Peracid Oxidation of an *N*-Hydroxyguanidine Compound: A Chemical Model for the Oxidation of N^{ω}-Hydroxy-L-Arginine by Nitric Oxide Synthase

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Arginine is oxidized by a class of enzymes called the nitric oxide synthases (NOS) to generate citrulline and, presumably, nitric oxide (•NO). N-Hydroxylation of a guanidinium nitrogen of arginine to generate *N*-hydroxyarginine (NOHA) has been shown to be a step in the biosynthesis of •NO. In an effort to elucidate the mechanism by which further oxidation of NOHA occurs, the oxidation of a model *N*-hydroxyguanidine compound by several peracids was studied in depth. This oxidative chemistry is a possible model for the enzymatic process since the corresponding urea (or citrulline equivalent product) is obtained along with an oxidized nitrogen species. The oxidized nitrogen product was, however, not •NO but rather HNO. •NO generation in this chemical system and in the enzymatic process would require another one-electron oxidation. The mechanistic details of this are further discussed.

Introduction

As a class of enzymes, the nitric oxide synthases (NOS) have been shown to oxidize L-arginine by an NADPH and oxygen-dependent process to give citrulline and a vasoactive and/or cytotoxic nitrogen oxide product. This oxidized nitrogen species has been reported to be nitric oxide (•NO) by a variety of indirect methods.[1-4] Recent studies have demonstrated that NOS converts L-arginine to •NO and citrulline in steps, forming the *N*-hydroxyguanidine species, *N*-hydroxy-L-arginine (NOHA) as a biosynthetic intermediate (Scheme I).[5-7] Therefore, NOS enzymes appear to be a class of monooxygenases that N-hydroxylate the guanidinium function of arginine as a first step.

The further oxidation of NOHA by NOS generates citrulline and presumably •NO. This reaction consumes NADPH and oxygen and proceeds with C–N bond scission and incorporation of an atom from molecular oxygen into citrulline[8,9] and •NO.[10] The mechanism of this novel three-electron oxidation has remained relatively unexplored. NOS oxidation of NOHA is postulated to follow any of several similar pathways.[11,12] One distinct mechanistic possibility for further oxidation of NOHA is *via* nucleophilic attack by an enzyme-derived peroxy species of either tetrahydrobiopterin (H_4B) or heme (both of which are present in the active enzyme).[11] In an effort to explore this possibility and facilitate our understanding of the NOS reaction mechanism, we have developed a chemical model of the proposed NOHA oxidation step that involves reaction of an *N*-hydroxyguanidine compound with peracids in organic solvents. Our results show that *N*-hydroxyguanidine oxidation by peracids generates primarily the expected ureido product (or citrulline equivalent product). The immediate nitrogen oxide product was, however, not •NO but rather HNO. The mechanistic implications of this are discussed later.

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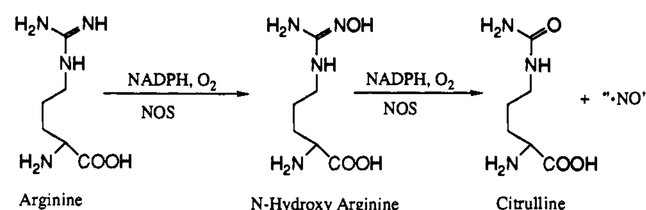
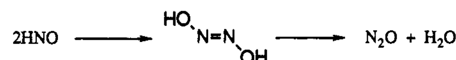
Scheme I. Pathway for the Conversion of Arginine to Citrulline and Nitric Oxide



