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Mechanisms of NO Release by N^1-Nitrosomelatonin: Nucleophilic Attack versus Reducing Pathways

Pablo M. De Biase, Adrián G. Turjanski, Darío A. Estrin, and Fabio Doctorovich*

Departamento de Química Inorgánica, Analítica y Química Física/INQUIMAE, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires/CONICET, Ciudad Universitaria, Pab. II, P. 3, C1428EHA Buenos Aires, Argentina

doctorovich@qi.fcen.uba.ar

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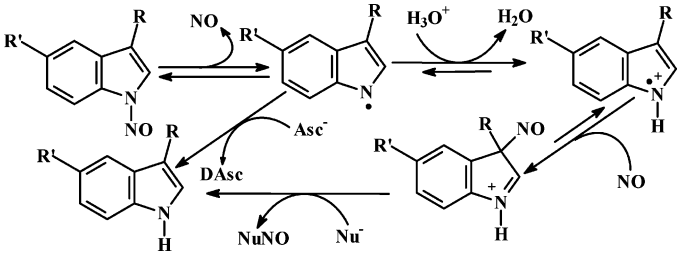

A new type of physiologically relevant nitrosamines have been recently recognized, the N^1-nitrosoindoles. The possible pathways by which N^1-nitrosomelatonin (NOMel) can react in physiological environments have been studied. Our results show that NOMel slowly decomposes spontaneously in aqueous solution, generating melatonin as the main organic product ($k = (3.7 \pm 1.1) \times 10^{-5}$ s^{-1}, Tris−HCl (0.2 M) buffer, pH 7.4 at 37 °C, anaerobic). This rate is accelerated by acidification ($k_{\text{pH 5.8}} = (4.5 \pm 0.7) \times 10^{-4}$ s^{-1}, $k_{\text{pH 8.8}} = (3.9 \pm 0.6) \times 10^{-6}$ s^{-1}, Tris−HCl (0.2 M) buffer at 37 °C), by the presence of O_2 ($k_o = (9.8 \pm 0.1) \times 10^{-5}$ s^{-1}, pH 7.4, 37 °C, [NOMel] = 0.1 mM, $P(O_2) = 1$ atm), and by the presence of the spin trap TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl; $k_o = (2.0 \pm 0.1) \times 10^{-4}$ s^{-1}, pH 7.4, 37 °C, [NOMel] = 0.1 mM, [TEMPO] = 9 mM). We also found that NOMel can transnitrosate to L-cysteinate, producing *S*-nitrosocysteine and melatonin ($k = 0.127 \pm 0.002$ M^{-1} s^{-1}, Tris−HCl (0.2 M) buffer, pH 7.4 at 37 °C). The reaction of NOMel with ascorbic acid as a reducing agent has also been studied. This rapid reaction produces nitric oxide and melatonin. The saturation of the observed rate constant ($k = (1.08 \pm 0.04) \times 10^{-3}$ s^{-1}, Tris−HCl (0.2 M) buffer, pH 7.4 at 37 °C) at high ascorbic acid concentration (100-fold with respect to NOMel) and the pH independence of this reaction in the pH range 7−9 indicate that the reactive species are ascorbate and melatonyl radical originated from the reversible homolysis of NOMel. Taking into account kinetic and DFT calculation data, a comprehensive mechanism for the denitrosation of NOMel is proposed. On the basis of our kinetics results, we conclude that under physiological conditions NOMel mainly reacts with endogenous reducing agents (such as ascorbic acid), producing nitric oxide and melatonin.

Introduction

Nitric oxide (NO) represents the biologically important form of the endothelium-derived relaxing factor (EDRF).[1] Under physiological conditions, NO directly activates soluble guanylyl cyclase (sGC) to transform guanosine triphosphate (GTP) into cyclic guanosine monophosphate (GMP), followed by kinase-mediated signal transduction. The endogenous formation of NO plays a key role in many bioregulatory systems including platelet inhibition, vasodilation, and smooth muscle relaxation.[2,3] Even though the normal concentration of NO in mammals is on the order of nanomolar,[4] the local concentration can be increased substantially, facilitating the formation of

* To whom correspondence should be addressed. Fax: 54-11-4576-3341.

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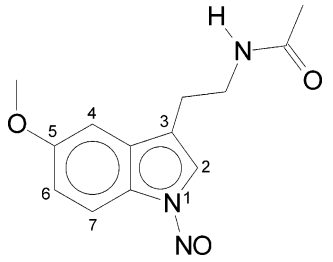

FIGURE 1. N^1-Nitrosomelatonin (NOMel) structure.

N_2O_3, a potent nitrosating agent.[5] The nitrosation reaction introduces a nitroso group into an organic molecule, leading to the formation of *C*-nitroso, *N*-nitroso, *O*-nitroso, or *S*-nitroso derivatives of the parent molecule. The formation of *S*-nitrosothiols is now generally believed to be of high physiological importance in vivo due to the capability of these compounds to activate sGC.[6]

Recently, a new type of physiologically relevant nitrosamines have been recognized, the N^1-nitrosoindoles. The nitrosation of tryptophan has been studied in proteins and model peptides, and it has been proposed that it can compete with cysteine for NO.[5] In previous works we have characterized the novel compound N^1-nitrosomelatonin (NOMel) (Figure 1)—formed by the reaction of NO with the naturally occurring hormone melatonin (Mel).[7−9] The decomposition of NOMel under normoxic solutions has been described to be a pH-dependent first-order reaction with a rate constant in aqueous phosphate buffer at pH 7.4 and 25 °C of $(7.0 \pm 0.5) \times 10^{-5}$ s^{-1}.[10] NOMel and N^1-nitroso-*N*-acetyltryptophan (NOTrp) are able to release NO in the presence of reducing agents such as ascorbic acid and NADH (reduced nicotinamide adenine dinucleotide).[5,10] This was also observed for *S*-nitrosoglutathione.[5,11] On the other hand, transnitrosation reactions of N^1-nitrosamines with thiols[12,13] and *S*-nitrosothiols with amines[14] have been reported. In view of the physiological significance of these compounds, their stability and the possible mechanisms of NO release deserve further investigation.

In the present work we have studied the denitrosation mechanism of NOMel as a model for N^1-nitrosoindoles. We have thoroughly characterized both theoretically and experimentally the spontaneous decomposition of NOMel, its reaction with reducing agents such as ascorbic acid, and the transnitrosation reaction between NOMel and the thiol L-cysteine (Cys). Our results provide a complete picture of the mechanisms involved in NO release by this new type of physiologically relevant nitroso compound.

