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Water-Soluble Organocatalysts for Hydrazone and Oxime Formation

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S Supporting Information

ABSTRACT: The formation of oximes and hydrazones is widely used in chemistry and biology as a molecular conjugation strategy for achieving ligation, attachment, and bioconjugation. However, the relatively slow rate of reaction has hindered its utility. Here, we report that simple, commercially available anthranilic acids and aminobenzoic acids act as superior catalysts for hydrazone and oxime formation, speeding the reaction considerably over the traditional aniline-catalyzed reaction at neutral pH. This efficient nucleophilic catalysis, involving catalyst−imine intermediates, allows rapid hydrazone/oxime formation even with relatively low concentrations of the two reactants. The most efficient catalysts are found to be 5-methoxyanthranilic acid and 3,5-diaminobenzoic acid; we find that they can enhance rates by factors of as much as 1−2 orders of magnitude over the aniline-catalyzed reaction. Evidence based on a range of differently substituted arylamines suggests that the *ortho*-carboxylate group in the anthranilate catalysts serves to aid in intramolecular proton transfer during imine and hydrazone formation.

■ INTRODUCTION

The formation of hydrazones and oximes is a versatile reaction for chemistry and biochemistry, enabling attachment of functional moieties (tags, labels, and other markers) to polymers and biomolecules, and facilitating the linkage of a wide variety of molecules. For reactions involving biomolecules, it presents a versatile bioorthogonal approach to conjugation chemistry, as a result of the fact that biomolecules contain few aldehydes or ketones and virtually no hydrazine or aminooxy functional groups.[1] Moreover, since certain hydrazones and oximes can be exchanged, such linkages have gained attention in dynamic combinatorial chemistry as well.[2,3] Despite being thermally and hydrolytically stable compounds under biological conditions, the formation of these products suffers from slow kinetics, particularly under neutral conditions.[3,4] For faster bond formation, reactions between carbonyl compounds and hydrazines or aminooxy groups require acidic conditions that are not compatible with biological systems and can damage biomolecules.[5] Recently, Dawson and co-workers revisited and expanded upon an earlier study by Jencks describing catalysis by anilines of the hydrazone formation between carbonyls and thiosemicarbazide.[3,4,6,7] Indeed, these recent studies confirmed that aniline could substantially accelerate both hydrazone and oxime formation under neutral pH conditions, broadening the utility of the reaction for bioorthogonal purposes.[4]

However, multiple problems still exist with the use of aniline as a catalyst for this reaction. Foremost is the need for very high concentrations of catalyst to enable complete reaction over reasonable time periods.[3,4,7] Hydrazone formation at low aldehyde/hydrazine concentrations (e.g., 10 μM reactants) requires upward of 100 mM aniline, or approximately 10 000 equiv of catalyst. Moreover, aniline is toxic to cells, presenting an added problem for possible applications in living systems.[8] With these considerations, and keeping an eye toward the mechanism of aniline-catalyzed hydrazone formation, we undertook efforts to discover new catalysts that could more rapidly form the desired adducts at reduced concentrations under neutral pH conditions. Surprisingly, only a few studies have attempted to find alternative catalysts for hydrazone formation, with 4-aminophenylalanine being the only recently reported catalyst to perform comparably to aniline.[9] Here, we report that anthranilic acids and 3,5-diaminobenzoic acid (Figure 1) serve as efficient, water-soluble nucleophilic catalysts for this reaction, substantially improving upon the long-known aniline catalysis.

Figure 1. Structures of a chromogenic hydrazone-forming substrate (**1**) and of selected catalysts of hydrazone/oxime formation (**2**−**5**).

■ RESULTS AND DISCUSSION

Catalyst Discovery and Optimization. To screen new candidate catalysts conveniently, we adopted a chromogenic reaction that enables simple spectrometric monitoring. Nitrobenzoxadiazole (NBD) hydrazine **1** is known to undergo distinct red shifts in its absorption upon reaction with aryl aldehydes.[10,16] We found that 4-nitrobenzaldehyde (yielding the hydrazone adduct of **1**) gave a further bathochromic shift to 504 nm, where **1** has virtually no absorbance (Figure S1,

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Supporting Information). Using this screen, we tested a variety of commercially available aliphatic and aromatic amines with 18 μM NBD hydrazine, 1 mM 4-nitrobenzaldehyde, and 1 mM catalyst in 10:1 phosphate buffered saline (pH 7.4):DMF, at 23 °C. Since the transition states of hydrazone formation involve acid and/or base catalysis, we were especially interested in amines having proximal acidic or basic groups, and in amines with pK_a values higher than that of aniline (see Table S1 in the Supporting Information).