Scheme II. Pathway for the Decomposition of HNO To Generate N_2O



Results

Oxidation of *N*-(*N*-hydroxyamidino)piperidine (NHAP) by *m*-chloroperbenzoic acid (mCPBA) in benzene under anaerobic conditions gave significant amounts of piperidinecarboxamide (PCA) and relatively minor amounts of 1-piperidinecarbonitrile (PCN). NHAP oxidation by hydrogen peroxide or *tert*-butyl hydroperoxide alone gave no reaction (data not shown). Analysis of the evolved gases from the peracid-mediated reaction by gas chromatography–thermal conductivity detection (GC/TCD), (for N_2O generation) and by chemiluminescence detection (for •NO generation) indicated that N_2O was the major gaseous product. Only trace amounts of •NO were seen (typically <0.1%) under any reaction condition. It should be mentioned here that N_2O generation from these reactions is actually an indication of HNO release. HNO is a metastable species and decomposes to give N_2O under the conditions of the reaction (discussed in greater detail later) (Scheme II). Also, only trace amounts of either NO_3^- or NO_2^- were found. Tabulation of the organic and inorganic product yields are given in Table I. Oxidation of NHAP by other peracids (or related oxidants) such as monoperoxyphthalic acid or potassium peroxymonosulfate under anaerobic conditions also gave PCA and PCN as the major organic products and N_2O as the major gaseous product. The source of N_2O in these reactions was not

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Table I. Product Analysis of the Oxidation of NHAP by Peracids

oxidant	product yields (%)[a]					
	PCA	PCN	HNO[b]	NO	NO_x[c]	NH_2OH
mCPBA[d] (in benzene)	61.3	5.6	26.5	<1	2.9	ND[e]
mCPBA (in methanol)	ND	ND	16.0	<1	ND	<0.1
MPPA[f] (in benzene)	12.4	10.7	4.7	<1	ND	ND
MPPA (in methanol)	23.0	7.64	18.6	<1	<0.1	<0.1
oxone[g]	36.2	8.9	2.4	<1	ND	ND

[a] Based on NHAP. [b] Detected and measured as N_2O. [c] Includes both nitrite (NO_2^-) and nitrate (NO_3^-). [d] *m*-Chloroperbenzoic acid, room temperature, anaerobic, in benzene (for further details see Methods section). [e] ND = not determined. [f] Monoperoxyphthalic acid, magnesium salt, hexahydrate. [g] Potassium peroxymonosulfate.

likely to be from the oxidation of released NH_2OH since attempted oxidation of NH_2OH by mCPBA in benzene gave no detectable N_2O (<0.5% conversion would have been easily detected). Also, NH_2OH was not detected from the reaction of NHAP with MPPA or mCPBA in methanol.

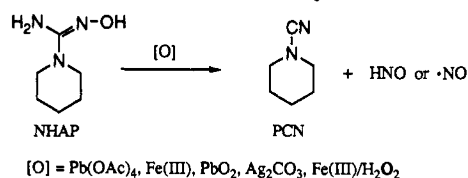
The stoichiometry of the organic products (PCA and PCN) and nitrogen oxide (primarily N_2O) was not 1:1 as might be expected. Although we have rigorously looked for other possible nitrogen oxide products, we were unable to fully account for the lack of 1:1 stoichiometry. In spite of this quantitative inconsistency we can say that N_2O is the major gaseous nitrogen oxide product. It should be considered, however, that the amounts of HNO generated from these reactions may be higher than the reported values since only headspace N_2O was determined and significant amounts of N_2O may remain in the reaction solvent. However, heating the reaction mixture to 60 °C prior to headspace gas analysis did not result in significant changes in the amount of N_2O detected. It is also possible that all the HNO was not converted to N_2O under the conditions of the reactions and therefore N_2O quantitation is not entirely indicative of the absolute amounts of HNO formed. It is clear, however, that HNO and *not* •NO was the major nitrogen oxide product of these reactions.

To assure that PCA and PCN were not GC-related thermal decomposition products of NHAP, HPLC analysis of the reaction mixture was also performed. The results of HPLC analysis revealed that PCA and PCN were a result of NHAP oxidation by peracids and not artifactual thermal decomposition products of NHAP. Also, to determine that PCA was not formed from the oxidation of the cyanamide function, PCN was reacted with mCPBA under identical reaction conditions as those for NHAP. Little or no PCA was formed under these conditions. Thus, PCA formation was a direct result of peracid oxidation of NHAP and not from further reaction of PCN with the oxidant. Also, a similar study indicated that PCN did not result from PCA decomposition under the reaction conditions.