Methods

Computational Procedures. The calculations were performed at the DFT level using the GAUSSIAN 98 software package.[15] All species where fully optimized at the B3LYP/6-31G** level. Solvent effects were modeled using the polarized continuum model (PCM) scheme. The PCM implementation given in ref 16, in which the self-consistency between the solute wave function and solvent polarization is achieved during the self-consistent field cycle, has been employed. The Gibbs free energy for solvation in water was obtained by making single-point calculations using PCM at vacuum-optimized geometries. The thermal contributions at 37 °C to the free energies were computed using standard statistical mechanics, the harmonic oscillator, rigid rotor, and ideal gas approximations. The frequency analysis predicted the absence of imaginary frequencies for all the optimized species.

Experimental Procedures. i. General Comments. Unless otherwise noted, all manipulations were performed with exclusion of oxygen using standard Schlenk procedures or septa; Ar was used as an inert gas. ^{1}H NMR spectra were recorded using 500 and 200 MHz spectrometers. Nitric oxide was detected with a specific electrode.

ii. Reagents. Distilled acetonitrile and ethanol and recrystallized ascorbic acid were used. TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl), NADH, L-cysteine, and deuterated solvents (benzene, acetonitrile, methanol, and water) were used as received. N^1-Nitrosomelatonin was prepared from melatonin purchased from Drogueria Saporiti SACIFIA, Buenos Aires, Argentina. The water used in all reactions was MilliQ water obtained from deionized water.

iii. N^1-Nitrosomelatonin Preparations. Method A. N^1-Nitrosomelatonin was generated by bubbling a 0.2 M melatonin solution in acetonitrile or ethanol with nitric oxide and oxygen. Nitric oxide was generated by the reaction of sodium nitrite and ferrous sulfate in acidic medium. The solution was bubbled for 30 min, adding NO and O_2 in stoichiometric amounts ($NO:O_2 = 4:1$). Finally, the system was purged with argon and cooled to 0 °C. After the addition of cold water the product precipitated. The so-obtained yellow powder was filtered, washed with water, and dried. The isolated yield was about 70%. The purity was checked by TLC and ^{1}H NMR (CD_3CN) (ppm): 1.81 (s), 1.84 (s), 2.10 (s), 2.12 (s), 2.86 (m), 3.50 (m), 3.81 (s), 3.87 (s), 6.47 (sa), 6.87 (s), 6.96 (dd), 7.08 (dd), 7.18 (dd), 7.63 (s), 7.81 (s), 8.01 (s), 8.05 (s), 8.22 (s), 8.26 (s).

Method B. NOMel was synthesized by adding a 2-fold excess of ethyl nitrite to a deoxygenated 0.2 M melatonin solution in ethanol. Finally, the same procedure as in method A was followed to obtain the purified product. The isolated yield was about 80%.

iv. Kinetics. Rate measurements involving N^1-nitrosomelatonin were done in spectrophotometric cells at 37 °C, measuring the disappearance of the absorption at 346 nm (ϵ

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TABLE 1. Observed Rate Constants (k_o) for NOMel Decomposition in Diverse Media at 37 °C

k_o (10^{-5} s^{-1})	pH	medium
2.2 ± 0.1	6.80[a]	distilled and degassed water with 1.5% v/v acetonitrile
3.7 ± 1.1	7.40	0.2 M Tris−HCl buffer in MilliQ water with 0.8% v/v ethanol
14.0 ± 0.1	7.40	0.2 M phosphate buffer in water with 0.8% v/v ethanol
85.2 ± 1.3	7.22	1.0 M phosphate buffer in water with 1.7% v/v acetonitrile

[a] Initial pH, measured at 25 °C, all others at 37 °C.

= 8616 ± 300 M^{-1} cm^{-1}). The observed rate constants (k_{obs}) for the spontaneous decay process, the reaction with TEMPO, O_2, cysteine, and ascorbic acid, were determined by initial rates or by the integral method, showing that all kinetics were first-order with respect to NOMel concentration. These reactions were carried out in Tris−HCl or phosphate aqueous buffered solutions with 0.8% v/v ethanol or less than 1.7% v/v acetonitrile, respectively.

v. *E*° Determinations. Voltammetry was carried out at 100 mV/s in water−ethanol (1:1) using potassium nitrate as the supporting electrolyte, and a three-electrode configuration (Pt working and counter electrodes, Ag/AgCl reference electrode). The working temperature was 25 °C, and the NOMel concentration used was about 1.5 mM. The cyclic voltammograms showed that the reduction was chemically irreversible.

Results and Discussion

Reaction of NOMel with Water and Other Nucleophiles. It has been reported that 3-substituted N^1-nitrosoindoles decompose spontaneously through a first-order reaction,[5,10,17] in the pH 0−7 range, yielding nitrous acid and the corresponding indole.[17] Moreover, it was found that in aqueous solution at about pH 7 the reactant is able to slowly release NO;[5] in the presence of oxygen NOMel decomposes, producing melatonin and oxidized melatonin derivatives (eq 2).[10] These last two pieces of evidence suggest that the reaction may involve a N−N homolytic bond dissociation step (eq 1).

$$\text{NOMel} \rightleftharpoons \text{NO} + \text{Mel}^{\bullet} \quad (1)$$

$$\text{Mel}^{\bullet} + \text{O}_2 \rightarrow \text{oxidized melatonin derivatives} \quad (2)$$

In Tris aqueous buffer, the spin trap compound TEMPO accelerated the observed rate constant (k_o) for NOMel decay (eq 3) ($k_o = (2.0 \pm 0.1) \times 10^{-4}$ s^{-1}, pH 7.4, 37 °C,

$$v = k_o[\text{NOMel}] \quad (3)$$

[NOMel] = 0.1 mM, [TEMPO] = 9 mM) as compared with the rate constant without TEMPO (Table 1). The decay rate in the presence of molecular oxygen was also accelerated ($k_o = (9.8 \pm 0.1) \times 10^{-5}$ s^{-1}, pH 7.4, 37 °C, [NOMel] = 0.1 mM, $P(O_2)$ = 1 atm). This rate was not significantly affected by raising the pH to 9. The above results support the intermediacy of free radicals in the reaction and therefore the homolytic bond dissociation

