{rel.}}(\text{aldehyde } \mathbf{149}) = 1$].

Figure 21.

Time course NMR analysis for the conjugation of phthalaldehyde (**159**) and *O*-benzylhydroxylamine. The isoindoline intermediate **160** forms rapidly, whereas the oxime **161** is generated rather slowly. Reprinted in part with permission from ref 83. Copyright 2014 Wiley-VCH.

Figure 22.

Effect of hydrazine structure on rate of hydrazone formation.¹⁴¹ The relative reaction rates refer to the hydrazone formation between the corresponding hydrazine **166–171** and 2-formylpyridine (**152**) [$k_{\text{rel}}(\text{hydrazine } \mathbf{166}) = 1$].

Figure 23.

Time course plots for the hydrazone formation of 2-formylpyridine (**152**) and DMAEH (**171**, green trace) or ethylhydrazine (blue trace). Reprinted in part with permission from ref 141. Copyright 2014 American Chemical Society.

Scheme 1. Formation of Oximes 4 or Hydrazones 5 from Alkoxyamines 1 or Hydrazines 2 and Aldehydes or Ketones 3

Scheme 2. Periodate-Mediated Oxidative Cleavage of Vicinal Diols **6** or 1,2-Amino Alcohols **7**

Scheme 3. Periodate-Mediated Oxidative Cleavage of 3'-Ribonucleotide 12 and the Equilibrium between the Resulting Dialdehyde 13 and 1,4-Dioxane 14

Scheme 4. Site-Selective Modification of Tyrosine Residues by Azo Coupling with Diazonium Ion 29

Scheme 5. Enzymatic Aldehyde-Modification of Cysteine Residues 31 with Pyrophosphate 32 and Protein Farnesyltransferase (PFTase)⁷⁶

Scheme 6. Pyridoxal Phosphate (35)-Mediated Oxidation of N-Terminal Amino Acids 34 via Biomimetic Transamination⁷⁹

Scheme 7. Selective Oxidation of N-Terminal Amines 34 with Oxone⁸⁰

Scheme 8. Solid-Phase Synthesis of Peptidyl Hydrazide 55 via Cleavage from Resin 54, 56, or 57

Scheme 9. Standard Mechanism for the Formation of Oximes 4 or Hydrazones 5 from Carbonyl Compound 3 and Alkoxyamines 1 or Hydrazines 2, Respectively

Scheme 10. Proposed Transition State 73/74, Which Leads to the Formation of the Protonated Intermediate 65 or 66^{103 a}

^aHA = generic acid.

Scheme 11. Equilibrium between the α -Nucleophiles 1 and 2 and the Respective Protonated Species 75–78

Scheme 12. Proposed Mechanism for the Hydrolysis of Trimethylhydrazonium Ions **96^{35a}**

^aProtonation of the imine nitrogen atom is disfavored due to the presence of an additional positive charge.

Scheme 13. Protonation of the *s*-Triazinyl Hydrazone **99, Which Lowers the Basicity of the Imine Nitrogen Due to the Adjacent Delocalized Positive Charge¹²³**

Scheme 14. Pictet-Spengler Ligation between Alkoxyamine 102 and Aldehyde 11¹²⁴

Scheme 15. Formation of 4,3-Borazaroisoquinolines (BIQs) 107 from Aromatic Boronic Acids 105 and Hydrazines 106^{126a}

^aBIQ 108 with extended delocalized π -electron system displays fluorescence emission.

Scheme 16. Synthesis of Stable 1,2,4-Oxadiazinan-5-ones 110 from Aldehydes 11 and α -Aminooxy Acetohydrazides 109¹²⁷

Scheme 17. Generally Recognized Mechanism for the Aniline-Catalyzed Formation of Oximes 4 or Hydrazones 5

Scheme 18. Proposed Transition State 136, Which Leads to an Activated Imine Intermediate¹⁶²

Scheme 19. Conjugation of Aromatic Aldehydes 159 and 105, Which Display Exceptionally Fast Reaction Rates Due to the Presence of an Electrophilic Group That Stabilizes the Tetrahedral Intermediate