Our experiments confirmed the slow rate of uncatalyzed hydrazone formation (yielding only 0.7% after 2 h, Table 1, entry 1), and the rate enhancement afforded by aniline (10.4% yield at the same time point, entry 2). A variety of other amines showed poor catalytic activity, and few were as effective as the parent aniline (see full reaction curves in the Supporting Information). For example, methyl glycinate, with a pK_a of 7.6 (near the buffer pH), showed negligible catalysis. Similarly, a variety of heterocyclic compounds with a more basic amine than aniline showed poor catalytic performance. Substituted anilines with increased electron density either were unstable in air-saturated water (e.g., 4-(dimethylamino)aniline), showed strong propensity for stable imine formation (4-methoxyaniline, 2,4-dimethoxyaniline, 3,4,5-trimethoxyaniline), or displayed only a negligible increase in rate versus aniline (*o*-anisidine).

Table 1. Yields of Hydrazone Formation by 1 + *p*-Nitrobenzaldehyde Using Varied Arylamine/Acid Catalysts at pH 7.4[a]

entry	catalyst	conversion (2 h)	yield (relative)
1	no catalyst	0.7 ± 0.2%	1.0
2	aniline (**2**)	10.4 ± 1.3%	14
3	anthranilic acid (**3**)	21.5 ± 0.8%	29
4	anthranilonitrile	0.8 ± 0.1%	1.0
5	anthranilamide	1.6 ± 0.1%	2.1
6	ethyl anthranilate	1.5 ± 0.3%	2.1
7	benzoic acid	1.8 ± 0.4%	2.4
8	benzoic acid + aniline 1:1	18.1 ± 1.9%	25
9	3-aminobenzoic acid	14.3 ± 1.2%	19
10	4-aminobenzoic acid	7.4 ± 0.4%	10
11	3,5-diaminobenzoic acid (**4**)	34.7 ± 2.4%	47

[a]Conditions: 18 μM **1**, 1 mM 4-nitrobenzaldehyde, 1 mM catalyst in phosphate buffered saline (pH 7.4) containing 10% DMF. Conversion was monitored by increase in absorbance at 504 nm.

However, one compound in our initial screen, anthranilic acid (**3**), provided improved conversion (to 21.5% after 2 h, Table 1, entry 3), offering a substantial increase of approximately 2.1-fold in yield relative to aniline and 29-fold relative to the uncatalyzed reaction. Anthranilic acid is only moderately more basic than aniline, with a pK_a measured between 4.8 and 5.0 compared to that of aniline (4.6),[17,18] but we hypothesized that the presence of the carboxylic acid *ortho* to the amine might provide an intramolecular general acid catalyst in the mechanism of both imine and hydrazone formation. To examine the importance of this carboxyl group, a variety of simple anthranilic acid derivatives lacking this acid group (anthranilonitrile, anthranilamide, ethyl anthranilate) were tested (Table 1, entries 4−6) and shown to be relatively inactive, performing only slightly better than the uncatalyzed reaction and considerably worse than aniline.

We further investigated the role of the *ortho*-carboxylic acid in anthranilic acid by comparisons with 3-aminobenzoic acid and 4-aminobenzoic acid (Table 1, entries 9 and 10), which have pK_a values similar to that of anthranilic acid.[19] The 4-isomer showed poor reactivity, performing worse than even aniline itself, whereas 3-aminobenzoic acid displayed a slight increase versus aniline. We considered the possibility that the increased rate for anthranilic acid might have resulted from its slight acidification of the PBS buffer; thus we tested benzoic acid as a control. In the absence of aniline catalyst, this weak acid slightly improved conversion (Table 1, entry 7) and also improved conversion somewhat in the aniline-catalyzed reaction as well (Table 1, entry 8). These results indicated that acidification may be playing a minor role; however, anthranilic acid alone provided improved catalysis over these and the other aminobenzoic acid controls. Taken together, the results suggest a special role of the *ortho*-carboxylic acid group in catalysis.

