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Mechanistic Studies on the Reaction between R_2N-NONOates and Aquacobalamin: Evidence for Direct Transfer of a Nitroxyl Group from R_2N-NONOates to Cobalt(III) Centers**

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The gaseous radical nitric oxide (•NO, NO) is a signaling molecule that plays a vital role in biology. It facilitates vasodilation and inhibits platelet aggregation in the cardio-vascular system, initiates the pro-inflammatory immune response, and regulates neurotransmission.[1, 2] Impaired NO bioavailability is associated with a wide variety of vascular pathologies, including endothelial cell dysfunction.[3] Conse-quently, there is considerable interest in NO donor molecules, such as 1-(N,N-dialkylamino)diazen-1-ium-1,2-diolates (R_2N-NONOates; Figure 1), which spontaneously decompose by first-order acid-catalyzed processes to release up to two NO molecules and the corresponding amine.[4–6] R_2N-NONOates are widely used as NO precursors in studies of NO-dependent biological processes[5,7] and as NO prodrugs with applications in NO-releasing biomaterials, wound healing, organ protection, and

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chemotherapy.[5,8] R_2N-NONOates can also be combined with anti-inflammatory drugs[9] and incorporated into dendrimers, nanoparticles, or microspheres to optimize their delivery.[10–12] These species are typically synthesized by reacting amines with NO(g) at high pressures.[6,13,14] O2-alkylated R_2N-NONOate conjugates with considerably enhanced stability have also been developed.[15–17]

One important distinction between R_2N-NONOates and the majority of other NO donor species is that they are generally regarded as being insensitive to the reaction medium and unreactive with most biomolecules, including thiols.[5,8,18] Reports on the reactions between R_2N-NONOates and transition metal complexes are rare. X-ray structures of Cu^{II}/R_2N-NONOate complexes show that NON-Oate can coordinate to metal centers through one or both oxygen atoms.[19–21] For a series of metal cations, it was also shown that the NO release rates from the NONOate adduct of the polyamine spermine is relatively insensitive to a range of metal cations (Fe^{3+}, Al^{3+}, La^{3+}, Ca^{2+}, Zn^{2+}, Mg^{2+}; rate of spontaneous NONOate decomposition up to 60 % slower, presumably via binding of the NONOates to the metal center[4]). However, by functionalizing one alkyl substituent of the R_2N-NONOate dialkylamine moiety to provide additional donor atoms to the metal center, the rate of NO release from the metal-coordinated R_2N-NONOate can be slowed by over an order of magnitude.[21]

Cobalamins (Cbls, vitamin B_{12} derivatives) are octahedrally coordinated cobalt(III) macrocycles, and can incorporate a range of ligands at the upper (β) axial site. These include aqua/hydroxy (H_2OCbl^+/HOCbl; $pK_a(H_2OCbl^+)=7.76\pm0.02$ at 25.0°C, total ionic strength I=0.50 M (KNO_3)[22]), cyanide (CNCbl), methyl (MeCbl), 5′-deoxyadenosyl (AdoCbl), nitro (NO_2Cbl), and nitrosyl (NOCbl) ligands. In searching for an efficient method to synthesize nitroxylcobalamin (also referred to as nitrosylcobalamin, NOCbl) for X-ray diffraction studies, we found that it can be synthesized in high yield and purity by reacting HOCbl with diethylamine-NONOate (DEA-NONOate, Figure 1) under anaerobic conditions.[23] Like other R_2N-NONOates, DEA-NONOate decomposes under anaerobic conditions by a clean, first-order, acid-catalyzed process to release NO and the corresponding amine, namely diethylamine (DEA).[4,24,25] Control experiments showed that DEA-NONOate decomposes cleanly into DEA alone (+ NO; pD 9.50 and 10.40; ^{1}H NMR spectroscopy). It is, however, well established that neither H_2OCbl^+ nor HOCbl react with NO.[26] We therefore carried out kinetic and mechanistic studies on this intriguing reaction.

