Direct Reaction of Amides with Nitric Oxide To Form Diazeniumdiolates

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* Supporting Information

ABSTRACT: We report the apparently unprecedented direct reaction of nitric oxide (NO) with amides to generate ions of structure R(C≡O)NH–N(O)−NO−, with examples including R = Me (1a) or 3-pyridyl (1b). The sodium salts of both released NO in pH 7.4 buffer, with 37 °C half-lives of 1−3 min. As NO-releasing drug candidates, diazeniumdiolated amides would have the advantage of generating only 1 equiv of base on hydrolyzing exhaustively to NO, in contrast to their amine counterparts, which generate 2 equiv of base.

Many secondary and primary amines react with nitric oxide (NO) to generate products of structure R'N−N(O)=NO−, known as diazeniumdiolate ions. Extensive structure/reactivity studies involving a wide variety of amines were carried out by the Drago research group a half a century ago, showing the remarkable generality of this amine-derivatizing reaction. Interestingly, they did not describe any attempts to react the corresponding amides with NO, nor have we seen any such reports in the intervening literature. We have long taken this absence of relevant prior literature as a tacit suggestion that attempts to diazeniumdiolate an amide by directly reacting it with NO would probably be fruitless, until now continuing our focus on the amine/NO adducts that have proven so useful as biomedical research tools and potential clinical entities.

Here we show that this skepticism was unfounded by reporting on the direct reaction of NO with amides under basic conditions to produce novel ionic diazeniumdiolates, an apparently unprecedented transformation (see eq 1).

Treatment of methanolic acetamide with 3 atm of NO in the presence of sodium methoxide led to deposition of a white solid with an ultraviolet absorbance maximum at 252 nm, consistent with the presence of a diazeniumdiolate group [i.e., a group of structure X−N(O)=NO−] and suggesting that the reaction had led to diazeniumdiolate ion 1a, as in eq 1.

The product proved relatively stable in basic solutions but decomposed on lowering the pH to 7.4 with a half-life at 37 °C of 1.2 min. Abundant NO was seen as a gaseous product of this reaction. In addition, significant yields of nitrite ion were observed. The results for the nitrogenous products are summarized in Table 1. The organic products were analyzed by NMR of the spent reaction mixture. Acetamide was detected in 78% yield, and the sodium acetate yield was close to 22%.

Similar results were seen when the experiment was repeated using nicotinamide instead of acetamide. Again, the product was stable in base but on neutralization smoothly lost its UV peak at 250 nm, suggestive of the diazeniumdiolate structure 1b with a half-life of 2.8 min at pH 7.4 and 37 °C. The relative distribution of nitrogenous products was similar to that seen in the case of acetamide in that they are primarily NO and nitrite, though in this case, some N2O was seen (Table 1). Nicotinamide was recovered in 55% yield, similarly to that of the NO (52%) and nicotinic acid (45% of theory).

The two ionic amide diazeniumdiolates can be compared to the primary amine analogue IPA/NO (Scheme 1), where the acyl groups of ions 1 are replaced with an alkyl residue. Although the

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Table 1. UV Spectral Data Plus Rates and Yields of Nitrogenous Products on Hydrolyzing Ions 1 at pH 7.4 and 37 °C: Comparison with IPA/NO

| compound | R | NO| N₂O | nitrite | t₁/₂ (min) | λmax (nm) 0.1 M NaOH | ε (mM⁻¹ cm⁻¹) 0.1 M NaOH |
|----------|---|---|-----|--------|-----------|---------------------|------------------------|
| 1a       | Me | 88% (±1%) | <0.01 | 9% (±0.3%) | 1.2 | 252 | 9.7 |
| 1b       | 3-pyridyl | 52% (±1%) | 7% (±0.7%) | 10% (±1%) | 2.8 | 250 | 11.8 |
| IPA/NO   | 30% | 70% | 3% | 2.3₁⁵ | 252²⁵ | 8.7²⁵ |

"Yield as a percent of theoretical plus or minus the standard deviation of three independent measurements. ²From Maragos et al.¹²"