Next, we explored the effects of modifications of the aromatic ring of anthranilic acid (Table 2). Both 3-methyl and 6-methyl substitutions of anthranilic acid were found to strongly diminish catalytic activity, providing conversions even lower than the aniline-catalyzed reaction (entries 4 and 7). We surmise that 3-methyl substitution inhibits imine/hydrazone formation by steric perturbation of the adjacent amine position. Similarly, the detrimental effect of the 6-methyl substitution may arise from steric clashing with the carboxylate group, which would force it to twist. This suggests the importance of the orientation of the

Table 2. Yields of Hydrazone Formation of 1 with Varied Anthranilic Acid Catalysts at pH 7.4[a]

Entry	Catalyst	Structure	Conversion (2 h)	Relative Yield
1	no catalyst	-	0.7 ± 0.2%	1.0
2	aniline (**2**)		10.4 ± 1.3%	14
3	anthranilic acid (**3**)		21.5 ± 0.8%	29
4	3-methylanthranilic acid		1.2 ± 0.4%	1.6
5	4-methylanthranilic acid		28.4 ± 4.4%	38
6	5-methylanthranilic acid		32.6 ± 0.5%	44
7	6-methylanthranilic acid		3.6 ± 0.3%	4.9
8	3-amino-2-naphthoic acid		31.5 ± 1.5%	43
9	4-chloroanthranilic acid		11.5 ± 0.3%	16
10	4-nitroanthranilic acid		4.0 ± 0.7%	5.4
11	4-methoxyanthranilic acid		21.7 ± 2.2%	29
12	4,5-dimethoxyanthranilic acid		37.1 ± 2.5%	50
13	5-iodoanthranilic acid		12.1 ± 1.0%	16
14	5-nitroanthranilic acid		1.1 ± 0.2%	1.5
15	5-methoxyanthranilic acid (**5**)		55.2 ± 1.1%	75
16	3-amino-4-methylbenzoic acid		1.4 ± 0.1%	1.9
17	4-amino-2-methylbenzoic acid		16.1 ± 2.6%	22
18	4,6'-bianthranilic acid		57.8 ± 7.3%	78
19	2-aminophenylacetic acid		3.7 ± 0.9%	5.1

[a]Conditions: 18 μM **1**, 1 mM 4-nitrobenzaldehyde, 1 mM catalyst in phosphate buffered saline (pH 7.4) containing 10% DMF. Conversion was monitored by increase in absorbance at 504 nm.

carboxylic acid to facilitate proton transfer during imine/hydrazone formation. The 4-methyl and 5-methyl derivatives, which would not undergo unfavorable steric interactions with either the amine or the carboxylate, showed greatly improved catalytic activity, with 5-methylanthranilic acid performing the best, at a 3.1-fold higher conversion than the aniline-catalyzed reaction and 44-fold better than the uncatalyzed reaction (Table 2, entries 5 and 6). Other isomers that deviated from the anthranilic acid structure were considerably less active than their anthranilic acid counterparts (Table 2, entries 16 and 17).

These results established that the 4- and 5-positions of the anthranilic acid ring could be modified to improve catalytic reactivity. We further altered these positions to increase or decrease the electron density of the ring and the amino group. At both positions, addition of an electron-donating group increased the reaction rate, whereas an electron-withdrawing group reduced the catalytic activity. 5-Methoxyanthranilic acid (5MA, **5**) yielded the greatest increase in conversion to hydrazone product after 2 h (55.2%), providing a 5.3-fold increase in yield relative to aniline and a 75-fold improvement over the uncatalyzed reaction. Although 4,6′-bianthranilic acid displayed an even greater performance than 5MA in our initial screen, it was found to be poorly soluble at reduced pH or higher concentrations, limiting further studies and applicability.

Two of the best-performing catalysts were studied under pseudo-second-order conditions at pH 7.4 to determine the apparent second-order rate constants and allow for comparison to aniline and simple anthranilic acid (Table 3, Figure 2). The data show that 5-methoxyanthranilic acid (5MA) yields an 84-fold higher rate constant than the uncatalyzed reaction, whereas aniline provides a considerably lower 15-fold enhancement in the rate constant. Further studies were done by determining rates at pH values ranging from 7.4 down to 4.5 (Table 4). Consistent with prior studies on the aniline-catalyzed reaction and hydrazone formation in general,[3,20] decreasing the pH of the reaction resulted in a faster reaction and a larger second-order rate constants and both new catalysts performed better than aniline at each pH tested (Table 4). Significantly, the relative advantage of the anthranilic acids over aniline decreased at the lower pH values, consistent with the notion that the *ortho*-carboxylic acid group acts as a general acid for proton transfer; at low pH values, specific acid catalysis (by buffer protonation of the amino leaving group) apparently begins to take over. At pH 5.5 or below, 3,5-diaminobenzoic acid (3,5-DABA) was found to perform yet better than anthranilic acid derivatives and considerably superior to aniline, which is consistent with the idea that this catalyst does not perform intramolecular proton transfer at the transition state of the reaction.