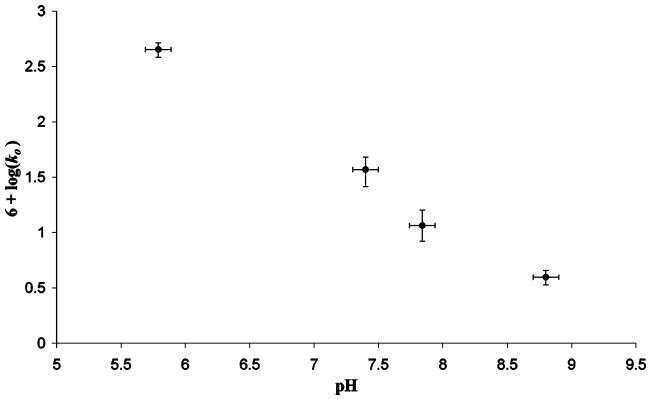

FIGURE 2. Effect of pH on the observed rate constant (k_o) for NOMel decay in Tris−HCl buffer at 37 °C. The solution has less than 1% ethanol to facilitate the dissolution of NOMel in water.

of NOMel in aqueous solution. However, the main products of the reaction (HNO_2 and Mel) cannot be explained in terms of this mechanism alone, indicating that other pathways are also operative.

NOMel spontaneous decomposition was carried out anaerobically in deuterated water−methanol mixtures (1:1), methanol, or benzene, avoiding light exposure. Products were characterized by ^{1}H NMR. In water−methanol mixtures, NOMel decomposed completely and only melatonin (Mel) was detected as the organic product by NMR (Figure SI1 in the Supporting Information). In contrast, in benzene and methanol, no reaction was observed after the same time (6 days). This result indicates that water plays an essential role in the anaerobic reaction, producing Mel and presumably nitrous acid (eq 4). In benzene and in methanol, the only operative reaction seems to be NOMel reversible N−N homolytic bond dissociation, so this equilibrium (eq 1) is displaced to the reactant side.

$$\text{NOMel} + \text{H}_2\text{O} \rightarrow \text{Mel} + \text{NO}_2^- + \text{H}^+ \quad (4)$$

The spontaneous decomposition of NOMel in water was followed by the decrease in NOMel absorbance at 346 nm under an argon atmosphere and avoiding light exposure. The reaction was studied in different buffer media to test the influence of the media over the reaction rate (Table 1). We found that in phosphate buffer the reaction is almost 4 times faster than in Tris−HCl buffer under the same conditions. This kind of catalysis by phosphate was also observed for the hydrolysis of N_2O_3.[18] We determined the observed rate constant (k_o) between pH 6 and pH 9 in Tris−HCl-buffered solutions at 37 °C (Figure 2). The reaction rate increases at low pH, indicating that protons play a role in NOMel decomposition. Similar results were seen for N^1-nitroso-*N*-acetyltryptophan (NOTrp) between pH 0 and pH 7.[17]

Furthermore, Meyer et al. studied the effect of nucleophiles (Nu^-'s) on the NOTrp decay rate at pH 6.[17] They observed that I^-, N^{3-}, SCN^-, Br^-, and Cl^-, in decreasing order of effectiveness, catalyze NOTrp decomposition. For the more powerful nucleophiles, the first-order depen-

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SCHEME 1. NOTrp Denitrosation Mechanism Proposed by Castro et al.[19]

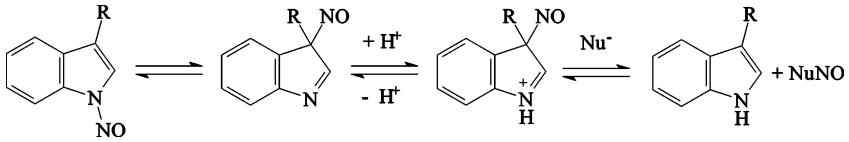

TABLE 2. Optimized Bond Distances and Mulliken Charges Obtained from DFT Calculations

	distance N−O (Å)	X	distance X−NO (Å)	δ^a
NO	1.159			0
NO^+	1.073			+1
NO^-	1.279			−1
1-NOMMIndole	1.220	N1	1.363	−0.227
1	1.145	N1	1.685	+0.275
2	1.199	N1	1.417	−0.082
3	1.134	C3	2.146	+0.276
4	1.135	C2	2.169	+0.258
5	1.129	N1	2.277	+0.311
6	1.227	N1	1.308	−0.086
7	1.340	N1	1.272	−0.205

[a] δ = Mulliken net charge over the NO group in water.

dence is soon lost when the nucleophile concentration is increased, and k_o tends to the same limiting value for each nucleophile. This indicates that an existing previous step limits the nucleophilic attack. Moreover, they observed that between pH 2 and pH 4 there is little change in k_o. This saturation was attributed to a protonation of NOTrp with a pK_a value of ca. 5.5;[17] however, this pK_a is much larger than expected for any possible protonation site in NOTrp that can lead to denitrosation. In another work, Castro et al. explained this effect, proposing a translocation of the NO moiety from position 1 to position 3 of the indolic ring (Scheme 1).[19] The 3-nitrosoindole so formed was suggested to be more susceptible to protonation than its precursor, the 1-nitrosoindole. In that way, the limiting step responsible for the saturation would be translocation. Finally, this protonated adduct loses NO^+, in a pathway catalyzed by Nu^-'s.

To get additional insight about the spontaneous decomposition mechanism, we performed DFT calculations on 1-nitroso-3-methyl-5-methoxyindole (1-NO-MMIndole) as a model compound. The *Z*-isomer and *E*-isomer of NOMMIndole are almost isoenergetic (data not shown). We searched for the existence of a protonated nitrosoindolic intermediate, optimizing various initial structures; the most relevant ones are depicted in Figure 3. In Table 2 there are some selected bond distances for these intermediates.

