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Supporting Information

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Mechanistic Studies on the Reaction between R_2N-NONOates and Aquacobalamin: Evidence for Direct Transfer of a Nitroxyl (NO^-) Group from NONOates to Cobalt(III) Centers

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Experimental Section

General procedures and chemicals. Hydroxycobalamin hydrochloride (HOCbl•HCl); stated purity by manufacturer is ≥96%, was purchased from Fluka BioChemica. The percentage of water in HOCbl•HCl (•nH_2O) (batch-dependent; typically 10-15%), was taken into account when calculating the amount of HOCbl•HCl used in the reactions, and was determined by converting HOCbl•HCl to dicyanocobalamin, $(CN)_2Cbl^-$ (0.10 M KCN, pH 10.0, $\varepsilon_{368} = 3.04 \times 10^4$ M^{-1} cm^{-1} [1]). 1-[N-Methyl-N-(6-(N-methylammoniohexyl)amino)]diazen-1-ium-1,2-diolate (MAHMA-NONOate, ≥ 98%), 1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate, ≥ 98%) and 1-[N-(3-aminopropyl)-N-(3-ammoniopropyl)amino]diazen-1-ium-1,2-diolate (DPTA-NONOate, ≥ 98%) were purchased from Cayman Chemical. 2-(N,N-Diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate, Na^+ salt, ≥98%), N-nitrosodiethylamine (DEA-NO, ≥ 99.5%), and diethylamine (DEA, ≥99.5%) were obtained from Sigma Aldrich. TES (≥ 99%) and TAPS buffers (≥99.5%) were purchased from Sigma and CHES (≥ 99%) and CAPS buffers (≥ 99%) were obtained from Acros. Sodium trifluoromethanesulfonate was prepared by adjusting a solution of trifluoromethanesulfonic acid (diluted to ~4 M; Acros, ≥ 99%) to pH 7.0 using NaOH, the solvent removed by rotary evaporation followed by drying under vacuum at 60 °C. The final product was stored in a dessicator.

Anaerobic solutions were degassed by at least three freeze-pump-thaw cycles under argon using standard Schlenck techniques. Preparation of stock solutions and mixing of the reactants was carried out in a MBRAUN Labmaster 130(1250/78) glove box operating under argon atmosphere. pH measurements were made outside the glove box at room temperature with an Orion model 710A pH meter equipped with a Wilmad 6030-02 pH electrode. The electrode was filled with 3 M KCl/saturated AgCl solution, pH 7.0, and standardized with standard BDH buffer solutions at pH 10.01 and 6.98. The accuracy of the measurements is ± 0.03 pH units.

UV-visible spectroscopy measurements. UV-visible spectra were recorded on a Cary 5000 spectrophotometer equipped with a thermostated (25.0 °C) cell holder, operating with WinUV Bio software (version 3.00). All kinetic experiments were performed under anaerobic conditions in air-free cuvettes with at least 10-fold excess of R_2N-NONOate in the presence of 0.30 M buffer, maintaining a constant ionic strength of 1.0 with $NaCF_3SO_3$. R_2N-NONOate solutions were freshly prepared immediately prior to use and protected from ambient light and air. The reaction was initiated by mixing an aliquot of a R_2N-NONOate stock solution in the appropriate anaerobic buffer solution with HOCbl•HCl (0.050 mM) dissolved in the same anaerobic buffer solution. Absorbance versus time data was collected at 356 nm. Control experiments showed identical rate constants at 437 nm. An excellent fit to a single first-order rate equation was found for all kinetic data for at least 5 half-lives (Microcal Origin, v 7.5).

NMR spectroscopy measurements. ^1H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer equipped with a 5 mm thermostatted (25 °C) probe. Buffered solutions were prepared in D_2O and TSP (3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, Na^+ salt) used as an internal reference. J. Young NMR tubes (Wilmad, 535-JY-7) were used.

Determination of rate constants for spontaneous decomposition of R_2N-NONOates. The rate constants for the spontaneous decomposition of R_2N-NONOates were measured spectrophotometrically by monitoring the decrease in absorbance of the NONOates at 250 nm. The reaction was initiated by mixing an aliquot (0.050 mL) of a R_2N-NONOate stock solution in anaerobic 0.010 M NaOH solution with the appropriate anaerobic buffer solution (2.95 mL). The final R_2N-NONOate concentration was 0.250 mM.

Determination of the percentage of nitrite impurity in DEA-NONOate and MAHMA-NONOate. The percentage of nitrite impurity present in DEA-NONOate and MAHMA-NONOate were determined using the Griess assay[2,3] under strictly anaerobic conditions. In order to generate a calibration curve, an aliquot of Griess reagent (1.50 ml) was added to an equal volume of buffer (1.50 mL, 0.10 M CHES buffer, pH 9.50) containing varying concentrations of nitrite (0, 20.0, 40.0, 60.0, 80.0 and 100.0 μM) and the absorbance at 586 nm determined.