Scheme 1. Structural Comparison of Ionic Diazieniumdilated Amides 1a and 1b to the Primary Amine Diazieniumdilate IPA/NO

three compounds have similar stability profiles, they differ greatly in the relative yields of their nitrogenous products (Table 1). As presented above, both 1a and 1b primarily generate NO and nitrite with very little N₂O production. In contrast, IPA/NO decomposes via two pathways running in parallel; the minor pathway regenerates starting materials from its synthesis (isopropylamine + 2 equiv of NO), while the major pathway produces a mole each of HNO and isopropanediazoate ion.¹¹

The ionic primary amine diazeniumdilate IPA/NO can be dialkylated in two successive steps, first at the terminal O and then at the amine N.¹³ Ion 1a can also be dialkylated, but it differs from IPA/NO in that the intermediate monoalkyl species could not be isolated. An additional difference between 1a and IPA/NO is that the former could theoretically be alkylated at the amide oxygen, a functionality absent in IPA/NO (Scheme 2). No evidence for alkylation at that position was seen, however, as X-ray crystallography confirmed the O,N-dialkylated structure 2 in Scheme 2.

Scheme 2. Benzylation of Ion 1a Produced the O,N-Dialkylated Derivative 2 (O,O'-Dialkylated Structure Was Not Observed)

Salt 1b could be dialkylated with methyl iodide and benzyl bromide (Scheme 3). To our surprise, the dialkylation products isolated after normal aqueous workup, which we expected to have structure 3, contained no halide ion, as evidenced by mass spectrometry and absence of a precipitate on addition of the silver(I) ion to their aqueous solutions. Molecular weight measurements of the dibenzylation products suggested that the N−H proton had been lost during workup, forming zwitterions 4b and 4b', as shown in Scheme 3, and this was confirmed by X-ray crystallography of the dibenzyl derivative.

We were surprised to find no prior literature documenting a successful reaction of NO directly with an amide. The only product of such a hypothetical reaction that we could locate had the constitution of N-diazieniumdilated acetanilide ion, but it was reportedly generated in a radical-mediated side reaction in the decomposition of N-nitrosacetanilide.¹⁴ (Several N-diazieniumdilated carbamates have also been prepared by routes that bypassed the ionic structure 1 by first O-alkylating the terminal oxygen of IPA/NO to make a neutral species and then acylating the NH group.¹⁵,¹⁶)

Mechanistically, by analogy with the pathways established for the primary amine diazeniumdolates, we assume that the decomposition of ions 1 consists of two general mechanisms, one being a major, or in the case of 1a exclusive, pathway leading to regeneration of the two NO molecules, with the starting amide being the organic product. A concurrent pathway, minor for 1b but not seen for 1a, begins with cleavage of HNO (followed by its dimerization to N₂O) and production of an acyldiazoate ion (which hydrolyzes to the carboxylic acid/carboxylate with loss of N₂), as shown in Scheme 4.

From the observed stoichiometry, 1a appears to dissociate exclusively by the NO release pathway shown at the top of Scheme 4, as no N₂O was detected. The NO and acetamide yields were roughly similar, as anticipated (88 and 78%,

Scheme 3. Diazeniumdilation of Nicotinamide, Followed by Dialkylation To Form Zwitterions 4

Scheme 4. Proposed Mechanisms of Dissociation of Diazieniumdilated Amide Ions 1 at Physiological pH (See Salmon et al.¹³ for a Full Mechanistic Description of IPA/NO Hydrolysis, Including pH Effects)
diazonium dialylated amides produces a nonbasic amide, while exhaustive hydrolysis of secondary amine diazeniumdiallates produces a still basic amide that consumes an additional proton. This property would be especially beneficial in a biomedical setting, where careful pH control is essential.

The results suggest that a variety of useful NO-releasing hybrid drugs might be forthcoming from a systematic study of reactions of different bioactive amides with NO as described above.

**Experimental Section**

**Caution:** IPA/NO and certain other diazeniumdiallates have shown a tendency to dissociate rapidly and unexpectedly and hence should be handled with due care.

**General.** Ultraviolet (UV) spectra were recorded on a diode array spectrophotometer. Nuclear magnetic resonance (NMR) spectra were collected with a 400 MHz spectrometer using appropriate deuterated solvents with chemical shifts reported in parts per million downfield from tetramethylsilane or 2,2,3,3-tetradeuterotrimethylsilylpropionic acid. Ions for high-resolution mass spectrometry were generated with electrospray ionization and detected with a quadrupole time-of-flight mass analyzer.