Following the changes in the Mulliken populations over the NO moiety and the N−O distance, as the X−NO distance increases the NO moiety acquires more NO^+ character (X = N, C; see Table 2). The intermediates **3**−**5** have the NO^+ bound to the aromatic electron cloud as a sort of π-complex, as has already been proposed.[19]

The stability of these intermediates was determined by estimating the free energy changes at 37 °C for the protonation process (eq 5, Table 3). As expected, solvation effects are very important for the charged species. On the basis of the $\Delta G°$ of protonation (water, 37 °C), the most plausible intermediate is **3** (3-NO-1-H-MMIndole$^+$).

$$\text{1-NO-MMIndole} + H_3O^+ \xrightarrow{\Delta G°} \text{H-NO-MMIndole}^+ + H_2O \quad (5)$$

A direct protonation does not explain the previous results in which it was observed that a limiting step prior to protonation exists.[17,19] Moreover, the DFT-calculated pK_a of intermediate **3** (−9.3) does not seem to be compatible with the observed results. In the range of pH in which we worked (pH 5.8−9.0), we found that the species most susceptible to protonation is the indolic radical Mel$^\bullet$ (Table 4), suggesting that the previous limiting step could be homolytic N−N bond dissociation of NOMel to Mel$^\bullet$ and NO, followed by protonation of the indolic radical.

On the basis of these results, a new mechanism for the denitrosation of NOMel, which in principle could be extended to other 3-substituted-nitrosoindoles, can be proposed (Scheme 2). In Figure 4, the free energy profile for the proposed mechanism is compared with that corresponding to mechanisms b′ and c′ proposed by Castro et al.[19] While they proposed NO translocation followed by protonation of the indole, our suggested pathway involves indole protonation prior to NO recombination. The formation of their proposed intermediate, 3-NO-MMIndole, is thermodynamically disfavored. Considering its equilibrium concentration, this adduct would be undetectable. On the other hand, the equilibrium concentration of MMIndole$^\bullet$ is considerably higher (0.1 μM for 0.1 mM nitrosoindole). Energetic values for each step are shown in Table SI1 in the Supporting Information.

Nucleophilic attack would take place in step d. The energetic value of this step was calculated considering water as the nucleophile. At a pH greater than 5, NO^+ carriers undergo a change to nitrite, averting the denitrosation reversibility (Scheme 2, step g). This new mechanism seems to be compatible with our results and with those reported previously.

NOMel Transnitrosation to L-Cysteine (Cys). The kinetics for the reaction of NOMel and Cys was measured in Tris−HCl (0.2 M) buffer, pH 7.4 at 37 °C, under an excess of Cys ([Cys] = 1.9 mM/10 mM) with respect to NOMel ([NOMel] = 100 μM). We found a global second-order reaction, first-order in NOMel and in Cys with a rate constant $k_r = 0.127 \pm 0.002\ M^{-1}\ s^{-1}$ at 37 °C. The first-order in Cys result was also verified under true second-order conditions, measuring initial rates at pH 9.0 where NOMel spontaneous decay is less significant ([Cys] = 0.19 mM/0.38 mM, [NOMel] = 0.1 mM, 37 °C, $k_r = 0.91 \pm 0.03\ M^{-1}\ s^{-1}$ determined using eq 7).

S-Nitrosocysteine (NOCys) was identified as a reaction intermediate—finally producing cystine—with a maximum in the absorption UV−vis spectra at 544 nm

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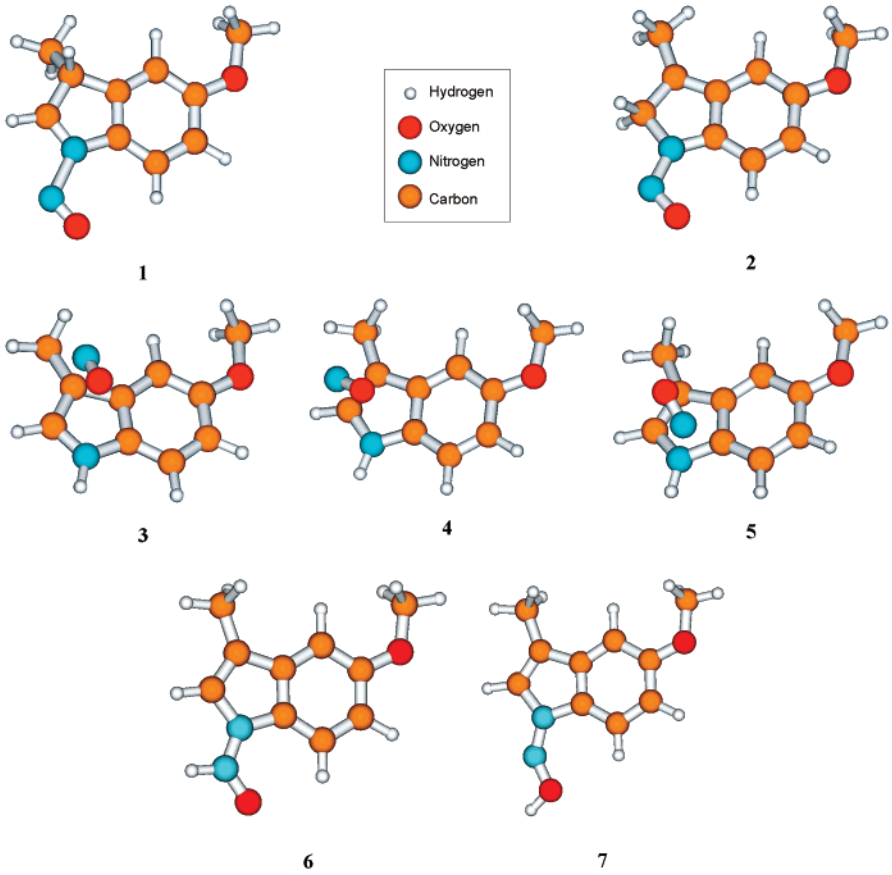

FIGURE 3. Optimized structures of the protonated intermediates: **1**, 1-NO-3-H-MMIndole$^+$; **2**, 1-NO-2-H-MMIndole$^+$; **3**, 3-NO-1-H-MMIndole$^+$; **4**, 2-NO-1-H-MMIndole$^+$; **5**, 1-NO-1-H-MMIndole$^+$; **6**, 1-NO-N-H-MMIndole$^+$; **7**, 1-NO-O-H-MMIndole$^+$.

TABLE 3. Computed DFT Energy and Free Energy Changes (kcal/mol) of the Protonation Process

HNOMMIndole$^+$	$\Delta E°$ + ZPE (vacuum, 0 K)	$\Delta G°$ (vacuum, 37 °C)	$\Delta G°$ (water, 37 °C)
1	−33.4	−33.5	25.2
2	−40.7	−41.1	13.0
3	−46.9	−47.0	10.7
4	−47.3	−47.8	11.6
5	−43.5	−43.4	16.8
6	−34.7	−33.7	26.4
7	−45.9	−44.9	19.8

TABLE 4. Experimental pK_a Values for Some Indole Radicals and Calculated pK_a Value for MMIndole•

radical	pK_a	radical	pK_a
indole	4.6[a]	tryptophan	4.3[a]
melatonin	4.5[a]	MMIndole	4.0[b]

[a] Experimental values from refs 20 and 21. [b] Calculated DFT value from $\Delta G°$ of Table SI1, step b for MMIndole.