The percentage of nitrite impurity in commercial DEA-NONOate was determined by adding 1.50 ml Griess reagent to a solution of DEA-NONOate (1.71 and 1.10 mM; carried out in duplicate) in 0.10 M CHES buffer, pH 9.50 and

the absorbance measured at 586 nm, giving 5.4% and 5.0% nitrite, respectively. In 0.10 M CAPS buffer, pH 10.80 a value of 4.1% nitrite was obtained. The absorbance at three nitrite concentrations (0, 20.0 and 40.0 μM) on the calibration curve were also checked in 0.10 M CAPS pH 10.80, to ensure that it is pH independent and were found to be the same within experimental error as those obtained at pH 9.50. Similar experiments with commercial MAHMA-NONOate (1.29 and 0.90 mM) showed 9.1% and 9.3% nitrite impurity at pH 9.50 and 10.8% and 11.7% at pH 10.80.

Both NONOates were also allowed to fully decompose under strictly anaerobic conditions at pH 9.50 prior to the addition of the Griess reagent and subjected to the same analysis procedure. The % nitrite in the decomposed NONOate solutions were the same within experimental error to that for the intact NONOates as expected, since NONOates decompose to form 2NO + the corresponding amine only. The nitrite is therefore not a consequence of partial decomposition of the NONOate during the Griess assay procedure, but arises from a nitrite impurity in the reagent itself.

Determination of the mole equivalents of nitrite produced in the reaction between HOCbl and 1.2 equiv. DEA-NONOate. The mole equiv. of NO_2^- present in the solution upon completion for the reaction between HOCbl and DEA-NONOate at pH 9.50 and 10.80 was also determined using the Griess assay under strictly anaerobic conditions. A calibration curve of absorbance versus nitrite concentration was generated in which an aliquot of Griess reagent (1.50 ml) was added to an equal volume of buffer (1.50 ml, 0.10 M CAPS buffer, pH 10.80) containing varying concentrations of nitrite (0, 20.0, 40.0, 60.0, 80.0 and 100.0 μM) and NOCbl (1.00×10^{-4} M) and the absorbance at 586 nm was determined. An aliquot of the product of the reaction between HOCbl (1.00×10^{-4} M) and DEA-NONOate (1.2 equiv.) at pH 10.80 (0.1 M CAPS) was subjected to the identical procedure, and the resulting absorbance found to correspond to 6.0 μM nitrite; that is, ~6% nitrite present in the DEA-NONOate reactant. Hence nitrite is not produced during the reaction between HOCbl and DEA-NONOate. The small amount of nitrite observed corresponds to the nitrite impurity in the DEA-NONOate reactant.

Figure S1. ^{1}H NMR spectra as a function of time after the addition of HOCbl (2.96 mM) to DEA-NONOate (4.44 mM) at pD 11.30 (25.0 ^{o}C 0.30 M Na_2CO_3, I = 1.0 M ($NaCF_3SO_3$)). Arrows indicate disappearance of HOCbl (↓, δ = 7.17, 6.70, 6.49, 6.23 and 6.04) or the appearance of NOCbl (↑, δ = 7.44, 7.27, 6.80, 6.35 and 6.25).[4,5]

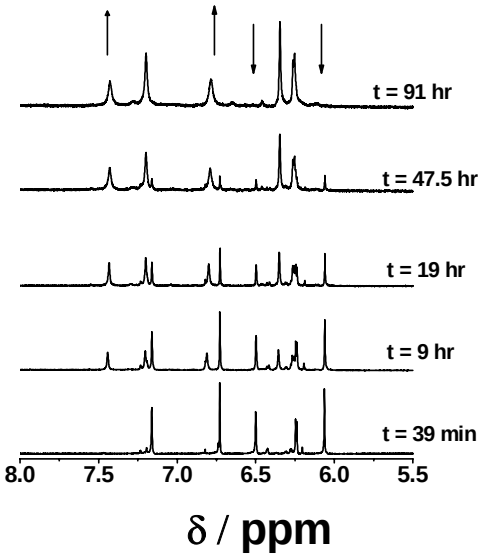


Figure S2. Plot of observed rate constant, k_{obs}, versus DEA-NONOate concentration for the reaction between HOCbl and DEA-NONOate (I = 1.0 M, $NaCF_3SO_3$, 25.0 °C). A) At pH 10.40. The best fit of the data to a line gives k_{app} (= slope) = 0.14 ± 0.01 M^{-1} min^{-1} and $10^3 k_{HOCbl}$ (= intercept) = 1.45 ± 0.01 min^{-1}. B) At pH 9.80. The best fit of the data to a line gives k_{app} = 0.29 ± 0.03 M^{-1} min^{-1} and $10^3 k_{HOCbl}$ = 1.97 ± 0.40 min^{-1}. C) At pH 9.50. The best fit of the data to a line gives k_{app} = 0.68± 0.02 M^{-1} min^{-1} with an insignificant intercept.