Table 3. Observed Second-Order Rate Constants for Hydrazone Formation with Different Catalysts[a]

catalyst	k_{obs} (M^{-1} min^{-1})	$k_{app(rel)}$
no catalyst	0.08 ± 0.01	1.00
aniline (**2**)	1.1 ± 0.2	14.6
anthranilic acid (**3**)	2.3 ± 0.2	29.5
5-methoxyanthranilic acid (**5**)	6.6 ± 0.2	83.6
3,5-diaminobenzoic acid (**4**)	3.2 ± 0.3	40.2

[a]Conditions: 18 μM **1**, 1 mM 4-nitrobenzaldehyde, 1 mM catalyst in phosphate buffered saline (pH 7.4) containing 10% DMF. Conversion was monitored by increase in absorbance at 504 nm.

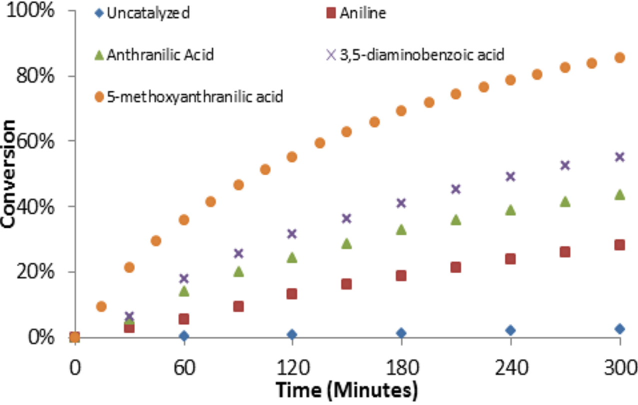

Figure 2. Conversion of NBD hydrazine **1** to hydrazone product in the presence of selected catalysts. Conditions: pH 7.4 buffer with 10% DMF, 23 °C.

Altering the concentration of catalyst provided further interesting results (Table 5; Figure S6, Supporting Information). As the concentration increased, the relative advantage of the new catalysts increased substantially. At 5 mM catalyst concentration, the second-order rate constant of 5MA was approximately 22-fold better than that of aniline, while at 10 mM catalyst concentration, an enhancement of almost 105-fold was observed. The most likely explanation is a synergistic enhancement resulting from not only the increased catalyst concentration but also the ability of 5MA to modulate the pH of the solution at this higher catalyst concentration. As the initial pH 7.4 PBS buffer contained 12 mM phosphates, addition of 10 mM catalyst was sufficient to decrease the pH to approximately 6. This also explains the ability of 4-aminobenzoic acid, a catalyst that performed worse than aniline at 1 mM concentration, to perform considerably better than aniline at 10 mM. This property of anthranilic acids as catalysts may prove useful in buffers commonly used for cellular labeling studies (PBS, DPBS, HBSS), which require exceedingly high concentrations of aniline or long incubation times to achieve labeling.[10,21−24] With a significant increase in the second-order rate constant for anthranilic acid catalysis at 10 mM concentration in PBS (similar to previously recommended conditions for aniline in cell-labeling studies[21−23]), the incubation time for such labeling techniques could likely be reduced from hours to minutes (Figure S6, Supporting Information).

We also tested 5MA and 3,5-DABA for their ability to catalyze oxime formation at pH 7.4, by labeling aminooxy-modified DNA with 7-(diethylamino)-3-formylcoumarin. Although 3,5-DABA showed approximately the same reactivity as aniline, 5MA was again observed to be a superior catalyst, providing a faster reaction than aniline and the expected large improvement over the uncatalyzed reaction (Figure 3). Second-order rate constants were determined for the reaction, and 5MA (60.2 M^{-1} min^{-1}) yielded rates that were approximately twice as fast as aniline (35.6 M^{-1} min^{-1}) and 3,5-DABA (37.2 M^{-1} min^{-1}) and 12 times faster than the uncatalyzed reaction (5.47 M^{-1} min^{-1}).