Figure 2 gives typical UV/Vis spectra for the reaction between HOCbl (0.050 mm) and excess DEA-NONOate (10.0 mm) as a function of time under anaerobic conditions at pH 10.80 (0.30 M CAPS buffer, 25.0°C, I=1.0 M ($NaCF_3SO_3$)). High buffer concentrations (0.30 M) were necessary to ensure a stable pH was maintained during the reaction. Figure 2, Inset a, shows a comparison between the initial and final spectrum of the reaction in which HOCbl is cleanly converted into NOCbl (λ_{max}=256, 278 (shoulder), 289, 315, and 478 nm) with sharp isosbestic points observed at 341, 370, and 498 nm, which is in agreement with literature values.[27, 28] In Figure 2, Inset b, the best fit of the absorbance data at 356 nm versus time to a first-order rate equation is superimposed upon the experimental data, giving $k_{obs}=(1.91\pm0.01)\times 10^{-3}$ min^{-1}. The rate of spontaneous acidcatalyzed decomposition of DEA-NONOate to NO and DEA was found to be more than one order of magnitude slower than the reaction of DEA-NONOate with HOCbl (half-life for the spontaneous decomposition $t_{1/2}\geq$ 1 week, pH 10.80; Table 1). This result suggests that HOCbl reacts directly with the DEA-NONOate complex to give NOCbl, and that decomposition of DEA-NONOate to form NO is not a prerequisite for the reaction to occur. The direct transfer of a nitroxyl group (NO^-) from R_2N-NONOate to a transition metal center to yield the corresponding nitroxyl complex is, to our knowledge, unprecedented.

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To further probe for formation of intermediate(s), the reaction was followed using ^{1}H NMR spectroscopy. Cbl complexes have five corrin and nucleotide protons that resonate in the aromatic region with chemical shifts dependent on the β-axial ligand. Observation of the reaction of HOCbl (2.96 mm) with DEA-NONOate (4.44 mm) at pD 11.30 by ^{1}H NMR spectroscopy showed that HOCbl (δ=7.17, 6.70, 6.49, 6.23, and 6.04 ppm) was cleanly converted into NOCbl (δ=7.44, 7.27, 6.80, 6.35, and 6.25, in agreement with literature values [27,29]), without any detectable Cbl intermediate (Supporting Information, Figure S1). Alkaline conditions were used to ensure that the spontaneous decomposition of DEA-NONOate is negligible. The reaction stoichiometry was determined by recording ^{1}H NMR spectra of the products of the reaction between HOCbl and 0.55, 1.1, 1.2, 1.5, or 2.2 mol equivalents of DEA-NONOate at pD 10.42. With 0.55 equivalents of DEA-NONOate, a mixture of NOCbl (ca. 55 %) and unreacted HOCbl (approx. 45 %) was observed, whereas with 1.2 equivalents of DEA-NONOate, HOCbl was essentially completely converted into NOCbl (Figure 3 a). Because approximately 1.2 equivalents of DEA-NONOate is required for the reaction to proceed to completion, this observation suggests that only one of the two nitric oxide moieties in the parent NONOate reacts with the cobalamin to form NOCbl.

Experiments were carried out to identify the non-cobalamin reaction product(s). It was determined that nitrite was not a reaction product (Griess assay; see Supporting Information). Two other potential non-Cbl reaction products, diethylamine (DEA) and *N*-nitrosodiethylamine (DEA-NO), are individually distinguishable by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the products of the reaction of HOCbl and 1.2 equivalents of DEA-NONOate in alkaline solution (pD 10.42) in the 3.5–4.3 ppm region revealed DEA-NO to be the non-Cbl reaction product (Figure 3 b). Therefore, DEA-NONOate reacts directly with HOCbl to produce NOCbl and DEA-NO. Formation of the corresponding nitrosamine is undesirable from a biological and pharmaceutical view point, given that many of these species, including DEA-NO, are carcinogenic.[30] Nitrosamines are also products of R_2N-NONOate photolysis and R_2N-NONOate decomposition under aerobic conditions.[30,32] Although it has been previously suggested that DEA-NO rapidly decompose to DEA and NO,[4,25] in this study it was a stable species.

To confirm that DEA-NONOate, rather than its decomposition products, react with H_2OCbl^+/HOCbl, H_2OCbl^+/HOCbl was added to a solution of DEA-NONOate that had fully decomposed to DEA and NO(g) at pH 7.40, 8.50, or 9.80, and the reaction was followed by UV/Vis spectroscopy. No reactions were observed after 16 h under any of the three pH conditions.