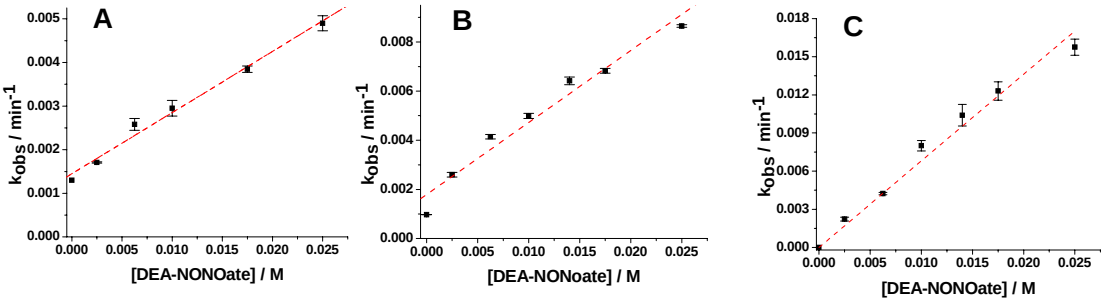


Figure S3. a) UV-vis spectra for the reaction between HOCbl (0.050 mM) and MAHMA-NONOate (5.00 mM) at pH 10.40, 25.0 °C (0.30 M CAPS, I = 1.0 M ($NaCF_3SO_3$)). Isosbestic points occur at 341, 370 and 498 nm. Inset: First and last spectra. The final product is NOCbl. b) Fit of the absorbance data at 356 nm versus time to a first-order reaction, giving k_{obs} = (7.85 ± 0.02) x 10^{-3} min^{-1}.

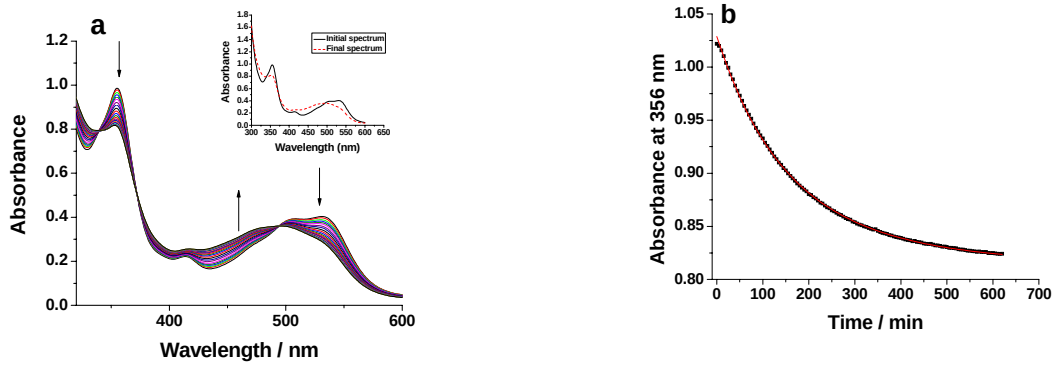
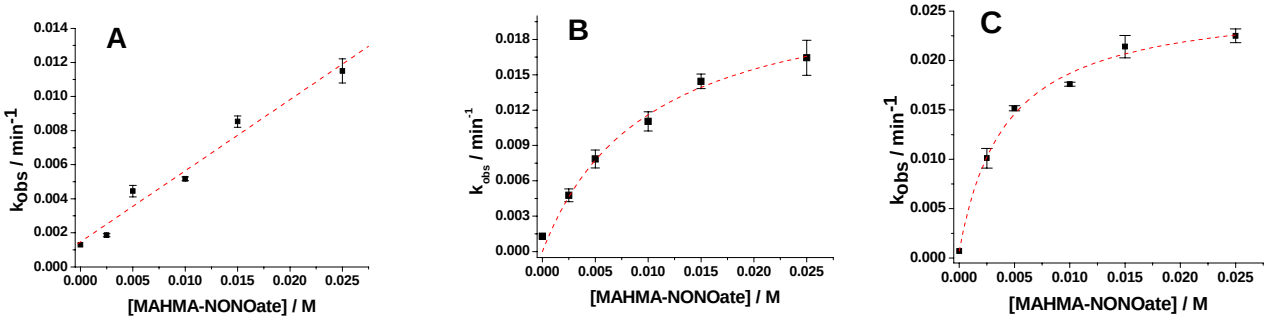