($\epsilon_{544\ nm}$ = 14.9 M^{-1} cm^{-1})[22] (Figure 5). The reaction was carried out in Tris−HCl buffer at pH 6.4. Although NOCys has another maximum at 335 nm, it is superimposed with a NOMel absorption band. On the other hand, NOMel is completely converted to melatonin as measured by ^{1}H NMR (Figure SI2 in the Supporting Information). EDTA must be added to the reaction vessel to avoid NOCys fast decomposition catalyzed by Cu^{2+}.[23] When the EDTA concentration is decreased, a faster decay of the NOCys absorption band can be observed (Figure SI3 in the Supporting Information). At higher thiol concentrations, the formed NOCys quickly disappears due to a secondary reaction in which it reacts with cysteine, producing cystine (Figure SI2) and various nitrogenated products (N_2O, NH_2OH, and NH_3).[24,25]

Considering our results, we propose that the reaction involves transnitrosation between NOMel and Cys as has recently been studied by Sonnenschein et al.:[13]

$$\text{NOMel} + \text{Cys} \rightarrow \text{NOCys} + \text{Mel} \qquad (6)$$

Cysteine has five different protonation species that can exist in aqueous solution depending on the pH (Scheme 3).[26] It has been proposed that the transnitrosation reaction between NOCys and Cys involves nucleophilic attack by the thiolate species **10** and **12**.[24,26] To find out

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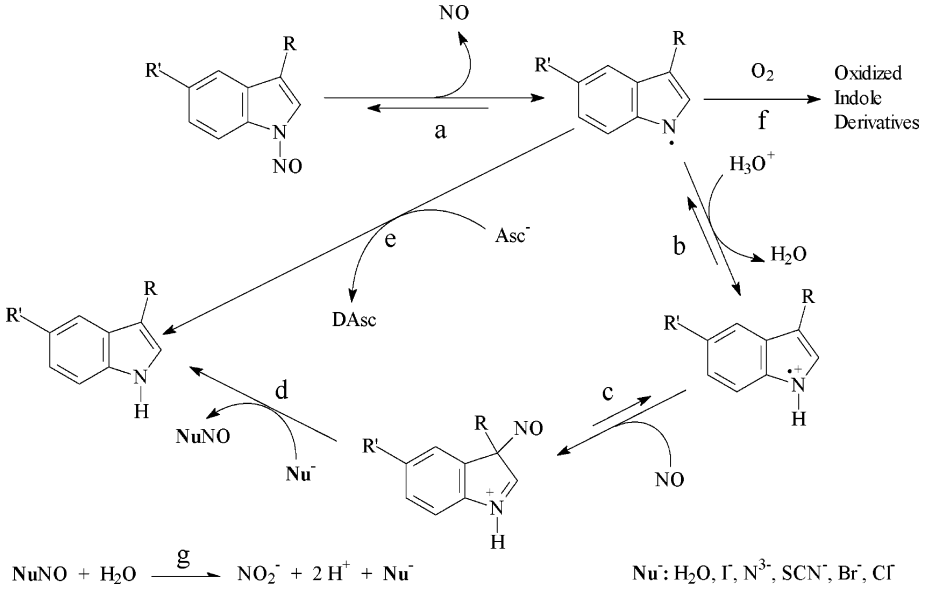
SCHEME 2. Proposed Denitrosation Mechanism for 3-Substituted 1-Nitrosoindoles

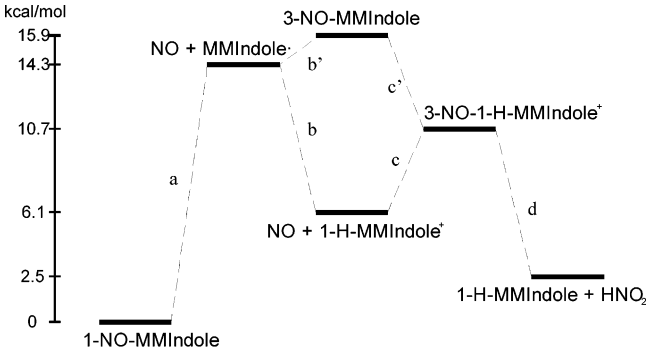


which thiol species are involved in the reaction, we studied the rate of reaction 6 as a function of pH. Cys solutions were prepared in phosphate-buffered solutions at pH 5.0, 7.4, 8.3, 9.9, and 12.4 at 37 °C; the NOMel: Cys ratio was about 1:600.

As under these conditions the spontaneous decomposition of NOMel competes with transnitrosation, we also measured the spontaneous decomposition of NOMel under the same conditions without Cys (Figure SI4 in the Supporting Information). The second-order rate constant for the reaction was established as follows:

$$k_r = (k_o - k_d)/[\text{Cys}] \qquad (7)$$

where k_r is the transnitrosation rate constant, k_{obs} is the observed pseudo-first-order rate constant, k_d is the spontaneous decomposition constant at the same conditions as k_{obs}, and [Cys] corresponds to the total cysteine concentration. The second-order rate constants (k_r) are plotted as a function of pH in Figure 6.



1-NOMMIndole + $H_3O^+ + H_2O$ • **NO** + **MMIndole•** + $H_3O^+ + H_2O$ (a)

NO + **MMIndole•** + $\mathbf{H_3O^+}$ + H_2O • NO + **1-H-MMIndole$^+$•** + 2 $\mathbf{H_2O}$ (b)

NO + **1-H-MMIndole$^+$•** + 2 H_2O • **3-NO-1-H-MMIndole$^+$•** + 2 H_2O (c)

NO + **MMIndole•** + $H_3O^+ + H_2O$ • **3-NO-MMIndole** + $H_3O^+ + H_2O$ (b')

3-NO-MMIndole + $\mathbf{H_3O^+}$ + H_2O • **3-NO-1-H-MMIndole$^+$** + 2 $\mathbf{H_2O}$ (c')

3-NO-1-H-MMIndole$^+$ + **2 H_2O** • **1-H-MMIndole** + $\mathbf{HNO_2}$ + $\mathbf{H_3O^+}$ (d)

FIGURE 4. Free energy profile in water at 37 °C from DFT calculations.