If indeed HOCbl reacts directly with DEA-NONOate, the observed rate constant should depend on the DEA-NON-Oate concentration. The dependence of k_{obs} on DEA-NON-Oate concentration (2.5–25.0 mm) at pH 10.80 was therefore determined (Figure 4). Measurements at higher DEA-NON-Oate concentrations were not possible owing to the limited solubility of DEA-NONOate. Fitting the data to a straight line gives a second-order rate constant, k_{app}= (0.056±0.002) L mol^{-1} min^{-1} (=*kK*'; see below) at pH 10.80 with an intercept of (1.31±0.02) × 10^{-3} min^{-1}. A control experiment showed that HOCbl itself slowly decomposes at pH 10.80 (k_{HOCbl}=(1.32±0.03) × 10^{-3} min^{-1}; Table 1, which, within experimental error, is the same as the intercept. Note that HOCbl decomposition is not strictly first-order and does not proceed to completion at lower pH values. The self-reduction of HOCbl in alkaline solution has been reported previously.[32,33]

The dependence of k_{obs} on DEA-NONOate concentration (2.50–25.0 mm) was studied at three other pH conditions (pH 9.50, 9.80 and 10.40). The data is plotted in Figure S2 in the Supporting Information and the rate constants are summarized in Table 1. It was necessary to take into

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account spontaneous DEA-NONOate decomposition in the treatment of the kinetic data at pH 9.50 and 9.80 at the lower DEA-NONOate concentrations by fitting to Equation (1):

$$A_{obs}=A_{\infty}+(A_0-A_{\infty})\exp\left(\frac{k_{app}[L]_0}{k_L}\left(e^{-k_L t}-1\right)\right) \quad (1)$$

where A_{obs}, A_0, and A_∞ are the observed, initial, and final absorbances respectively, k_{app} is the (pH-dependent) rate constant, k_L is the observed rate constant for spontaneous NONOate decomposition, and $[L]_0$ is the initial NONOate concentration. The derivation of this equation is given in the Supporting Information.

Above pH 10.80, the rate of reaction between HOCbl and DEA-NONOate is extremely slow, whereas at pH values below 9.50, the spontaneous decomposition of DEA-NON-Oate was found to be within one order of magnitude or even faster than the reaction of interest. For example, at pH 9.30, the observed rate constant for the reaction between DEA-NONOate (2.5 mM) and HOCbl (0.050 mM) is 3.0×10^{-3} min^{-1}, whereas that for spontaneous decomposition of DEA-NONOate is 1.6×10^{-3} min^{-1}. At higher NONOate concentrations, although the rate of the Cbl/NONOate reaction is faster, considerable interference occurs from gas evolution despite gentle stirring with stir bars at the bottom of the cuvettes. The gas arises from acid-catalyzed spontaneous DEA-NONOate decomposition to NO(g) and DEA, leading to unreliable data. Furthermore, a second reaction was observed below pH 10, which was subsequently shown to arise from excess NO(g) from decomposed DEA-NONOate reacting with NOCbl to form nitrocobalamin (NO_2Cbl). This reaction becomes increasingly important at lower pH values and higher DEA-NONOate conditions. Further details are given in the Supporting Information.

From Table 1, it can be seen that the second-order rate constant k_{app} increases with decreasing pH. The pK_a of DEA-NONOate is 5.0,[4] and a ^{1}H NMR titration experiment showed no further deprotonation for DEA-NONOate in the range pH 8.5–12.5. Control experiments showed that DEA-NONOate does not react with methylcobalamin (MeCbl), cyanocobalamin (CNCbl) or adenosylcobalamin (AdoCbl). This result suggests that a labile β-axial ligand, such as the aqua ligand of H_2OCbl^+, is required for the reaction between Cbls and NONOates to proceed. It is well established that HOCbl is inert to substitution.[22] Using the value of k_{app} at pH 9.50 (0.68 $Lmol^{-1}$ min^{-1}) and pK_a(H_2OCbl^+)=7.76,[22] a second-order rate constant for the reaction between H_2OCbl^+ and DEA-NONOate of approximately 38 $Lmol^{-1}$ min^{-1} (ca. 0.63 $Lmol^{-1}$ s^{-1}) was estimated. However, rate constants for ligand substitution of the β-axial ligand of H_2OCbl^+ are typically two to four orders of magnitude larger than this.[34] The simplest mechanism consistent with the experimental data is given in Scheme 1, in which a rapid pre-equilibrium to form the NONOate-Cbl complex precedes rate-determining nitrogen-nitrogen bond cleavage to give NOCbl and R_2N-NO. The corresponding rate equation is given in Equation (2) where $K'=K[H^+]/([H^+]+K_a(H_2OCbl^+))$:

$$k_{obs}=\frac{kK'[\text{NONOate}]}{1+K'[\text{NONOate}]}+k_{HOCbl} \quad (2)$$

Equation (2) reduces to $k_{obs}=kK'[\text{NONOate}]+k_{HOCbl}$ when K' is small, which is consistent with the Cbl reactant being predominantly HOCbl, not H_2OCbl^+, and a reaction intermediate not being observable. Although it was not possible to obtain rate data for the reaction between H_2OCbl^+/HOCbl at pH values close to the pK_a value of H_2OCbl^+, and therefore show that H_2OCbl^+ (pK_a 7.76[22]), not HOCbl, reacts with DEA-NONOate, the increase in k_{app} as the pH is decreased (Table 1) is consistent with this interpretation.

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The reactions between H_2OCbl^+/HOCbl and three other R_2N-NONOates were also investigated. DETA-NONOate (Figure 1) was chosen for studies at lower pH conditions to further probe whether the increase in the apparent rate constant k_{app} with decreasing pH arises from H_2OCbl^+/HOCbl speciation, because DETA-NONOate is remarkably stable to acid-catalyzed decomposition when compared with other R_2N-NONOates (Table 2). However, at pH 7.4, the reaction between H_2OCbl^+/HOCbl and DETA-NONOate was extremely slow and incomplete, with a rate constant of the same order as that for the spontaneous rate of decomposition of the NONOate ($k_{obs}\approx 8\times10^{-4}$ min^{-1}, $k_L=1.6\times10^{-4}$ min^{-1}; [DETA-NONOate]=7.5 mM, ca. 70% of HOCbl/H_2OCbl^+ converted into NOCbl over 2.5 days). Although the reaction was faster at pH 5.88, once again H_2OCbl^+ was only partially converted into NOCbl and k_L and k_{obs} were within a factor of two of each other. Similar results were obtained for the reaction of H_2OCbl^+/HOCbl with DPTA-NONOate (pH 9.30, 8.00, 7.40, and 6.80), with the extent of H_2OCbl^+/HOCbl conversion into NOCbl decreasing with decreasing pH. At pH 6.80, practically no reaction occurred between H_2OCbl^+ and DPTA-NONOate, with the latter species apparently decomposing before it could react with H_2OCbl^+. Therefore, it appears that having two sterically bulky substituents on the secondary amine of R_2N-NON-Oates leads to both thermodynamically and kinetically unfavorable reactions of these species with H_2OCbl^+/HOCbl, although further studies are required to confirm this. Sterically demanding substituents also stabilize R_2N-NONOates with respect to acid-catalyzed decomposition (Table 2).[6,35]

Kinetic studies on the reaction between H_2OCbl^+/HOCbl and MAHMA-NON-Oate were also carried out. As with DEA-NONOate above, HOCbl reacts with MAMHA-NONOate to produce NOCbl (Supporting Information, Figure S3). Interestingly, although the plot of k_{obs} versus NONOate concentration was linear at pH 10.80, curvature was observed at lower pH values (Supporting Information, Figure S4). Additional studies revealed that the most likely explanation for the curvature is a nitrite impurity (approx. 10 %) in commercial MAHMA-NONOate, which is not easily removed. Nitrite reacts rapidly with H_2OCbl^+ to form nitrocobalamin (NO_2Cbl),[36,37] and the formation of NO_2Cbl slows down the apparent rate of the reaction between HOCbl and MAHMA-NONOate. Further details are given in the Supporting Information.