Discussion

Since the discovery that NOHA is a biosynthetic intermediate in the oxidative conversion of arginine to •NO and citrulline, an understanding of possible mechanism(s) of oxidation of *N*-hydroxyguanidine compounds has become important in attempting to understand the enzymatic process.[5-7] A previous investigation of the chemical oxidation of *N*-hydroxyguanidines indicated that either •NO or nitroxyl (HNO) could be generated depending on the oxidant.[13] The relevance of that study to

Scheme III. Oxidation of NHAP by Various Oxidants[13]



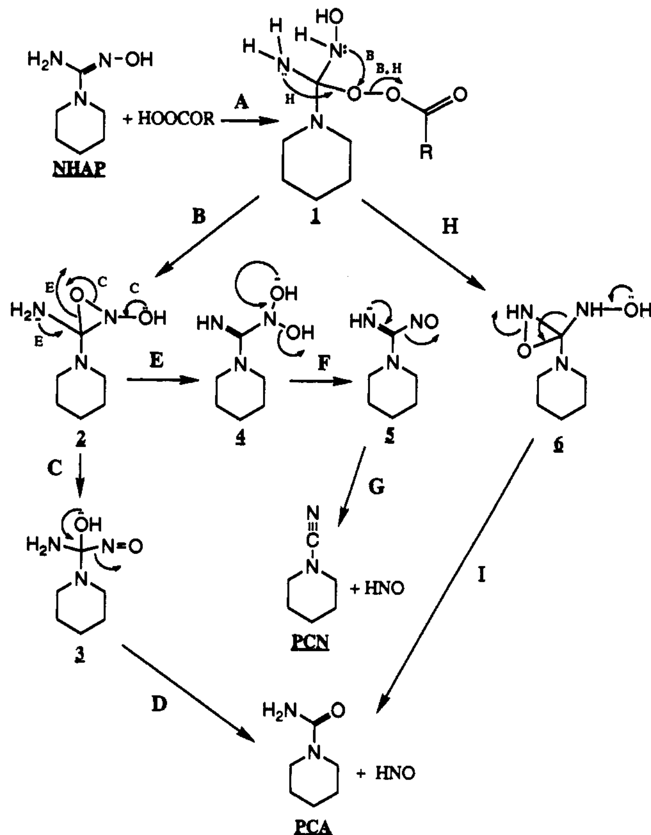
[O] = $Pb(OAc)_4$, Fe(III), PbO_2, Ag_2CO_3, Fe(III)/H_2O_2

the enzymatic process is somewhat questionable, however, since urea products (the citrulline equivalent coproduct in the case of arginine oxidation) were not detected. Only the corresponding cyanamide was found (Scheme III). Also, the oxidants utilized may or may not be related to the oxidant generated by the NOS enzyme. Significantly, it has been previously shown that NOS utilizes NADPH and O_2 as cofactors and contains FMN, FAD, and H_4B as prosthetic groups.[11] Furthermore, iron–heme has recently been shown to be a prosthetic group as well[14,15] (the relevance of which will be discussed later). Although the nature of the ultimate oxidant generated by NOS is not known, it has been postulated that other H_4B requiring oxygenases generate a pterin–hydroperoxy species which is involved in the oxidation of substrate (possibly with assistance from a reduced metal species). It has also been proposed that peracids, such as mCPBA, are more reasonable models for the pterin–hydroperoxy species than are alkyl hydroperoxides.[16] Thus, an investigation of the reaction of peracids with *N*–hydroxyguanidines may generate reasonable clues to possible mechanisms of enzymatic NOHA oxidation.

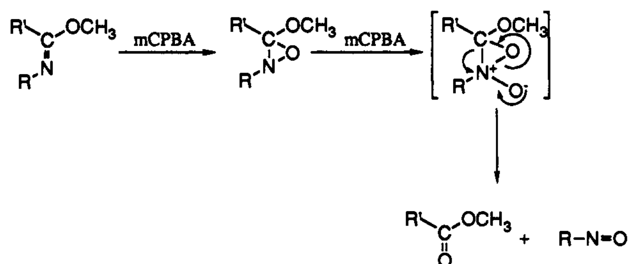
Unlike the previous study on *N*-hydroxyguanidine oxidation,[13] mCPBA oxidation of a representative *N*-hydroxyguanidine compound, NHAP, generated significant amounts of the corresponding urea product, PCA (>50%). This product corresponds to citrulline generation from arginine in the enzymatic reaction. Further, because PCA had formed in reactions run under inert atmosphere and in oxygen-free solvent, its oxygen must be derived from the peracid. Thus, the reaction mimics the proposed enzymatic oxidation in that the peroxy group was the source of the incorporated oxygen. The cyanamide product, PCN, was also found but was, in all cases, the minor reaction product (Table I).