The reaction rate constant (k_r) correlates with the thiolate concentration [RS^-] (Figure 6), consistently with a reaction mechanism which involves nucleophilic attack of the thiolate on the nitroso moiety of NOMel. This is in agreement with previous results for S-transnitrosation reactions between *S*-nitrosothiols and thiols[26] in which an intermediate formed by these two reactants has been recently reported.[29]

The activation barrier for the reaction of Cys with NOMel was determined by measuring k_r at 28, 37, and 46.5 °C in 1 M phosphate buffer, pH 7.4. The activation energy was found to be 22 ± 1 kcal/mol. We have obtained similar results for the NOCys/Cys transnitrosation reaction.[29] This value is 6 kcal/mol higher than that reported for NOTrp.[13]

It has been observed that, at a concentration of thiol 38-fold higher than 2 mM, k_r decreases from 0.127 to 0.033 M^{-1} s^{-1} (37 °C, pH 7.4). We have tested the effect of phosphate, Tris, and Cu^{2+} on this reaction, disregarding all of them as responsible for this effect. Comparing our k_r with that obtained by Sonnenschein et al.,[13] we observe this same trend. In less concentrated Cys solutions (20-fold lower) the k_r determined for NOMel and Cys was 57-fold higher (k_r = 7.2 ± 2.4 M^{-1} s^{-1}, [Cys] = 100 μM, [NOMel] = 100 μM).[13] This rate constant must be overestimated because NOMel spontaneous decay was not considered significantly appreciable at this condition. These results show that at increasing cysteine concentration the observed rate constant ($k_o = k_r$[Cys] + k_d) tends to a limiting value. Although it could be proposed that cysteine could be behaving as the above-mentioned

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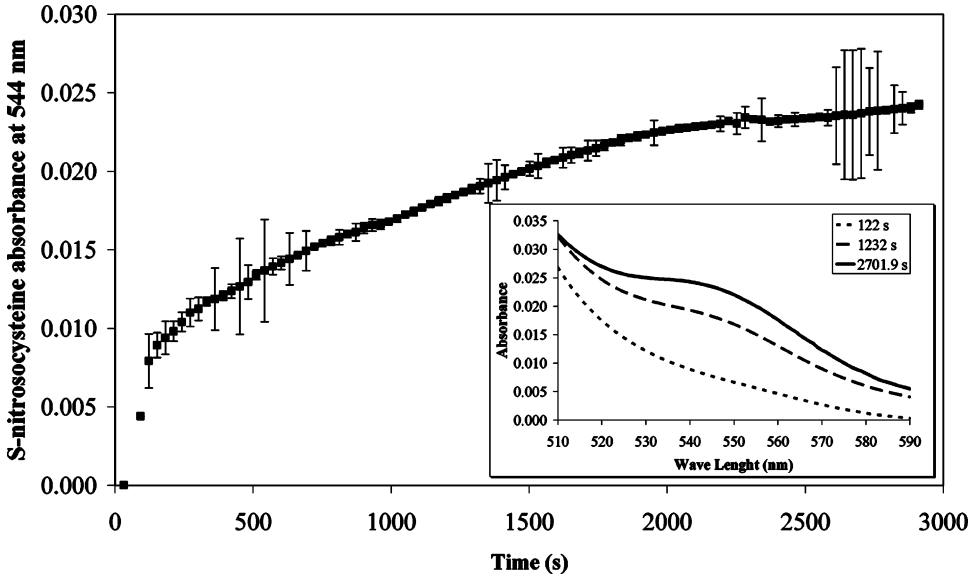
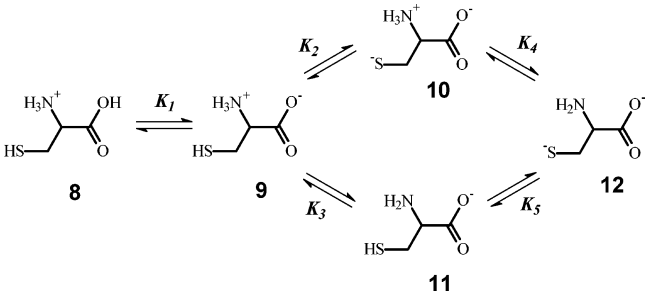


FIGURE 5. UV−vis detection of *S*-nitrosocysteine at 544 nm ($\epsilon_{544\,nm}$ = 14.9 M^{-1} cm^{-1}). Conditions: 32 °C, 0.15 M Tris−HCl buffer, pH 6.4, [EDTA] = 55.1 mM (Cu^{2+} chelant), [Cys] = 13.0 mM, [NOMel] = 9.0 mM. Inset: The visible spectrum for the reaction (from 510 to 590 nm) is shown at three different times (122, 1232, and 2702 s).

SCHEME 3. Cysteine Protonation Species[26]



nucleophiles (Cl^-, Br^-, etc.), this cannot be the main pathway since the maximum observed rate constant (k_o) for the NOMel−Cys reaction was found to be higher than that for the first limiting step of the nucleophile pathway (NOMel homolytic rupture, Scheme 2, step a; k_1, eq 11 below) and also because at alkaline pH the reaction does not decrease its rate as expected (Scheme 2, steps b−d). Thus, cysteine could be exerting a direct nucleophilic attack on NOMel in a reversible and nonconcerted way.

$$\text{NOMel} + \text{Cys}^- + \text{H}^+ \leftrightarrow \text{MelNOCys}^- + \text{H}^+ \leftrightarrow \text{MelHNOCys} \rightarrow \text{NOCys} + \text{MelH}$$

Therefore, the observed saturation could be attributed to a later limiting step just as the break off of a formed hypothetic NOMel−cysteine adduct, similar to the one we have found for nitrosocysteine.[29] We consider that this issue should be further studied.