Varied Substrates. Further studies varying the carbonyl partner with the best-performing catalysts showed that 3,5-DABA provided enhanced catalysis on a larger variety of substrates (Figure S8, Supporting Information). Although approximately equivalent to 5MA for electron-poor aldehydes, 3,5-DABA was more proficient with benzaldehyde and

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Table 4. Observed Second-Order Rate Constants (M^{-1} min^{-1}) for Hydrazone Formation at Varied pH

catalyst	pH 4.5	pH 5.5	pH 6.5	pH 7.4
no catalyst	31.4 ± 1.3	3.7 ± 1.0	0.52 ± 0.05	0.08 ± 0.01
aniline (**2**)	410 ± 2	83 ± 6	7.4 ± 0.3	1.1 ± 0.2
anthranilic acid (**3**)	550 ± 30	96 ± 5	10.3 ± 0.3	2.3 ± 0.2
5-methoxyanthranilic acid (**5**)	620 ± 90	140 ± 10	27.5 ± 1.7	6.6 ± 0.2
3,5-diaminobenzoic acid (**4**)	1180 ± 60	260 ± 60	23.2 ± 1.9	3.2 ± 0.3
3-aminobenzoic acid	650 ± 40	79 ± 6	8.9 ± 1.1	2.0 ± 0.3

[a]Conditions: 18 μM **1**, 1 mM 4-nitrobenzaldehyde, 1 mM catalyst in phosphate buffers (see the Supporting Information) containing 10% DMF. Conversion was monitored by increase in absorbance at 504 nm.

Table 5. Observed Second-Order Rate Constants (M^{-1} min^{-1}) for Hydrazone Formation at Different Catalyst Concentrations[a]

catalyst	5 mM	10 mM
aniline	3.5 ± 0.3	5.9 ± 0.3
anthranilic acid	36 ± 2	640 ± 20
4-aminobenzoic acid	9.0 ± 1.3	140 ± 20
5-methoxyanthranilic acid	79 ± 3	620 ± 30
3,5-diaminobenzoic acid	44 ± 3	270 ± 40

[a]Reactions contained 18 μM **1**, 1 mM 4-nitrobenzaldehyde, and 1 mM or 10 mM catalyst in 10:1 PBS (pH 7.4):DMF, monitoring absorbance at 504 nm.

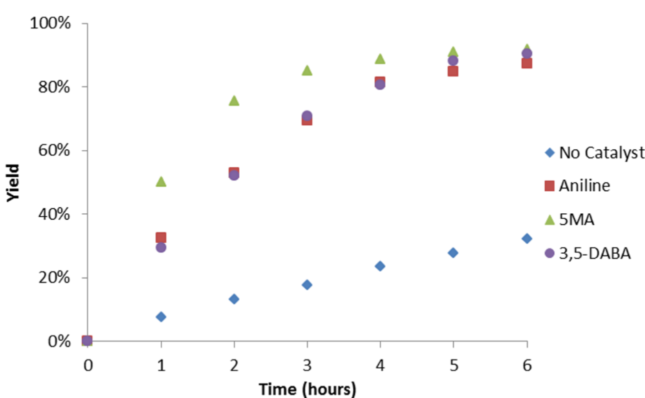

Figure 3. Oxime formation between 40 μM aminooxy functionalized DNA and 200 μM 7-(diethylamino)-3-formylcoumarin containing 1 mM catalyst at pH 7.4 (75 mM phosphates).

electron-rich aldehydes (e.g., *p*-anisaldehyde). It is likely that this catalyst operates by a different mechanism, as the carboxylic acid is not *ortho* to either amino group. There may still be some favorable effect of a local carboxylic acid, as seen by the fact that 3-aminobenzoic acid performed better than 4-aminobenzoic acid (but worse than anthranilic acid) (Table 1). In addition, the fact that 3,5-DABA contains two anilinic amine groups likely provides a simple statistical increase of imine, and subsequent product, formation.

We also tested ketones as possible substrates for catalysis. We found that acetophenone was a poor substrate for 5MA and 3,5-DABA relative to aniline, suggesting possible unfavorable steric effects of α-branching. Next, we tested an unbranched aliphatic ketone (2-butanone); because little absorbance change occurs with this substrate, we followed the reaction by fluorescence instead, following a published method.[10] Whereas aniline and 3,5-DABA both showed little reaction, 5MA was found to be a potent catalyst, providing complete reaction in only 5 min (Figure S9, Supporting Information). Thus, aromatic aldehydes and aliphatic ketones are good substrates for anthranilate catalysts, whereas an aromatic ketone is not. The origin of this difference is not yet clear; further experiments will be needed to test the broad scope of the catalysts.