Figure S4. Plot of observed rate constant, k_{obs}, versus MAHMA-NONOate concentration for the reaction between HOCbl and MAHMA-NONOate (I = 1.0 M ($NaCF_3SO_3$), 25.0 °C). A) At pH 10.80. The best fit of the data to a line gives k_{app} = 0.42 ± 0.04 M^{-1} min^{-1} and $10^3 k_{HOCbl}$ = 1.32 ± 0.03 min^{-1}. B) At pH 10.40. C) At pH 9.80. Individual k_{obs} values were obtained from the best fit of the absorbance versus time data to eqtn(1) in the text. Observed rate constants, k_L, for spontaneous decomposition of MAHMA-NONOate were (3.42 ± 0.05) x 10^{-4} min^{-1} (pH 10.80), (9.31 ± 0.01) x 10^{-4} min^{-1} (pH 10.40) and (2.94 ± 0.01) x 10^{-3} min^{-1} (pH 9.80).



Studies on the reaction between HOCbl and DEA-NONOate at pH < 10: evidence for a second reaction. A second reaction was observed which became more prominent at lower pH and higher DEA-NONOate concentrations. Figure S5 gives a plot of absorbance at 586 nm versus time for the reaction between HOCbl (2.40 mM) and DEA-NONOate (3.50 mM) at pD 9.54. From this data it is clear that more than one reaction is occurring. The isosbestic wavelengths are practically unchanged during the entire reaction, suggesting that the NOCbl product may react further to ultimately regenerate the HOCbl reactant. However, given that the visible spectra of HOCbl and NO_2Cbl are so similar (Figure S6), this means that the isosbestic points for conversion between HOCbl and NOCbl (341, 370 and 498 nm, Figure 2) are practically the same as those for NOCbl/NO_2Cbl conversion (338, 371 and 497 nm); hence another possibility is that NOCbl is instead converted to NO_2Cbl.

In order to distinguish between these two possibilities, the reaction between HOCbl (2.40 mM) and DEA-NONOate (3.50 mM) was followed by ^1H NMR spectroscopy. The data is shown in Figure S6. A spectrum obtained ~147 min after mixing the reagents shows complete conversion of HOCbl to NOCbl within this time period, and the appearance of small signals attributable to NO_2Cbl (δ = 7.20, 6.70, 6.40, 6.26 and 6.16 [6]), which become more intense as the second reaction progresses, leading to ~ 50% conversion to NO_2Cbl after 26 hr. Therefore, once formed, NOCbl reacts further to give NO_2Cbl under these pH conditions.

Experiments were carried out to identify the species which reacts with NOCbl product to produce NO_2Cbl. Control experiments established that NOCbl is stable in the pH range of these studies (0.1 M TAPS, pD 8.46; no reaction by ^1H NMR spectrum after 24 hr). Given that DEA-NO is the other product of the reaction between HOCbl and DEA-NONOate, the reactivity of DEA-NO (20 mM) with NOCbl (3.7 mM) was studied at pD 8.46. No reaction was observed by ^1H NMR spectroscopy after 24 hr. DEA and NO are the products of the spontaneous acid-catalyzed decomposition of DEA-NONOate; hence the reactivity of NOCbl with DEA (20 mM) at pD 8.46 was also determined; once again no reaction had occurred after 24 hr. However NOCbl is completely converted to NO_2Cbl upon reacting NOCbl (3.7 mM) and DEA-NONOate (20 mM) at pD 8.46 (24 hr). Importantly, in alkaline solution, no reaction is observed between NOCbl and DEA-NONOate (20 mM, pD 11.1, 0.1 M carbonate buffer). This suggests that NO(g), rather than DEA-NONOate itself, reacts with NOCbl to ultimately form NO_2Cbl. To probe this further, NOCbl was added to a solution of DEA-NONOate which had been allowed to completely decompose to NO and DEA prior to the addition of NOCbl, and once again NO_2Cbl was formed. In order to directly confirm this, the reaction of NOCbl with NO(g) from a cylinder was studied at pH 8.46 and 11.1. Under both conditions, considerable conversion of NOCbl to NO_2Cbl was observed upon bubbling the solution with excess NO(g). Hence the observation of a second reaction for pH values < 10 can be attributed to the reaction of NO(g) derived from spontaneous acid-catalyzed decomposition of DEA-NONOate with the NOCbl product of the direct reaction between DEA-NONOate and H_2OCbl^+/HOCbl. The reaction of NO-Co(III) complexes with excess NO(g) to form the corresponding nitro complex has been previously reported.[7-9]

Figure S5. Plot of absorbance at 586 nm versus time for the reaction between HOCbl (2.40 mM) and DEA-NONOate (3.50 mM) at pD 9.54 and 25.0 °C (0.50 M CHES, I = 1.0 ($NaCF_3SO_3$)). The Cbl product of the first reaction, a, is orange NOCbl, with the expected characteristic spectral changes and isosbestic points. Allowing the reaction to proceed for longer time, b, results in the formation of a second, red Cbl product (= NO_2Cbl).