With the purpose of studying the thermodynamic feasibility of this reaction, DFT calculations were made at the B3LYP/6-31G** level. We employed two structural analogues for NOMel and Cys, 1-nitroso-3-methyl-5-methoxyindole and L-cysteine ethyl ester, respectively. We found a standard free energy value at 37 °C in water solution of −4.6 and −22.5 kcal/mol for the reaction with the thiol and the thiolate, respectively, accordingly with a previous work on sulfur-to-sulfur transnitrosation.[30]

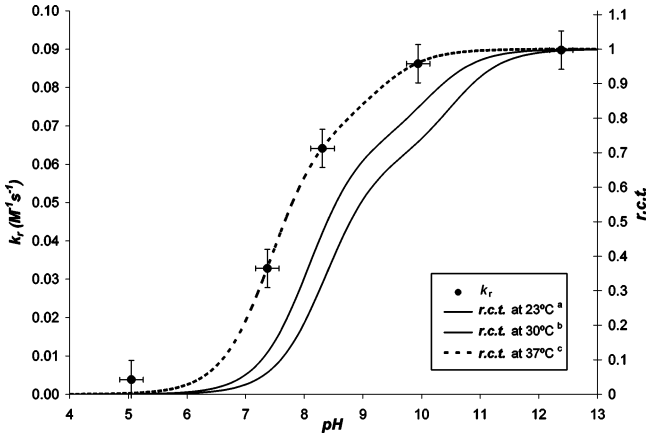


FIGURE 6. Relative concentration of cysteine thiolate species (rct) and second-order rate constant values of the reaction of NOMel with cysteine (k_r) as a function of pH. rct = $[RS^-]/([RS^-] + [RSH])$. $[RS^-]$ and [RSH] were obtained from the corresponding equilibrium constants (see the Supporting Information). Notes: a, ref 27; b, ref 28; c, fitted to data.

It has been proposed that indoles can compete with thiols for NO, thus forming *N*-nitrosoindoles.[5] Moreover, we propose that *N*-nitrosoindoles can transnitrosate to thiols as the common reaction between *S*-nitrosothiols and thiols. In this sense *N*-nitrosoindoles could act as NO carriers as well as *S*-nitrosothiols.

Reaction of NOMel with Reducing Agents. Previous studies suggest that the reaction of NOMel with reducing agents releases NO.[5,10] This reaction could have great biological relevance at the time of explaining guanilyl cyclase activation when a nitroso compound is introduced into a biological system. At first, the reaction of NOMel with NADH was tested, and NO concentration was monitored with a specific electrode (Figure SI5 in

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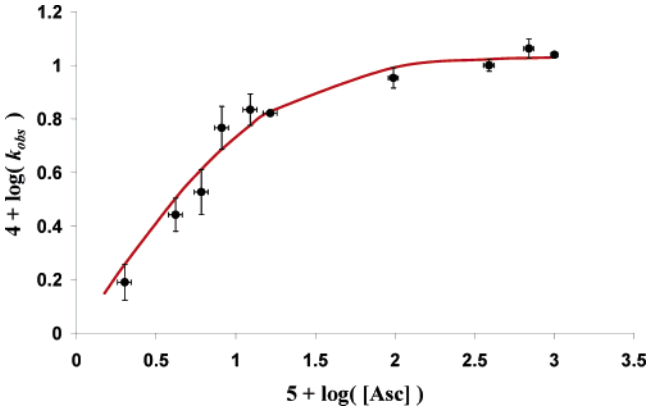

FIGURE 7. Dependence of ascorbate concentration on the observed rate constant (k_{obs}) for the reaction of NOMel with Asc ($[NOMel]_i = 0.13$ mM, 37 °C, pH 7.4): points, experimental data; curve, values fitted according to eqs 11 and 12.

the Supporting Information). This result showed that the reaction quickly releases NO. Similar results were obtained with another reducing agent, ascorbic acid (Asc), following first-order kinetics with respect to NOMel (eq 8, where v_0 is the initial rate). Figure 7 shows k_{obs}

$$k_{obs} = v_0/[NOMel] \tag{8}$$

dependence on $[Asc]_T$ (total ascorbic acid molar concentration), not observed in a previous study.[10] It can be seen that, starting from $[Asc]_T \approx 10^{-3}$ M (100:1 ratio with respect to [NOMel]), k_{obs} remains practically constant, suggesting that NOMel does not react directly with Asc; otherwise the reaction would not be zeroth-order in $[Asc]_T$ at high concentrations.

A mechanism that could explain the observed dependence involves homolytic rupture of NOMel as the first step (eq 9, as suggested above), and subsequent reaction of Asc with Mel• (eq 10)

$$NOMel \leftrightarrow NO + Mel^{\bullet} \tag{9}$$

$$Mel\bullet + Asc \rightarrow MelH + DAscH\bullet \tag{10}$$

($DAscH^{\bullet}$ = monodehydroascorbate radical). $DAscH^{\bullet}$ could further react with $Mel^{\bullet}$, producing melatonin and dehydroascorbate (DAsc). Anyhow, the destiny of this intermediate will not be considered, since its concentration is low with respect to $[Asc]_T$ at the initial reaction rates measured.

Considering that other decomposition pathways are very slow compared with this reaction (a valid assumption taking into account $[Asc]_T$), and using the steady-state approximation for $[Mel^{\bullet}]$, we can obtain the following expressions:

$$k_{obs} = k_1/(1 + a/[Asc]_T) \tag{11}$$

$$a = k_{-1}[NO]_i/k_2 \tag{12}$$

where k_1 and k_{-1} are the rate constants for the direct and reverse step 9, respectively, k_2 is the rate constant for step 10, and $[NO]_i$ is the nitric oxide concentration at the initial state of the reaction. We assume this amount to be constant because it corresponds to the NO amount that derives from a constant concentration of a NOMel ethanolic solution in equilibrium with NO; as in methanol the decomposition rate in ethanol is not appreciable.

TABLE 5. Effect of pH on the Observed Rate Constant (k_{obs}) for the Reaction of NOMel with Asc in 0.2 M Tris−HCl-Buffered Solutions at 37 °C[a]

pH	k_{obs} (10^{-3} s^{-1})	pH	k_{obs} (10^{-3} s^{-1})
7.0	1.27	8.4	1.05
7.4	1.12	9.2	0.85
7.7	1.06		

[a] $[NOMel]_i = 0.1$ mM, $[Asc]_i = 10$ mM.

TABLE 6. Experimental Activation Parameters Corresponding to the Homolytic Rupture of NOMel

$\Delta G^{\ddagger}$ (kcal mol^{-1})	22.51 ± 0.14
$\Delta H^{\ddagger}$ (kcal mol^{-1})	27.56 ± 0.02
$\Delta S^{\ddagger}$ (cal mol^{-1} K^{-1})	16.49 ± 0.07

Equation 11 adjusts reasonably well to the experimental data (Figure 7), with $k_1 = (1.08 \pm 0.04) \times 10^{-3}$ s^{-1} and $a = (1.0 \pm 0.1) \times 10^{-4}$ M. To support this consideration, we studied the pH dependence of the reaction at the saturation level, measuring k_{obs} between pH 7 and pH 9, finding that k_{obs} has a very small pH dependency (Table 5).