Finally, if a labile axial ligand is required for the transfer of NO^- from R_2N-NONOates to cobalt(III) corrinoids to produce the corresponding nitroxyl complex, then R_2N-NONOates should also react with the closely related Co^{III} cobinamide derivatives (Cbi) in which the nucleotide is cleaved at the phosphodiester.[38] In support of this, aquahy-droxycobinamide was found to react rapidly with DEA-NONOate to produce the corresponding NOCbi complex (k_{obs}=0.5 min^{-1}, [DEA-NONOate]=1.0 mM, pH 9.30, 25.0°C). Detailed kinetic studies on this system are currently underway.

To summarize, UV/Vis and ^{1}H NMR spectroscopy studies on the reaction between R_2N-NONOates and HOCbl/H_2OCbl^+ suggest that H_2OCbl^+ reacts directly and essentially stoichiometrically with DEA-NONOate to give NOCbl and the corresponding toxic nitrosoamine DEA-NO. Decomposition of the NONOate to produce NO is not a prerequisite for the reaction to occur. To our knowledge, a direct reaction between R_2N-NONOate and a transition metal complex to produce a nitroxyl complex is unprecedented. Given the widespread use of R_2N-NONOates as NO donors and the interest in these compounds as pharmaceuticals, further studies on the potential biological relevance of this type of reaction would be of great interest.

Supplementary Material

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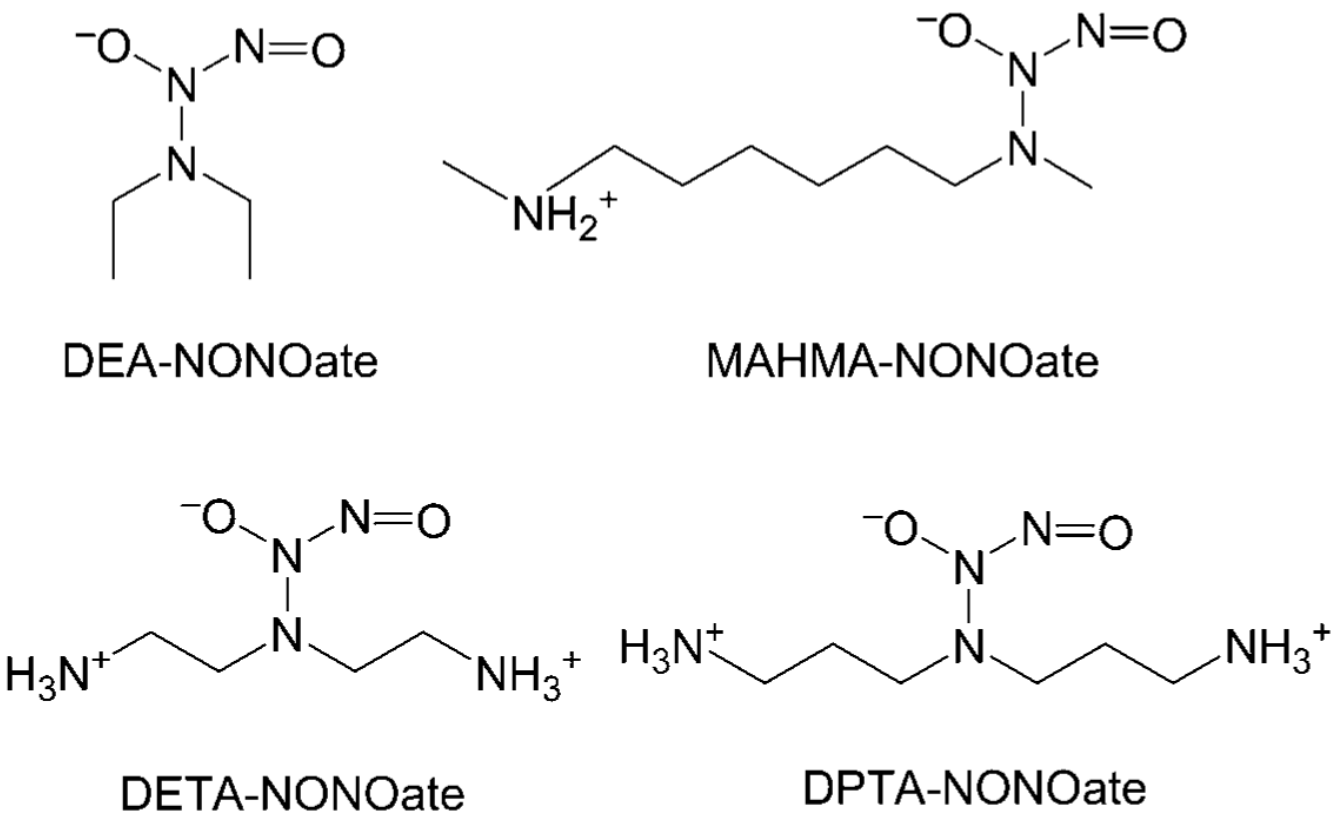