Analysis for the inorganic nitrogen oxide products revealed that •NO was not generated in significant amounts (0.1%). The major nitrogen oxide product was instead N_2O. Oxidation of NHAP with other peracids also resulted in significant PCA generation with N_2O again being the major, gaseous nitrogen oxide product. The source of N_2O is undoubtedly via initial generation of nitroxyl (HNO) which has been shown to undergo spontaneous dimerization to hyponitrous acid which then decomposes to give N_2O and water (Scheme II).[17] Direct analysis for HNO is extremely difficult as it is a self-reacting, metastable species with an expected short half-life. Thus, HNO is typically detected indirectly by analysis for its stable decomposition product, N_2O. Thus, peracid oxidation of NHAP generates the corresponding urea, cyanamide (a minor organic product), and HNO (the major nitrogen oxide product). Possible mechanisms which could account for the observed products are given in Scheme IV. These mechanisms all require initial nucleophilic attack of the peracid on the *N*-hydroxyguanidine function to give 1 (step A). In mechanism A–B–C–D, 1 is converted to the *N*-hydroxyoxazirine 2, which rearranges to generate a hydroxy–nitroso species 3, followed by elimination of HNO

Scheme IV. Possible Mechanisms for PCA, PCN, and HNO Generation from the Peracid Oxidation of NHAP.

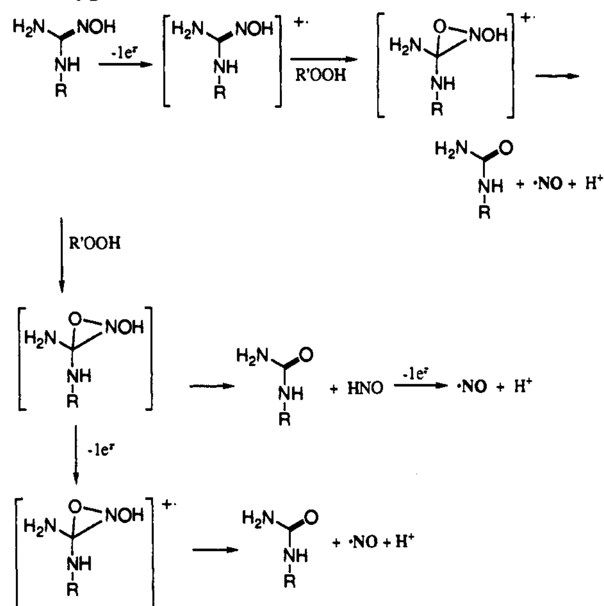


Scheme V. Proposed Mechanism for the Oxidation of Imino Ethers by Peracids To Generate an Alkyl Nitroso Species and Ester[20]



to give the urea (PCA). (The elimination of HNO from a species similar to **3** has been proposed as a crucial step to carbonyl formation in the Nef reaction.[18,19]) PCN formulation from **1** can be envisioned to occur *via* mechanism A–B–E–F–G. That is, amine-assisted oxazirine ring opening from **2** to give the dihydroxylated guanidinium species **4**, followed by elimination of water, step F, to give **5** and HNO loss, step G, to give PCN. Another possible mechanism for PCA formation, mechanism A–H–I, involves nucleophilic attack of the amino group, rather than the *N*-hydroxy nitrogen, from **1** to give the oxazirine **6**, step H, which then decomposes to give HNO and PCA, step I. In partial support of the proposed mechanism A–B–C–D, alkoxyoxazirines can be formed by the oxidation of imino ethers by peracids.[20] Further oxidation of this alkoxyoxazirine presumably results in the generation of an *N*-oxide which decomposes to give an alkylnitroso compound and the corresponding ester (Scheme V). The *N*-oxide species can be viewed as chemically similar to the *N*-hydroxy species proposed in this work and the formation of an alkylnitroso compound would be mechanistically similar to the generation of HNO

Scheme VI. Possible Mechanisms for •NO Generation from a Three-Electron Oxidation of an *N*-hydroxyguanidine[a].