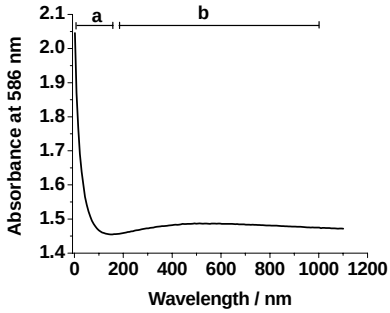


Figure S6. UV-visible spectra of (a) 0.050 mM H_2OCbl (solid line) and (b) 0.050 mM NO_2Cbl (0.3 M TAPS, pH 8, 25.0 °C).

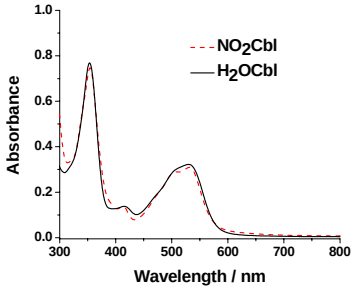
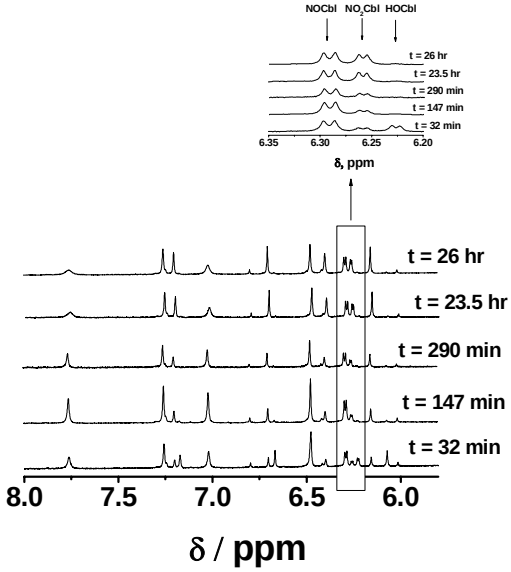


Figure S7. ^{1}H NMR spectra as a function of time after the addition of HOCbl (2.40 mM) to DEA-NONOate (3.50 mM) at pD 9.54 (25.0 °C 0.50 M CHES, I = 1.0, ($NaCF_3SO_3$)). Inset: 6.35-6.2 ppm region, showing the conversion of NOCbl (δ = 7.71, 7.27, 6.98, 6.45 and 6.30), to NO_2Cbl (δ = 7.20, 6.70, 6.40, 6.26 and 6.16).[6] The chemical shifts of the reactant HOCbl are at 7.17, 6.67, 6.48, 6.23 and 6.07 ppm.



Derivation of equation (1).

Let A = $HOCbl/H_2OCbl^+$ and L = R_2N-NONOate.

For A + L → A—L, $d[A]/dt = k_{app}[A][L]$

The ligand, R_2N-NONOate, also spontaneously decomposes:

L → product $\quad d[L]/dt = k_L[L]$

If [L] changes significantly during the reaction but the formation of the A—L complex does not contribute to its concentration change (i.e, the complex concentration is negligible compared to [L]), the following equation is valid for the decay of L:

$$[L] = [L]_0 e^{-k_L t}$$

Hence $$\frac{d[A]}{dt} = -k_{app}[A][L]_0 e^{-k_L t}$$

Integration of this equation leads to the following expression:

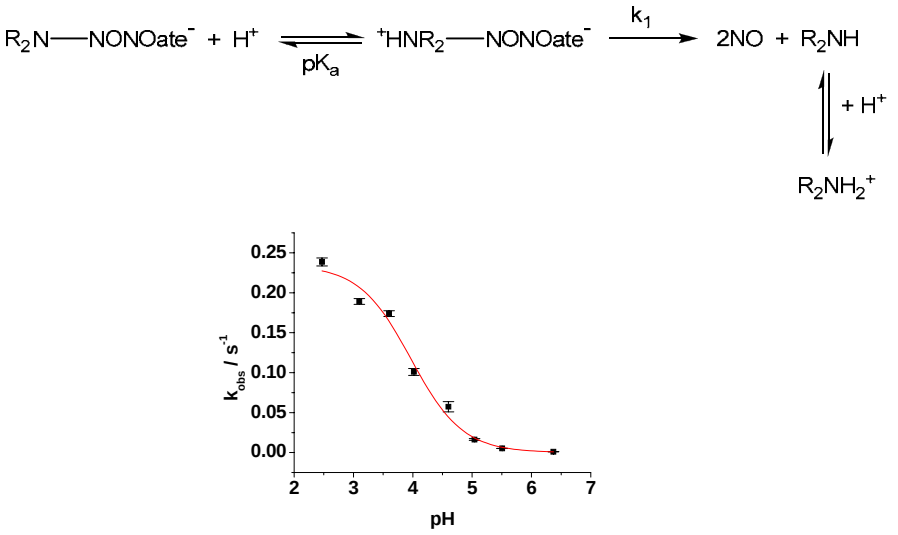
$$[A] = [A]_0 e^{\frac{k_{app}[L]_0}{k_L}(e^{-k_L t}-1)}$$

If the reaction is monitored by following the change in absorbance of the Cbl species as a function of time, then

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

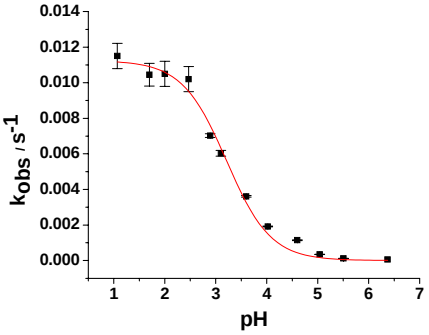
Determination of the rate constant and pK_a for spontaneous decomposition of DPTA-NONOate. Rate constants for the spontaneous decomposition of DPTA-NONOate as a function of pH (pH 2.47-6.37) at 37.0 °C were measured spectrophotometrically by following the decay in the DPTA-NONOate absorbance at 250 nm. Results of a control experiment indicated that the rate of decomposition was independent of the presence of air; hence these experiments were carried out under aerobic conditions. For pH > 3.1 the reaction was initiated by adding an aliquot (0.0500 mL, 6.00 mM) of a stock solution of DPTA-NONOate in 10 mM NaOH to a cuvette containing buffer (2.95 mL) which had been thermostated in the cell holder of the Cary 500 spectrophotometer. For pH < 3.1 rate constants were determined by stopped-flow spectroscopy by mixing equal volumes of a DPTA-NONOate solution in 1.0 mM NaOH, (0.200 mM) with buffer (0.1 M, I = 1.0 M with NaCl), using an Applied Photophysics RX.2000 Rapid Mixing Stopped-Flow unit in conjunction with the Cary 5000 spectrophotometer. NaH_2PO_4/Na_2HPO_4, acetic acid/sodium acetate and citric acid/sodium citrate buffers (0.10 M) were used, and the total ionic strength maintained at 1.0 M (NaCl). Absorbance versus time data fitted well to a first-order equation. The results are summarized in Figure S8.

Figure S8. Plot of observed rate constant versus pH for the acid-catalyzed spontaneous decomposition of DPTA-NONOate (37.0 °C, 0.10 M buffer, I = 1.0 M (NaCl)). The best fit of the data gives $k_1 = 0.23 \pm 0.01$ sec^{-1} and $pK_a = 3.96 \pm 0.13$.



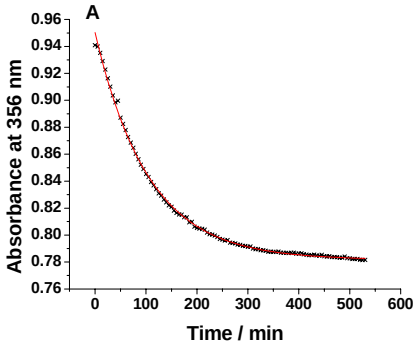
Determination of the rate constant and pK_a for spontaneous decomposition of DETA-NONOate. Rate constants for the spontaneous decomposition of DETA-NONOate as a function of pH (pH 1.07-6.37) at 37.0 °C were measured spectrophotometrically by following the decay in the DETA-NONOate absorbance at 252 nm under anaerobic conditions. The reaction was initiated by adding an aliquot (0.0500 mL, 6.00 mM) of a stock solution of DPTA-NONOate in 10 mM NaOH to a cuvette containing buffer (2.95 mL) which had been thermostated in the cell holder of the Cary 5000 spectrophotometer. NaH_2PO_4/Na_2HPO_4, acetic acid/sodium acetate, citric acid/sodium citrate buffers and HCl (0.10 M) were used, and the total ionic strength maintained at 1.0 M (NaCl). Absorbance versus time data fitted well to a first-order equation. The results are summarized in Figure S9.