**Purity Measurements for Salts 1a and 1b.** Ionic diazeniumdiallates are notoriously difficult to purify, so it is correspondingly problematic to provide quantitative data on the properties of the above-identified products. Nevertheless, rough purity estimates could be derived from NMR experiments using internal standards in alkaline solutions. The result for 1a using potassium phthalimide as internal standard was a purity level of 50%, with the remainder presumably consisting of NaOH and other UV- and NMR-silent constituents. The purity of the 1b sample was similarly determined to be 80% using tert-butyl alcohol as internal standard. These values were used as normalization factors to establish the extinction coefficients as well as theoretical yields of the various products summarized in Table 1.

**Sodium 1-(N-Acetylamino)diazene-1-ium-1,2-diolate (1a).** A solution of 5.9 g (0.1 mol) of acetamide in 21.7 mL (0.1 mol) of 4.6 M methanolic sodium methoxide, 10 mL of methanol, and 50 mL of diethyl ether was charged with 40 psi of nitric oxide and stirred under pressure at room temperature. A precipitate began to be visible within 2 h; stirring was continued overnight, giving a thick white precipitate. The precipitate was collected by filtration, washed with acetone, and dried under vacuum to give 3.07 g of a white powder that was 50% pure according to the NMR/internal standard method described above (10% yield). As a further purification step, 2 g of crude 1a (50% pure, 7.1 mmol) was dissolved in 100 mL of methanol and stirred at room temperature. The methanolic solution was treated with 100 mL of ether, giving a white solid that was collected by filtration and triturated with acetone, then dried in vacuo to give 1.7 g of product. This process removed most of the acetamide contaminant: UV (in 0.01 M NaOH) \( \lambda_{max} \) 252 nm (9.7 mM\(^{-1}\) cm\(^{-1}\)); \( ^1H \) NMR (D\(_2\)O/NaOD) \( \delta \) 1.68 (s); \( ^13C \) NMR (D\(_2\)O/NaOD) \( \delta \) 20.8, 171.

**Sodium 1-(N-Nicotinoylamino)diazene-1-ium-1,2-diolate (1b).** A solution of 9.1 g (0.075 mol) of nicotinamide in 30 mL of methanol was treated with 15.2 mL (0.07 mol) of 4.6 M methanolic sodium methoxide. To the solution was added 50 mL of ether, after which the vessel was degassed and charged with 50 psi of nitric oxide. The solution was stirred at room temperature; after 72 h, the pressure was released and the white precipitate was collected by filtration. The solid was washed with acetone and dried under vacuum to give 3.91 g of 1b (80% pure by the internal standard NMR method described above, 20% yield) as a powder: UV (0.01 M NaOH) \( \lambda_{max} \) 250 nm (11.8 mM\(^{-1}\) cm\(^{-1}\)); \( ^1H \) NMR (D\(_2\)O/NaOD) \( \delta \) 7.50–7.56 (m, 1H), 8.23–8.28 (m, 1H), 8.59–8.63 (m, 1H), 8.93–8.96 (m, 1H); \( ^13C \) NMR (D\(_2\)O/NaOD) \( \delta \) 123.8, 132.6, 136.4, 147.7, 150.0, 171.4.

**Reaction of 1a with Benzyl Bromide To Form 2a.** To a slurry of 2.52 g of 1a (0.089 mmol) and 2.08 g of anhydrous sodium carbonate in 10 mL of DMF at 0 °C was added 438 \( \mu L \) (3.6 mmol) of benzyl bromide; the resulting slurry was stirred at 0 °C with gradual warming to room temperature. After being stirred overnight, the reaction mixture was cooled to –80 °C and placed in a lyophilizer; on evaporation, the solid residue was extracted with dichloromethane. The organic layer was dried over sodium sulfate, filtered through a layer of magnesium sulfate, and evaporated to give 229 mg of oil. The crude product was chromatographed on a 25 g silica gel column and eluted with a gradient from 100% methylene chloride to 70:30 methylene chloride/ethyl acetate to give 102 mg (38%) of pure 2a.