[a] The one-electron oxidation step can occur from any of the three steps: from NOHA, from the intermediate oxazirine (or equivalent), or from HNO (also see ref 11). R' = biopterin or iron–heme.

proposed herein. Regardless of the route, PCA (or PCN) formation from the peracid oxidation of NHAP results in concomitant HNO generation and not •NO release.

Recent studies have demonstrated that the NOS isoforms are iron–heme-containing proteins.[14,15] This finding indicates that the NOS enzymes have distinct similarity to the family of monooxygenases known as the cytochromes P450. In fact, recent studies have postulated NO generation from *N*-hydroxyarginine or related amidoximes by a cytochrome P450 catalyzed process.[21,22] The heme group in NOS is likely to participate in the initial hydroxylation of arginine. This reaction consumes 1 mol each of oxygen and NADPH and represents a two-electron oxidation of the guanidinium nitrogen of arginine to generate NOHA. The subsequent oxidation of NOHA could be carried out by either a heme or tetrahydrobiopterin-related nucleophilic oxygen species with reactivity similar to that of the peracids used herein. Of note, iron–peroxo species have been shown to be nucleophilic agents which can react with electrophiles.[23] Our results suggest that such a reaction would lead to citrulline formation. However, •NO generation would require an additional one-electron oxidation. The identity of this one-electron oxidant is unknown but it is conceivable that the ferric–heme can accept a single electron from a metabolic intermediate, such as an oxazirine or HNO, which would then lead to •NO generation. In partial support of this concept, HNO has been shown to be capable of reducing ferric–heme proteins to generate •NO.[24] Alternatively, other redox cofactors on the enzyme may accept an electron from an oxidized nitrogen species resulting in •NO production. Scheme VI outlines the mechanistic possibilities regarding single-electron removal and •NO generation. In any case, NADPH stoichiometry studies support a one-electron removal by NOS at some point during this step[5] and may suggest that the electron obtained is utilized along with electrons derived from NADPH to activate oxygen for further catalysis. It is also possible that HNO itself is the immediate enzymatic

product and is responsible for at least some of the observed biological activity attributed wholly to •NO. Significantly, HNO has recently been found have biological activity like that of •NO in smooth muscle tissue.[24,25]

Conclusion

This study demonstrates that oxidation of *N*-hydroxyguanidines by peracids results in formation of the corresponding urea and HNO (possibly through an *N*-hydroxyoxazirine or equivalent intermediate). Thus, oxidation *via* attack of an enzyme generated nucleophilic oxygen species on *N*-hydroxyguanidine could account for the formation of citrulline from NOHA. •NO formation would, however, require another one-electron oxidation (unless HNO were the product).

Experimental Section

Chemical and Solutions. *N*-(*N*-Hydroxyamidino)piperidine (NHAP) was synthesized according to the method described previously.[26] Piperidinecarbonitrile (PCN) [1530-87-6], piperidinecarboxamide (PCA) [2158-03-4], Oxone, monopersulfate ($2KHSO_5{\cdot}KHSO_4{\cdot}K_2SO_4$) [37222-66-5], monoperoxyphthalic acid magnesium salt hexahydrate (MPPA) [846765-66-7], *m*-chloroperbenzoic acid (mCPBA) [C6,270-0], 1-cyclohexyl-2-pyrrolidinone [6837-24-7], 8-hydroxyquinoline [148-24-3], and benzaldehyde [100-52-7] were purchased from the Aldrich Chemical Company (Milwaukee, WI). All solvents were obtained from commercial sources and were of the highest purity available.

PCN/PCA Assay by Gas Chromatography (GC). The quantitation of PCA and PCN from the oxidation of NHAP was accomplished using the following method. An aliquot containing 0.5 mM 1-cyclohexyl-2-pyrrolidinone (internal standard) and the reaction sample being assayed was injected onto a Hewlett-Packard 5890 gas chromatograph equipped with a nitrogen-phosphorus (NPD) and a 5% phenylmethyl silicon column (0.2-mm i.d. 0.33-mm film thickness) with a flow rate of 3 mL/min. Injector and detector temperature were 200 °C. Initial oven temperature was 85 °C and increased at a rate of 30 °C/min until a final temperature 190 °C was reached. Quantitation of products was achieved by comparison with a standard curve using 1-cyclohexyl-2-pyrrolidinone as the internal standard. The retention times for PCA, PCN, and internal standard were 4.50, 6.25 and 8.86 min, respectively.

