

The Reaction of Peroxynitrite with Organic Molecules Bearing a Biologically Important Functionality. The Multiplicity of Reaction Modes as Exemplified by Hydroxylation, Nitration, Nitrosation, Dealkylation, Oxygenation, and Oxidative Dimerization and Cleavage

Nobuaki Nonoyama, Hiroshi Oshima, Chizuru Shoda, and Hitomi Suzuki*

Department of Chemistry, School of Science, Kwansei Gakuin University, Gakuen 2-1, Sanda 669-1337

(Received April 24, 2001)

The reactions of peroxynitrite with a variety of organic molecules which include a biologically important functionality have been examined to construct a simple model for the peroxynitrite-induced in vivo transformations as well as a chemical probe for the active species involved therein. Phenols were found to undergo hydroxylation, nitration, oxidative dimerization, and oxidation to cyclohexadienones and quinones. The ring nitration of catechol was confirmed for the first time in the in vitro reaction of peroxynitrite. Dealkylation and *N*-oxide formation were the major reaction modes observed for *N*,*N*-dimethyl-*p*-toluidine. 1,2-Phenylenediamine gave benzotriazole in high yield. The electron-deficient C–C double bond in 1,4-naphthoquinone underwent epoxidation, while the electron-rich C–C double bond in α-methylstyrene suffered oxidative cleavage to acetophenone. The activated double bond in *trans*-stilbene underwent oxidative cleavage and epoxidation in parallel to give benzaldehyde and *trans*-stilbene oxide as the major products. The triple bond in diphenylacetylene was simply oxygenated to form benzil, together with trace amounts of ring nitration products. 1-Phenylethanol, imidazole, 2′-deoxyadenosine, and 2′-deoxyguanosine were all quite slow to react, while uracil and cytosine were almost inert to peroxynitrite. The reaction modes exhibited by peroxynitrite are too widespread and complicated to explain the whole mechanistic pathway in terms of a single active species. All reaction modes observed for the peroxynitrite to date could be classified into five categories according to their types: i) electron transfer type, ii) *O*-electrophilic type, iii) *N*-electrophilic type, iv) *O*-nucleophilic type, and v) radical type. Some of these may compete under certain conditions. The active species involved in each of these types of reactions are as follows: i) NO^+, NO_2, and OH, ii) ONO***O***H, iii) O***N***OOH and NO^+, iv) ***O***OH^- and ONO***O***$^-$, and v) NO_2 and $OH^\bullet$.

In recent years, the peroxynitrite anion ($O{=}NOO^-$), which forms in vivo from nitrogen monoxide and superoxide anion,[1,2] has received considerable attention due to its potential to injure or destroy biological molecules or molecular systems, leading to the pathogenic process of human diseases. Typical phenomena that have been reported to involve peroxynitrite are the breaking of DNA strands[3,4] and the inactivation of enzymes.[5,6,7] However, chemical processes involved in these phenomena remain to be elucidated in many respects due to the lack of relevant information on the nature of peroxynitrite.

A number of papers have dealt with the reaction of peroxynitrite; such studies include the nitration and oxidation of phenols,[8,9,10,11] nitration and nitrosation of thiols,[12,13] oxidation of lipids,[14,15] nitration as well as other complex transformations of indole[16,17,18] and purine derivatives,[19,20] oxidation of aldehydes,[21,22] and one-electron oxidation of metal complexes.[23] In a recent communication,[24] we have reported some new modes of reactions exhibited by peroxynitrite, which included the epoxidation of an electron-deficient carbon-carbon double bond, dealkylation of tertiary amine, and formation of benzotriazole from 1,2-phenylenediamine. In the present paper, we have elaborated the above results further and have extended our study to include compounds bearing other functional groups of biological significance.

We have employed ^{1}H NMR as a major tool to monitor the progress of reaction; a known type of organic transformation occurring in situ in the reaction mixture could be directly observed. By assigning the peaks of each product, we could readily identify and quantify the products simultaneously without their isolation. The reactions were carried out in deuterium oxide (D_2O), acetonitrile-d_3 (CD_3CN), and chloroform-*d* ($CDCl_3$). The behavior of peroxynitrite was found to be quite similar in these solvent systems, showing a similar product distribution as well as a similar kinetic profile.[25] Hydrophobic substrates and products are soluble in the latter two solvents, so a variety of organic substrates were subjected to the action of peroxynitrite as a model for bio-transformations. The compounds chosen as simplified models for biomolecules included phenol (**1**), catechol (**5**), *p*-cresol (**7a**), 1,4-naphthoquinone (**24**), 1-phenylethanol (**35**), and imidazole (**36**), which may be regarded as the substructure of tyrosine (**7b**), vitamin K, norepinephrine, and histidine, respectively, as illustrated in Fig. 1. In addition, several compounds were examined as potential chemical probes for the active species involved in the peroxynitrite-induced transformations. They included 2,6-di-*t*-butylphenol (**12**), *N*,*N*-dimethyl-*p*-toluidine (**17**), 1,2-phenylenediamine (**21**), α-methylstyrene (**26**), *trans*-stilbene (**28**), and diphenylacetylene (**33**).