Figure 1.
Structures of selected R_2N-NONOates. (For definitions of the abbreviations, see the Supporting Information).

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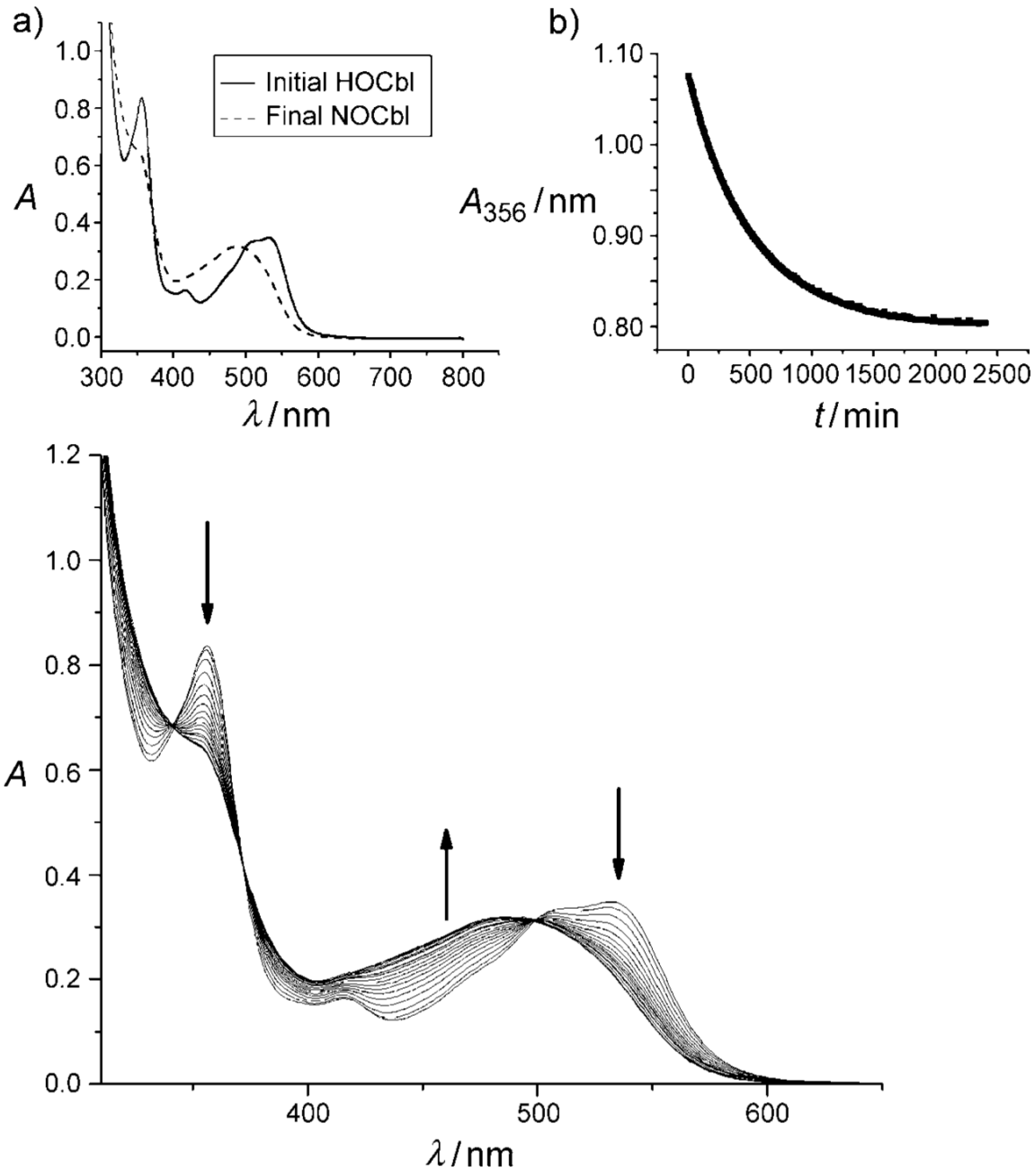