PCN/PCA Determination by High-Performance Liquid Chromatography (HPLC). Quantitative anlaysis of PCN and PCA was also accomplished by HPLC. Analysis was performed on a Rainin liquid chromatograph equipped with a 25 × 4.3 mm, 5-μm Beckman C-18 reversed-phase column and a Spectra-Physics 100 UV-vis detector operating at 220 nM. The mobile phase consisted of 35% acetonitrile/65% water. The retention times for PCA, PCN, and internal standard (benzaldehyde) were 1.78, 3.13, and 5.03 min, respectively. Quantitation was accomplished by comparison with a standard curve.

Nitric Oxide Detection by Chemiluminescence. The possible evolution of nitric oxide from NHAP oxidation was monitored utilizing an Antek 720 chemiluminescence detector. The reactions were typically run in septum-sealed 50-mL single-neck round-bottom flasks under a nitrogen atmosphere. All solvents were thoroughly degassed prior to use with a 10-20-min nitrogen sparge. After completion of the reaction, a small gas sample (100-500 μL) was taken out of the headspace through the septum. This sample was then injected directly into 50-mL nitrogen-filled flask which was swept into the chemiluminescence detector by a nitrogen stream. Quantitation of nitric oxide was accomplished by comparison of the detector response to a standard curve generated from authentic nitric oxide samples.

Nitrous oxide (N_2O) Detection. N_2O detection and quantitation was performed on a 5710 Hewlett-Packard gas chromatograph equipped with a thermal conductivity detector, 2-m × 9-mm Porapak Q column operating at 60 °C, with a flow rate of 20 mL/min. Quantitation was achieved by comparison with injections of known amounts of authentic N_2O. Retention time of N_2O was 2.0 min.

Determination of Nitrite and Nitrate. Levels of both nitrite (NO_2^-) and nitrate (NO_3^-) from the oxidation of NHAP by mCPBA in benzene and by MPPA in methanol were determined using the method of Braman and Hendrix.[27] Briefly, after the reaction was complete, the solvent was removed by rotary evaporation, and the residue was then taken up in a minimal amount of water and added, via syringe, to a solution of refluxing vanadium(III)/2 M HCl. The evolved gases from the reducing solution were swept into a Dasibi chemiluminescence detector. Quantitation was performed by comparing the detector response to a standard curve generated from the addition of known amounts of nitrite or nitrate to the reducing solution.

Assay for Possible Hydroxylamine (NH_2OH) Generation. Possible NH_2OH generation was tested by utilizing the method of Magee and Burris.[28] Briefly, 1 mL of the reaction solution was added to 1 mL of a 1% ethanolic solution of 8-hydroxyquinoline and 1 mL of 2 N Na_2CO_3. The mixture was vortexed and allowed to stand for 1 h. Absorbance at 710 nM was then determined versus a reference standard which contained 1 mL of the 1% 8-hydroxyquinoline solution, 1 mL of the 2 N Na_2CO_3, and 1 mL of the reaction solvent containing the oxidizing agent only (no NHAP). Also, possible interference by NHAP was tested by performing the above assay using a 30 mM solution of NHAP (no oxidizing agent). NHAP was found to slightly interfere with the assay. The 30 mM solution of NHAP gave an absorbance at 710 nM approximately equivalent to that of a 1 mM NH_2OH standard. Thus, the assay is approximately 30-fold more sensitive for NH_2OH than it is for NHAP. Minimum detection levels of this assay were determined to be approximately 0.01 mM (equivalent to a 0.07% reaction conversion) assuming no NHAP interference.