Mechanistic Considerations. To understand how anthranilic acid serves as a catalyst in hydrazone formation, we explored its role under various conditions. The mechanism for nucleophilic catalysis of hydrazone formation involves the initial formation of an imine species, followed by subsequent reaction with the hydrazine to afford the hydrazone product.[25] Previous studies have shown that the first step, imine formation, is rate-limiting at moderate catalyst concentrations.[6,25]

To further test whether anthranilic acid catalysis follows a similar mechanism, we varied the concentration of the NBD hydrazine from 9 to 36 μM and examined the effect on reaction rate. As with aniline catalysis, the second-order rate constant for the anthranilic acid catalyzed reaction showed little or no change across these different concentrations (Table 6; and Figure S10, Supporting Information), confirming that hydrazine replacement of the imine occurs after the rate-limiting imine formation step. This indicates that, at low to moderate catalyst concentrations, imine formation is still rate-limiting and that improved catalysis by the new compounds is explained by enhancement of imine formation rather than a change in mechanism.

Table 6. Second-Order Rate Constants (M^{-1} min^{-1}) at Varying Concentrations of NBD Hydrazine for the Aniline and Anthranilic Acid Catalyzed Hydrazone Formation[a]

concentration (μM)	aniline catalysis	anthranilic acid catalysis
9	1.3 ± 0.2	2.39 ± 0.07
18	1.1 ± 0.2	2.3 ± 0.2
27	0.93 ± 0.09	1.90 ± 0.07
36	0.91 ± 0.02	1.84 ± 0.03

[a]Reactions contained the indicated concentration of **1**, 1 mM 4-nitrobenzaldehyde, and 1 mM catalyst in 10:1 PBS (pH 7.4):DMF, monitoring absorbance at 504 nm.

To further explore imine formation, we studied the formation and hydrolysis of the imine intermediate with anthranilic acids to understand the imine's relative stability and susceptibility to conversion to the hydrazone product. We found that aniline and 4-nitrobenzaldehyde equilibrated to the imine:starting material mixture after approximately 2 h (Figure 4), whereas the preformed imine was found to be moderately stable, with a half-life for hydrolysis of 20 min. This relatively slow equilibration helps to explain the initial lag observed during early time points in the case of the aniline-catalyzed reaction (see the Supporting Information), as there must be a sufficient buildup of the concentration of the imine for the

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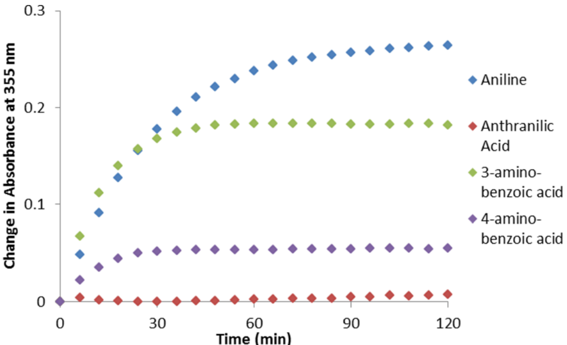

Figure 4. Monitoring imine formation between aldehyde and varied catalysts reveals that the equilibrium for imine formation with anthranilic acid lies to the side of the aldehyde. Shown are reactions of 4-nitrobenzaldehyde with aniline (blue), anthranilic acid (red), 3-aminobenzoic acid (green), or 4-aminobenzoic acid (black). The change in absorbance at 355 nm was monitored in 10:1 PBS (pH 7.4):DMF (1 mM reactants), at 23 °C.

reaction to proceed. The decrease in this initial lag under anthranilic acid catalysis indicates more rapid formation and equilibration to the imine intermediate, allowing for subsequent product formation during the faster second step of the reaction. This lag presents a further kinetic disadvantage to the aniline-catalyzed reaction as compared with the new catalysts. The reaction between anthranilic acid and 4-nitrobenzaldehyde, however, showed little or no observable amounts of imine, and the preformed imine itself was found to hydrolyze very rapidly with a half-life of 20 s (Table 7). Both 3- and 4-aminobenzoic acids were observed to equilibrate to the imine product more rapidly than aniline (30 min or less), but more slowly than anthranilic acid. The two isomers also displayed longer hydrolytic half-lives than the latter catalyst, which is again consistent with a beneficial role of the *ortho*-carboxylic acid in transferring protons during imine formation/breakdown.