Figure 2.

UV/Vis spectra for the reaction between HOCbl (0.050 mM) and DEA-NONOate (10.0 mM) at pH 10.80. (Spectra taken every 10.0 min, 0.30 M CAPS, I=1.0M ($NaCF_3SO_3$), 25.0°C.) Isosbestic points occur at 341, 370, and 498 nm. Inset a) First and last spectra. The final product is NOCbl. b) Fit of the absorbance data at 356 nm (A_{356}) versus time to a first-order rate equation, giving k_{obs}=(1.91±0.01)×10^{-3} min^{-1}.

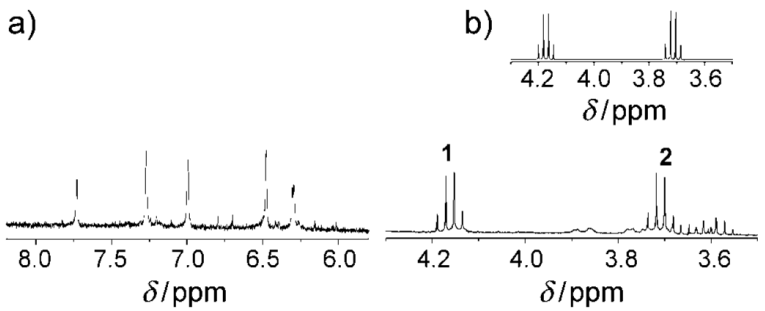

Figure 3.

^{1}H NMR spectrum of the products of the reaction between HOCbl and 1.2 equiv DEA-NONOate after 5 days (pD 10.42, 0.50 M CAPS). a) Aromatic region showing the 5 characteristic signals of NOCbl at δ=7.73, 7.27, 7.00, 6.48, and 6.31 ppm. The small peaks are impurities arising from Cbl decomposition at the high pD conditions. b) 4.3–3.5 ppm region, showing signals attributable to DEA-NO (δ=4.20, 4.18, 4.16, 4.15, 3.74, 3.72, 3.71, and 3.69 ppm) overlapping with NOCbl signals. These signals were not observed in the ^{1}H NMR spectrum of the reactant HOCbl. Inset: ^{1}H NMR spectrum of authentic DEA-NO (0.50 M CAPS, pD=10.42).