Oxidation of NHAP by m-Chloroperbenzoic Acid (mCPBA). A solution of NHAP (0.150 mmol) in benzene (10 mL, 15 mmol) was placed in a 50-mL round-bottom flask covered with a septum. Nitrogen gas was bubbled through the solution for 15 min. mCPBA solution in benzene or methanol (10 mL degassed, 30 mM) was then injected into the NHAP solution through the septum and the mixture stirred for 2 h. Gas samples were taken from the headspace and analyzed for nitric and nitrous oxide. GC analysis for reaction products (PCA and PCN) was performed by direct injection of a sample of the reaction solution into the GC. (Analysis of PCA and PCN from the reaction carried out in methanol was difficult since a reaction byproduct cochromatographed with the PCA peak). Analysis for NO_3^- and NO_2^- was performed as described above. HPLC analysis of the reaction mixture in benzene was performed as follows: the 20-mL reaction solution was transferred to a separatory funnel and washed with 2 × 25 mL of 10% Na_2SO_3 (to remove unreacted mCPBA). The organic fraction (containing the PCN) was analyzed using the HPLC method described above. The aqueous layer was saturated with NaCl and extracted with 20 mL of ethyl acetate. The organic fraction was then washed with a 5% sodium bicarbonate solution (to remove the *m*-chlorobenzoic acid). The organic solvent was then removed by rotary evaporation and the residue resuspended in acetonitrile. The acetonitrile solution was then assayed for PCA using the HPLC method described above.

Attempted Oxidation of Hydroxylamine (NH_2OH) by mCPBA. The possibility that mCPBA oxidation of NH_2OH may release HNO was tested by reacting mCPBA (50 mmol), hydroxylamine hydrochloride (40 mmol), and sodium bicarbonate (48 mmol) together in dry benzene (distilled from sodium) in a sealed 25-mL round-bottom flask under an atmosphere of nitrogen. Headspace analysis for N_2O was performed as described above. After 0.5, 1, and 3 h, no N_2O was detected. The detection system would have easily detected 0.5% conversion of NH_2OH to N_2O if it had occurred.

Oxidation of NHAP by Oxone. Into a 50-mL round-bottom flask was added 0.184 g (0.300 mmol) of Oxone and 10 mL of benzene. The flask was then covered with a septum and flushed with nitrogen for 5 min. Then 10 mL (0.150 mmol) of a 15 mM NHAP solution taken up in benzene was added with a syringe. The heterogeneous mixture was stirred for 24 h at room temperature. After the reaction was stopped, the headspace was analyzed for N_2O and the solution analyzed for the organic products PCA and PCN by GC-NPD. The reaction was also carried out in THF, benzene/acetone, and THF/acetone (all reactions gave the same products in similar yields). Possible

•NO evolution was monitored by analyzing the reaction headspace utilizing the chemiluminescence detector.

Oxidation of NHAP by Monoperoxyphthalic Acid, Magnesium salt Hexahydrate (MPPA). MPPA (0.148 g, 0.300 mmol) and NHAP (0.020 g, 0.14 mmol) were placed into 50-mL, three-neck round-bottom flask. The flask was then swept with nitrogen for 15 min. The reaction was started by injection of 10 mL of dry, degassed solvent (benzene or methanol) using a syringe. The flask was sealed under nitrogen and stirred for 14 (benzene) and 2.5 (methanol). Gaseous and organic products (PCA and PCN) were then analyzed using the GC methods described previously. Analysis for NO_3^- and NO_2^- were performed as described above.

Analysis for the Possible Conversion of PCA to PCN with mCPBA. A solution of PCA (100 000 nmol) in benzene (10 mL, 20 mM) and 10 mL of a 30 mM mCPBA solution in benzene were mixed and stirred for 2 h. The reaction mixture was then extracted with 10% Na_2SO_3 (to remove any mCPBA). The organic layer was then assayed for PCN using the HPLC procedure described above.

Analysis for the Possible Conversion of PCN to PCA with mCPBA. A PCN solution (10 mL, 1 mM) and mCPBA (10 mL, 30 mM) were stirred for 2 days. The solution was washed with 2 × 25 mL of 10% Na_2SO_3. The aqueous phase was saturated with NaCl and extracted with 25 mL of ethyl acetate. The organic solvent was removed by rotary evaporation. The residue was resuspended and acetonitrile and assayed by HPLC.

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