**Reaction of 1b with Dimethyl Sulfate To Form 4b.** Compound 1b (1.34 g, 80% pure, 5.3 mmol) was dissolved in 50 mL of cold methanol (0 °C) and reacted with dimethyl sulfate (1.52 mL, 16 mmol) as above to give 1.8 g of an orange oil. The resulting crude product was chromatographed on silica gel using a gradient from 100% dichloromethane to 50:50 dichloromethane/methanol as the eluent. A fraction containing 296 mg of product was concentrated, whereupon the residual oil crystallized on standing. Recrystallization from methanol gave 259 mg of product. The resulting white precipitate was collected by filtration, washed with acetone, and dried under vacuum to give 93 mg of a white solid that was 30% pure by the internal standard NMR method described above, 5% yield) as a powder: UV (0.01 M NaOH) \( \lambda_{max} \) 244 nm (8.0 mM\(^{-1}\) cm\(^{-1}\)); \( ^1H \) NMR (D\(_2\)O/NaOD) \( \delta \) 7.50–7.56 (m, 1H), 8.23–8.28 (m, 1H), 8.59–8.63 (m, 1H), 8.93–8.96 (m, 1H); \( ^13C \) NMR (D\(_2\)O/NaOD) \( \delta \) 123.8, 132.6, 136.4, 147.7, 150.0, 171.4.

**Scheme 5. Hydrolysis of Diazonium Diallylated Amines To Produce NO Consumes Two Protons, While That of the Corresponding Amide Derivatives Consumes Only One**

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**Kinetic Studies.** Kinetic experiments were performed at 37 °C using a standard UV–visible spectrophotometer. Reactions were initiated by addition of substrate to a preheated cuvette containing the buffer. Analyte concentration was measured in 100 μM 0.1 M phosphate buffer, pH 7.4, containing 50 μM diethylentriamine pentaacetic acid (DTPA). In each experiment, the data were analyzed at the λₘₜₐₓ and the rate was derived by fitting the data to an exponential curve typical for first-order processes.

**Analysis for NO.** Chemiluminescence detection and quantification of NO evolving from the reactions were conducted using a commercial nitric oxide analyzer. A pH 7.4 solution of 0.1 M phosphate buffer with 50 μM DTPA was sparged with inert gas until a steady detector response was established. Compound 1a, 1b, or IPA/NO was added to a final concentration of 100 μM and the NO release profile was followed over time after injection. The resulting curve was integrated to quantify the amount of NO released/mol of compound.

**Griess Assay Test for Nitrite Detection.** Each reaction was allowed to proceed to completion, according to the protocol above, in the absence of purging with inert gas. We then added 100 μL of Griess reagent, 300 μL of sample, and 2.6 mL of deionized water together in a spectrophotometer cuvette. We incubated the mixture for 30 min at room temperature and prepared a reference sample by mixing 100 μL of Griess reagent and 2.9 mL of deionized water. The absorbance of the nitrite-containing sample at 548 nm relative to the reference sample was measured.

**N₂O Measurements by Gas Chromatography.** The gas chromatographic reactions were run according to the conditions stated above. The gas chromatography was performed using an electron capture detector, equipped with a 370 MBq source. A packed column (2 m x 2.0 mm i.d.) was used with helium as the carrier gas. The GC operation conditions were as follows: injector and detector temperatures were at 250 °C; oven temperature was programmed from 90 to 200 °C at 20 °C/min and held at 200 °C for 1.1 min; helium flow was 30 mL/min; and nitrogen was used as makeup gas at 2 mL/min.

**Organic Product Analysis.** The organic products of the aqueous decomposition of 1a and 1b were analyzed by NMR upon exhaustion of the nitrosating reagents in a deuterated phosphate buffer under the conditions listed above.

**X-ray Crystal Data on Compounds 2 and 4b.** Single-crystal X-ray diffraction data on compounds 2 and 4b were collected using Mo Kα radiation and a Bruker APEX-2 CCD area detector. Crystals were prepared for data collection by coating with high viscosity microscope oil. The oil-coated crystal was mounted on a micromesh mount and transferred to the diffractometer. The structures were solved by direct methods and refined by full-matrix least-squares on F² values using the programs found in the SHELXTL suite (Bruker, SHELXTL v6.01/2000, Bruker AXS Inc., Madison, WI). Corrections were applied for Lorentz, polarization, and absorption effects. Parameters refined included atomic coordinates and anisotropic thermal parameters for all non-hydrogen atoms. Hydrogen atoms on carbons were included using a riding model (coordinate shifts of C applied to H atoms) with the C–H distance set at 0.96 Å. Complete information on data collection and refinement is available in the Supporting Information.

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