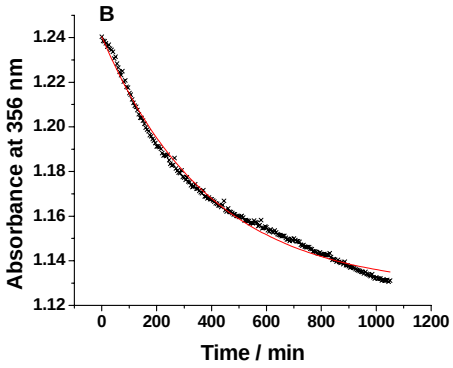
Figure S9. Plot of observed rate constant versus pH for the acid-catalyzed spontaneous decomposition of DETA-NONOate (37.0 °C, 0.10 M buffer, I = 1.0 M (NaCl). The best fit of the data gives $k_1 = (1.12 \pm 0.03) \times 10^{-2}$ s^{-1} and $pK_a = 3.21 \pm 0.10$.



The effect of nitrite on the rate of the reaction between HOCbl and MAHMA-NONOate.
To study the effect of nitrite on the reaction of HOCbl with MAHMA-NONOate, the rate of the reaction between HOCbl (0.050 mM) and MAHMA-NONOate (5.0 mM) was monitored at 356 nm in the absence and presence of 10.0 mM $NaNO_2$ at pH 9.80 (0.30 M CHES, I = 1.0 M ($NaCF_3SO_3$), 25 °C). In the absence of nitrite the data fits well to a first-order rate equation, giving $k_{obs} = (1.30 \pm 0.01) \times 10^{-2}$ min^{-1} (Figure S10). However in the presence of nitrite (10.0 mM), the fit of the data to a first-order equation is poor (Figure S16), and the reaction is much slower ($k_{obs} \sim 2.6 \times 10^{-3}$ min^{-1}). Hence, the presence of free nitrite slows down the apparent rate of reaction between HOCbl and MAHMA-NONOate.

Figure S10. Absorbance at 356 nm versus time for the reaction between A) HOCbl (0.050 mM) and MAHMA-NONOate (5.0 mM). The best fit of the data to a 1st-order equation gives $k_{obs} = (1.30 \pm 0.01) \times 10^{-2}$ min^{-1} and B) HOCbl (0.050 mM) and MAHMA-NONOate (5.0 mM) in the presence of 10 mM $NaNO_2$. The best fit of the data to a 1st-order equation gives $k_{obs} \sim (2.56 \pm 0.05) \times 10^{-3}$ min^{-1}; note that the fit is poor.