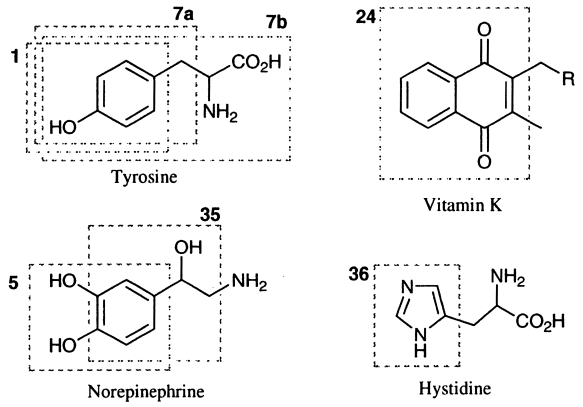
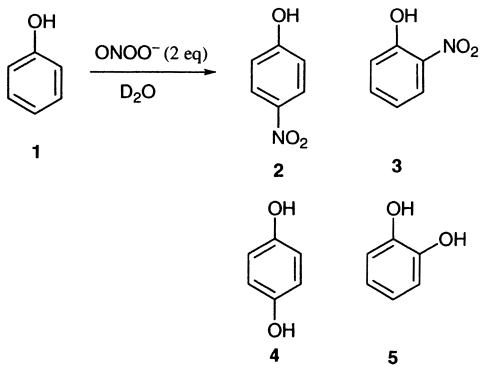


Fig. 1.

Results and Discussion

Reaction of Phenols and Catechol. There are many types of biomolecules that contain a phenolic substructure. Recent works have shown this structural unit to be quite susceptible to the action of peroxynitrite, giving a mixture of nitration and hydroxylation products.[9,10,11] In the present work, we have taken up phenol (**1**) as a simplified model for phenolic biomolecules and have examined its behavior toward peroxynitrite in detail according to the procedures **A**–**C** (see Experimental). The results obtained were compared with the previously reported ones.

^{1}H NMR spectra of the reaction mixtures, obtained according to the procedures **A** and **B**, showed several sets of new peaks, some of which were assigned to *p*-nitrophenol (**2**), *o*-nitrophenol (**3**), and hydroquinone (**4**) by direct comparison with the authentic specimens (Eq. 1).



(1)

In addition, small amounts of catechol (**5**) and *p*-benzoquinone (**6**) were detected by HPLC. The latter two products could not be detected by ^{1}H NMR spectra due to extensive overlapping of their characteristic peaks. According to the procedure **C** as a control experiment, the original peak of **1** remained unchanged in intensity and no other peaks appeared even after prolonged standing. The relative yield of each product was calculated on the basis of their peak area relative to that of an internal standard (Table 1). The major reaction modes observed for phenol were nitration and oxidation; in the former reaction, *ortho*- and *para*-nitration products **2** and **3** were formed in ca. 1:1 ratio, while in the latter the hydroxylation products **4**, **5** and quinone **6** were obtained. Table 1 shows a comparison with the previous data reported for the reactions under similar conditions.[9–12] Despite the differences in way of manipulation, our results bear close resemblance to the reported ones, endorsing the validity and reliability of our procedure.