This information suggests that an Asc species whose concentration does not change with pH (between pH 7 and pH 9) could be participating. Asc pK_a values[31] show that ascorbate (Asc^-) is the most relevant and the only pH-independent species of all Asc species (Asc, Asc^-, Asc^{2-}) at these pHs. So we can modify eq 10 likewise, as depicted in Scheme 2, path e ($DAsc^{\bullet-}$ = unprotonated monodehydroascorbate radical):

$$Mel^{\bullet} + Asc^- \rightarrow MelH + DAsc^{\bullet-} \tag{10'}$$

To verify that NOMel homolytic rupture is the limiting step, we measured k_{obs} under saturation conditions ($[NOMel] = 0.1$ mM, $[Asc]_T = 4.3$ mM) at different temperatures (26, 31, 37, and 42 °C). The experimental activation parameters obtained from these results are shown in Table 6. The experimental activation enthalpy for this reaction agrees very well with the DFT-estimated value of 27.6 kcal mol^{-1} for NOMMIndole. This result is consistent with the fact that NOMel homolytic rupture is the limiting step of this reaction.

With the purpose of analyzing the thermodynamical feasibility of the reaction, we performed DFT calculations for the global reaction, using 3-methyl-5-methoxyindole as a model compound (1-NO-MMIndole = 1-nitroso-3-methyl-5-methoxyindole, 1-H-MMIndole = 3-methyl-5-methoxyindole):

$$2(1\text{-NO-MMIndole}) + Asc^- + H^+ \rightarrow 2NO + 2(1\text{-H-MMIndole}) + DAsc \tag{13}$$

The 2:1 stoichiometry of the global reaction was determined experimentally by measuring NOMel and Asc concentrations spectrophotometrically. The free energy value for this reaction (eq 13) in water at 37 °C (−11.9 kcal/mol) points out that NOMel reduction at this condi-

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TABLE 7. Redox Free Energies and Potentials of Some Relevant Species at pH 7 vs NHE (Normal Hydrogen Electrode)

hemireaction	$\Delta G°$ (kcal/mol)	$E°$ (mV)	hemireaction	$\Delta G°$ (kcal/mol)	$E°$ (mV)
NOMel/NOMel$^-$	15.7	−680[a]	Cu^{2+}/Cu^+	−3.7	+161
NOIndole/NOIndole$^-$	13.5	−587[b]	O_2/H_2O_2	−13.0	+281
cystine/cysteine	7.8	−340	indole•/indole$^-$	−7.5	+327[b]
NAD^+/NADH	14.8	−320	NO_2^-/NO	−8.3	+360
DAsc/Asc	−3.7	+80			

[a] This work. [b] Reference 32 (measured in acetonitrile). All others from refs 33−37.

TABLE 8. Estimated Half-Lives for the Reactions of NOMel with Various Agents at pH 7.4 and 37 °C

NOMel reaction	plasma concn (μM)	expected rate constant (k_{obs})[a] (s^{-1})	NOMel half-life (min)
reaction with ascorbate	50[b]	3.6×10^{-4}	32
spontaneous decomposition		3.7×10^{-5}	312
reaction with cysteine	10 (ref 13)	1.27×10^{-6} [c]	9096

[a] Considering that ascorbate and cysteine remain constant during the reaction. [b] Although ascorbic acid circulates in human plasma at approximately 30−50 μM, it accumulates in millimolar concentrations in host defense cells. Mononuclear leukocytes, for example, may have intracellular ascorbic acid concentrations of 3.5−6 mM.[38] In those cases k_{obs} is almost saturated, and the half-life is limited to 11 min. [c] Calculated using our second-order rate constant $k_r = 0.127 \pm 0.002$ M^{-1} s^{-1} at 37 °C, pH 7.4.

tion is thermodynamically feasible (see the Supporting Information). Other reducing agents such as H_2O_2 (Table SI2 in the Supporting Information) and Cu^+ (data not shown) also react with NOMel, although the mechanism of these reactions might be different than in the case of Asc.

Conclusions

We consider that, under the experimental conditions of our work, direct NO^+ transfer from NOMel was not compatible with our results except for the case of the catalytic hydrolysis of NOMel by phosphate where NO^+ is probably transferred directly to phosphate. In the other reactions we discarded the direct NO^+ transfer by NOMel because it would involve the formation of melatonin anion (Mel^-) that is significantly unstable at the pH conditions used ($pK_a(Indol^-) = 20.9$).[32] In the case of NOMel−phosphate, concerted exchange of NO^+ and H^+ may be involved, avoiding the formation of Mel^-. The nitrosyl phosphate formed would be rapidly hydrolyzed.

Direct electron transfer from NADH, Asc, H_2O_2, or Cu^+ to NOMel is not thermodynamically feasible at standard concentrations (Table 7). However, the indole radical has a high enough redox free energy to accept an electron from any of these reducing agents. Considering the above-mentioned results, homolytic rupture of NOMel followed by reduction (Scheme 2, paths a and e) seems to be the most likely pathway for NO release by reaction with reducing agents.

Under conditions compatible with biological media, it is likely that NOMel will mainly react with reducing agents, releasing NO (Table 8). Which of these reactions predominate will depend on the relative local concentration of these reactants. Taking into account the known concentrations of Asc[34] and cysteine[19] in plasma, the reaction with Asc would be the predominant pathway.

A comprehensive mechanism for NO release by NOMel in physiological media involves initial homolytic rupture of NOMel (Scheme 2, path a) followed by reduction (path e), oxidation (path f), or proton-aided NO translocation followed by nucleophilic attack (paths b−d). The suggested mechanism explains all our experimental and theoretical results, and could be in principle extended to other *N*-nitrosoindoles such as NOTrp.

Acknowledgment. Thanks are due to ANPCyT, Antorchas, CONICET, and UBA for financial support. Thanks are also due to Laura L. Perissinotti for the provided data.

Supporting Information Available: DFT energetic values, selected NMR spectra, kinetic data, rate law derivation, and geometries. This material is available free of charge via the Internet at http://pubs.acs.org.

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