Table 7. Hydrolytic Half-Lives of Imines of Different Catalysts in 10:1 PBS (pH 7.4):DMF, 23°C

imine	$t_{1/2}$ (hydrolysis) (min)	k_{rel}
aniline	20.0 ± 0.9	1.0
anthranilic acid	0.35 ± 0.01	57
3-aminobenzoic acid	14.0 ± 0.2	1.4
4-aminobenzoic acid	13.0 ± 0.1	1.6

These results indicate that the equilibrium for imine formation between anthranilic acid and 4-nitrobenzaldehyde lies more strongly to the side of starting materials than for imine equilibration with aniline, indicating that the anthranilic acid imine intermediate is higher in energy than the aniline. The energetic barrier for imine formation, however, must be lower than that for aniline to explain the observed catalytic enhancement. Our observations support the notion that the intermediate catalyst–imine adduct equilibrates rapidly and is present in relatively low concentrations, consistent with previous studies of aniline-catalyzed hydrazone formation.[3,6] In the anthranilic acid catalyzed case, the more rapid hydrolysis coupled with the diminished initial lag confirms that the imine is formed more rapidly, with a lower energetic barrier. This results in both a suppression of the lag and an overall faster rate.

Understanding the specific role of the *ortho*-carboxylic acid of anthranilic acid in catalysis is complicated by the inherent amphoteric, bifunctional nature of anthranilic acid. At pH 7.4, it is expected that the compound should exist largely in the deprotonated, anionic form (i.e., with both the amine and the carboxylic acid deprotonated).[18] During imine formation between the catalyst and aldehyde, initial attack of the amine results in formation of a tetrahedral hemiaminal, which ultimately breaks down (eliminating water) to form the imine. Since imine formation is more rapid with anthranilic acid catalysis, elimination of water appears to be more rapid as a result of the *ortho*-carboxylic acid. We hypothesize that this group acts as a general acid catalyst, by being transiently protonated and transferring its proton at the transition state to the neighboring hemiaminal to aid in elimination of water. Multiple lines of evidence support this notion. First, 3-amino- and 4-aminobenzoic acid are not as efficient as anthranilic acid in catalysis despite having acidic groups that are similar in pK_a.[19] The second is the finding that substitution at the 4-position by electron-donating groups enhances catalysis (Table 2); this is expected to raise the pK_a of the acid, resulting in a larger fraction of carboxylate in the protonated state. Indeed, the most reactive catalysts have electron-donating groups in the ring, whereas electron-withdrawing substituents greatly diminish catalysis; this is the opposite trend that would be expected if a simple pH-lowering effect were responsible for catalysis. Finally, methyl substitution adjacent to the carboxylic acid abolishes catalysis, whereas more remote methylation enhances it. This is consistent with orientation of the carboxylate being important to proton transfer at the transition state.

In addition to this proposed intramolecular cataysis, the reaction is also catalyzed by protons from solution, as evidenced by the general acceleration of all reactions (including those with catalysts lacking an *ortho*-carboxylate) at lower pH values. The added benefit of the current catalysts is to speed the reaction at neutral pH values where protons from solution exist at very low concentrations. Because the pK_a of the carboxylic acid in these catalysts is significantly lower than the pH of solution, only a very small fraction of the carboxylate is in the active (protonated) form, which suggests that (a) the acid form is highly effective in this catalysis and (b) that future catalyst designs with higher pK_a may be more effective than the anthranilic acids. Future studies will test this possibility and will explore the applications of the new catalysts in conjugation reactions.

■ CONCLUSIONS

We have determined that anthranilic acids and 3,5-diaminobenzoic acid present a new class of efficient water-soluble catalysts for hydrazone and oxime formation with aldehydes. At pH 7.4, 1 mM 5-methoxyanthranilic acid shows a second-order rate constant over 6-fold greater than that of aniline, while higher concentrations of catalyst can provide greater than 2 orders of magnitude enhancement in the rate constant relative to aniline. The results are likely to be widely useful in formation of hydrazones and oximes for bioorthogonal conjugations and for more general chemical applications as well. Moreover, with anthranilic acid serving as an intermediate in the biosynthesis of tryptophan,[26,27] it is likely that it and related derivatives will be considerably less toxic for cellular applications than aniline.