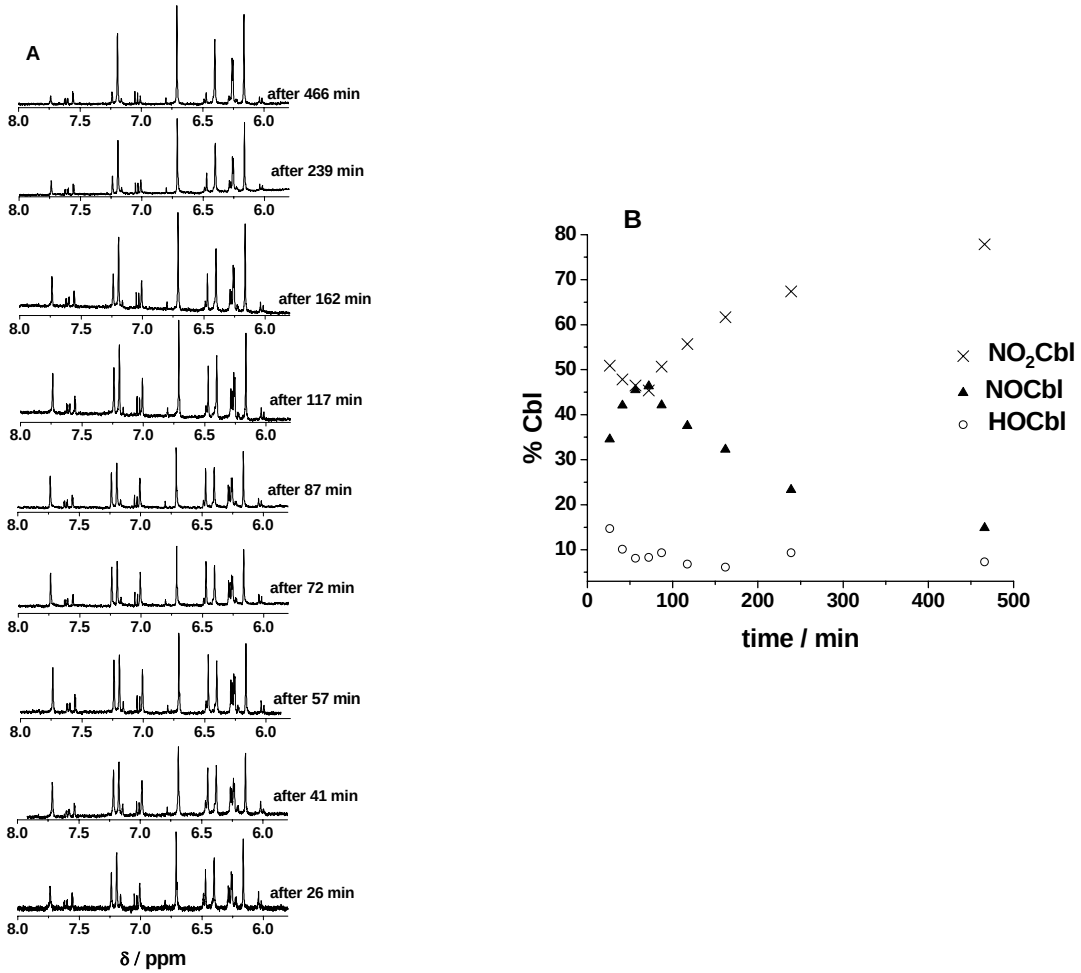
The effect of replacing HOCbl with NO_2Cbl on the rate of the reaction with MAHMA-NONOate.
To further probe the effect of nitrite on the reaction and to determine if NO_2Cbl reacts with MAHMA-NONOate under the pH conditions of this study, the rate of the reaction between authentic NO_2Cbl (0.050 mM) and MAHMA-NONOate (5.0 and 15 mM) was determined (pH 9.80, 0.30 M CHES, I = 1.0 M ($NaCF_3SO_3$), 25 °C)). In both cases the data fitted well to a 1st-order rate equation ($k_{obs} = 0.009 \pm 0.002$ min^{-1} and 0.013 ± 0.002 min^{-1} for 5.0 and 15 mM MAHMA-NONOate, respectively). However, the rate constants were significantly slower compared to those obtained using HOCbl (0.050 mM) as a reactant under the same conditions (0.015 and 0.022 min^{-1} for 5.0 and 15 mM MAHMA-NONOate, respectively). Hence substituting HOCbl for NO_2Cbl results in a significant decrease in the rate of the reaction.

Kinetic studies on the reaction between HOCbl and MAHMA-NONOate at pD 9.80 using ^{1}H NMR spectroscopy: evidence that HOCbl is rapidly converted to NO_2Cbl, which reacts further to give NOCbl.

The reaction between HOCbl (2.0 mM) and MAHMA-NONOate (10 mM) at pD 9.80 (0.30 M CHES buffer) was followed by ^{1}H NMR spectroscopy (Figure S11). A spectrum obtained ~26 min after mixing the reagents shows a mixture of ~ 15% HOCbl, ~50% NO_2Cbl and ~35% NOCbl. During the next ~50 min the amount of NOCbl increases at the expense of NO_2Cbl and HOCbl. This suggests that the majority of HOCbl reacts with nitrite ($t_{½} \leq 7$ min) to form NO_2Cbl in addition to reacting with MAHMA-NONOate to form NOCbl. At longer times the NOCbl product reacts with NO(g) produced by spontaneous NONOate decomposition to re-form NO_2Cbl.

The reaction mixture was also simultaneously monitored using UV-vis spectroscopy. The absorbance at 590 nm (which was chosen since the Cbl concentration is so high) first decreases due to the conversion of the HOCbl/NO_2Cbl mixture to NOCbl, followed by the slower reaction of the NOCbl product with NO(g) to re-form NO_2Cbl.

Figure S11. A) ^{1}H NMR spectra as a function of time for the reaction between HOCbl (2.0 mM) and MAHMA-NONOate (10 mM) at pD 9.80 (25 °C, 0.30 M CHES, I = 1.0 M ($NaCF_3SO_3$)). **1** = HOCbl (δ = 7.16, 6.71, 6.49, 6.23 and 6.04 ppm); **2** = NO_2Cbl (δ = 7.19, 6.71, 6.40, 6.25 and 6.16 ppm); **3** = NOCbl (δ = 7.73, 7.24, 7.01, 6.47and 6.29 ppm). **B)** Relative amounts of the 3 Cbl species determined by integration of the peaks in the δ 7.25-7.15 region.



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