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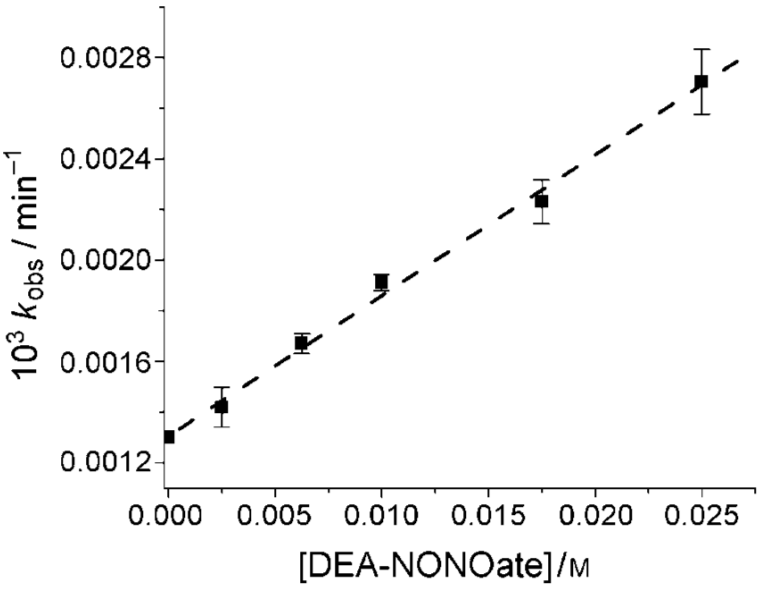
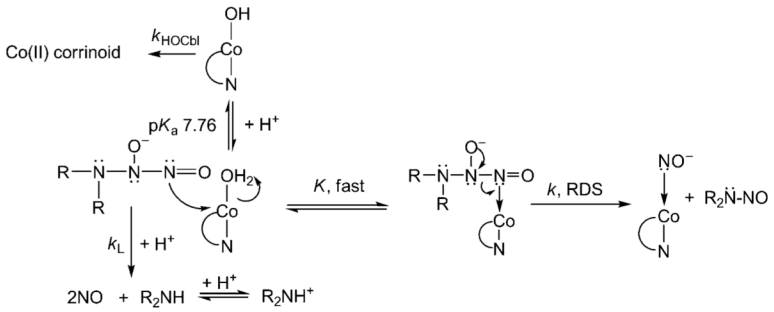


Figure 4.

Plot of observed rate constant k_{obs} versus NONOate concentration for the reaction between HOCbl and DEA-NONOate at pH 10.80. The best fit of the data gives k_{app} =(0.056±0.002) Lmol^{-1} min^{-1} (slope) and k_{HOCbl} =(1.32±0.03)×10^{-3} min^{-1} (intercept, 0.30M CAPS, I=1.0M ($NaCF_3SO_3$), 25.0°C).



Scheme 1.

Proposed mechanism for the reaction of H_2OCbl^+/HOCbl with R_2N-NONOate.

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Table 1

Apparent rate constants k_{app} for the reaction between HOCbl and DEA-NONOate, and observed rate constants for the spontaneous decomposition of HOCbl (k_{HOCbl}) and DEA-NONOate (k_L)

pH	k_{app} [L mol^{-1} min^{-1}]	$10^3 k_{HOCbl}$ [min^{-1}]	$10^3 k_L$ [min^{-1}]
9.50	0.68±0.02	0.33±0.01	1.24±0.01
9.80	0.29±0.03	0.97±0.02	0.555±0.008
10.40	0.14±0.01	1.43±0.05	0.197±0.001
10.80	0.056±0.002	1.32±0.03	$t_{½}$≥1 week

[a]I=1.0M ($NaCF_3SO_3$), 0.30M buffer, 25°C.

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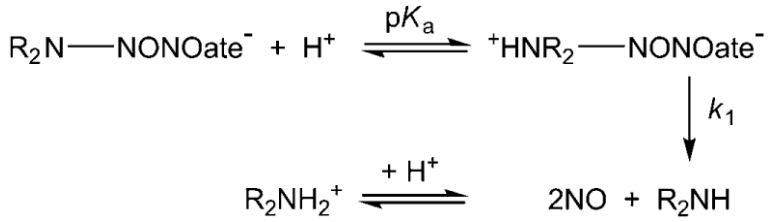
Table 2

pK_a values and rate constants for spontaneous, acid-catalyzed decomposition of R_2N-NONOates (37°C)



R_2N-NONOate	**k_1 at 37°C [s^{-1}]**	**pK_a(HN^+R_2-$NONOate^-$)**
DEA-NONOate	1.1±0.4[a]	5.0±0.2[a]
MAHMA-NONOate	0.52±0.39[a]	5.9±0.3[a]
DPTA-NONOate	0.23±0.01[b]	3.96±0.13[b]
DETA-NONOate[c]	$(3.3\pm3.1)\times10^{-2}$[a]	3.1±0.4[a]
	$(1.12\pm0.03)\times10^{-2}$[b]	3.21±0.10[b]

[a] Reference [4].

[b] This work.

[c] See Supporting Information for details. A second decomposition pathway for DETA-NONOate at higher pH was not observed.[4] UV spectra results provide evidence that protonation occurs at the amine nitrogen.[4]