■ EXPERIMENTAL SECTION

Synthesis. NBD hydrazine **1** and 7-(diethylamino)-3-formylcoumarin were prepared according to literature procedures.[10,11]

The preparation of the hydrazone between NBD hydrazine and 4-nitrobenzaldehyde was adopted from an analogous literature procedure.[10] A 75 mg portion of NBD hydrazine and 581 mg of 4-nitrobenzaldehyde (10 equiv) were stirred in 30 mL of methanol at room temperature overnight. The resulting dark red solid was filtered, washed with methanol, and dried to afford 121 mg of pure product (96%). An analytically pure sample was obtained by flash column chromatography over silica gel eluting with ethyl acetate. Melting point > 230 °C.

^{1}H NMR (500 MHz, DMF-*d7*): 8.94 (s, 1H), 8.45 (d, 2H, *J* = 8.5 Hz), 8.41 (d, 1H, *J* = 9 Hz), 8.28 (d, 2H, *J* = 8.5 Hz), 8.24 (d, 1H, *J* = 9 Hz)

^{13}C NMR: Solubility in common NMR solvents was too poor to obtain a spectrum; a saturated sample in DMF-*d7* showed only solvent peaks.

ESI-MS (M-H): 327.25 (calcd 327.05).

Imines were prepared by dissolving 2 mmol of the aniline derivative and 2 mmol of 4-nitrobenzaldehyde in 10 mL of ethanol and stirring overnight at room temperature to provide a precipitate. Ethanol was removed by rotary evaporation, and the resulting solid was crystallized in ethanol, filtered, and washed with ethanol to afford the imine product.[12] The 4-nitrobenzaldehyde-based imines from aniline, anthranilic acid, 3-aminobenzoic acid, and 4-aminobenzoic acid are previously reported compounds.[12−15]

General Screening Procedure. A 590 μL portion of 10:1 PBS (pH 7.4):DMF was added to a UV−vis cuvette. A 1.2 μL portion of a 500 mM solution of catalyst in DMF was added (final concentration 1 mM), and the baseline was collected. A concentrated solution of NBD hydrazine **1** (1.4 μL) was added (final concentration 18 μM). The reaction was initiated by the addition of 7.6 μL of a concentrated solution of 4-nitrobenzaldehyde (final concentration 1 mM), and the absorbance at 504 nm was monitored for 2 h. The absorbance data were converted to concentration (in μM) of hydrazone **6** by dividing by the extinction coefficient of **6** (14 100 L mol^{-1} cm^{-1}; path length, 1 cm), and yields were determined by dividing the concentration at the given time point by 18 μM (starting concentration of **1**). The yield was assumed to be zero at the starting point. All experiments were performed in triplicate and averaged.

Equations used for second-order kinetic fits can be found in the Supporting Information. R^2 values for fits were, in most cases, greater than 0.98.

Reactions at Varied pH. The same conditions as above were used, but PBS was substituted by the appropriate buffer: pH 4.5 (50 mM sodium phosphates), pH 5.5 (100 mM sodium phosphates), and pH 6.5 (100 mM sodium phosphates).

Reactions with Varied Concentrations of Catalyst or Hydrazine. The same conditions as those in the general section were used, but the concentration of catalyst was increased to either 5 or 10 mM with all other concentrations kept constant. For reactions with different amounts of hydrazine compound, the same conditions as those in the general section were employed, with the hydrazine concentration varied at 9, 27, or 36 μM.

DNA Conjugation. DNA (H_2NO-5′-TTT-GTA-TAT-TAT-GCC-3′) was synthesized DMT-On and cleaved from the solid support using incubation in concentrated aqueous ammonia for 17 h at 55 °C. The crude DNA was purified by PolyPak II (Glen Research) to afford the aminooxy modified DNA. Conjugation was performed with 40 μM DNA and 200 μM 7-(diethylamino)-3-formylcoumarin in 75 mM Na·phosphate buffer (pH 7.4) containing 1 mM catalyst (total volume 400 μL). Aliquots (40 μL) were removed and injected into an analytical HPLC at 1 h time points for 6 h. MALDI-TOF data: Starting Aminooxy 15mer: 4816.9 (calcd 4816.9). Oxime product: 5048.3 (calcd 5044.0)

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and further kinetics information supplied in the Supporting Information file. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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