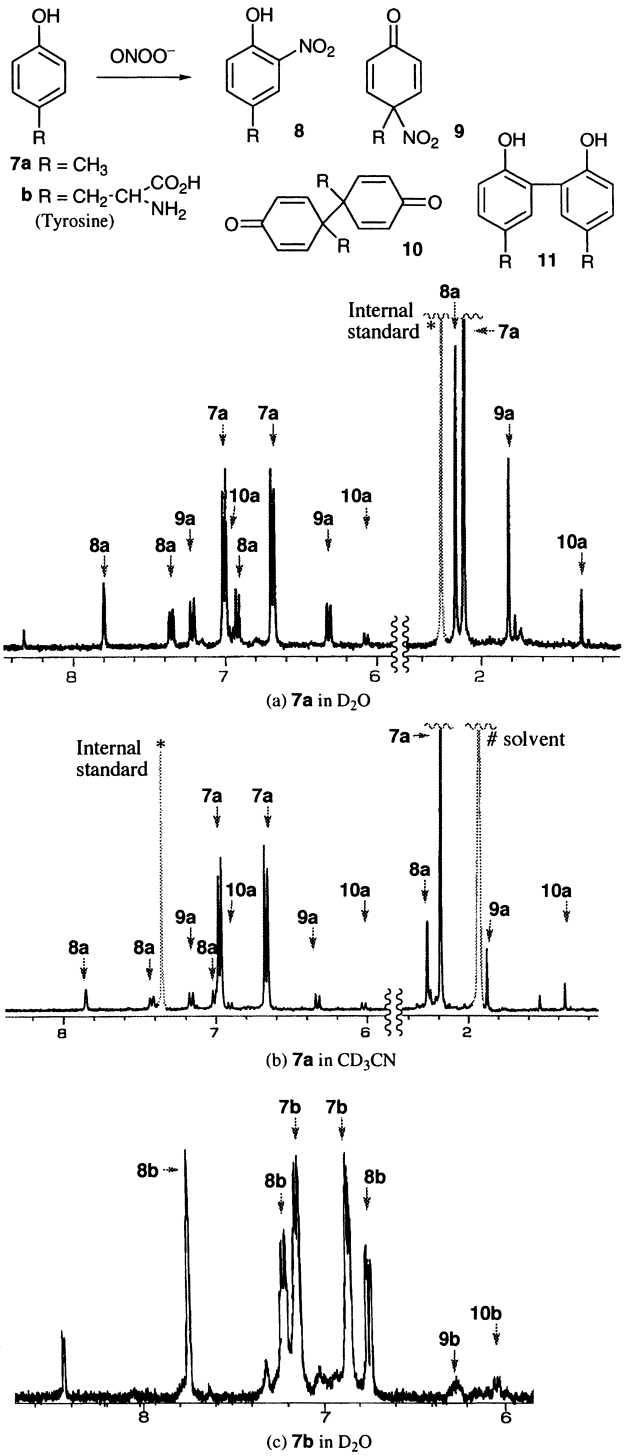


Fig. 2. ^{1}H NMR (δ/ppm, 400 MHz) spectra from the reaction of *p*-cresol **7a** (a) in buffered D_2O, (b) in CD_3CN, and (c) from the reaction of tyrosine **7b** in buffered D_2O. All reactions were carried out according to procedure **A** and product yields are listed in Table 2.

Table 1. Reaction of Phenol **1** with Peroxynitrite

		$ONOO^-$	Yield[a)]/%					Conversion[b)]
Entry	Conditions	equiv	**2**	**3**	**4**	**5**	**6**	%
1	Procedure **A**, D_2O	4	3	4	1	—	—	26
2	Procedure **B**, D_2O	4	7	8	0	—	—	36
3	Procedure **C**, D_2O, control	4	—	—	—	—	—	< 5
4	H_2O buffer (pH 7)[c)]	1	0.5	0.8	0.2	0.1	trace	10
5	CH_3CN[c)]	1	3.0	3.4	0	trace	0.1	19
6	H_2O buffer (pH 7)[9]	—	1	3	1	2.5	1	—[d)]
7	H_2O buffer (pH 7)[10]	—	2	2	3	3	—	—[d)]
8	H_2O buffer (pH 7)[11]	—	2	3.5	1	1	—	—[d)]
9	H_2O buffer (pH 7), CO_2 [12]	—	5	7	2	0	—	—[d)]

a) Yields in entries 1–3 were calculated on phenol employed, while those in entries 4–9 were based on peroxynitrite added.
b) Percent ratio against the consumed phenol.
c) Yields were determined by HPLC.
d) Conversion data are not available.

In order to see the generality of the present reaction, we further examined the behavior of related phenol derivatives toward peroxynitrite under similar conditions.

The reaction of *p*-cresol (**7a**) with peroxynitrite was carried out in buffered D_2O and CD_3CN according to the procedures **A** and **C**. Regardless of the buffered solutions (Figs. 2a and 2b), both reaction systems showed a close similarity in the peak pattern of ^{1}H NMR spectra. Three sets of major peaks were observable, one of which showed an aromatic feature and the other two non-aromatic ones. Of three major products, one was readily identified as 4-methy-2-nitrophenol (**8a**) by direct comparison with the authentic specimen. Two non-aromatic compounds showed two doublet peaks with a large coupling constant J = 9.6–10.0 Hz (mostly < 9.0 for aromatic ones) and their methyl peaks shifted upfield from the benzylic (2.2 ppm) to the aliphatic regions (< 2.0 ppm). On the basis of the spectral data, one of these was identified as 4-methyl-4-nitro-2,5-cyclohexadienone (**9a**) and the other tentatively assigned as a 4,4′-dimeric structure of oxidized *p*-cresol, e.g., 1,1′-dimethyl-4,4′-dioxo-1,1′,4,4′-tetrahydrobiphenyl (**10a**). However, compounds **9a** and **10a** were comparatively unstable under the conditions employed and decomposed within several hours. A flask-scale reaction was performed for *p*-cresol (**7a**) and subsequent chromatography of the resulting product mixture gave nitration product **8a**, together with small amounts of a 2,2′-dimer (**11a**). The peaks of **11a** were hidden by those of compound **7a** in the ^{1}H NMR spectrum of the reaction mixture. In Table 2, the product yields are presented in conversion yield, which refers to the percent yield of a product against the consumed amount of a given substrate. The modification of reaction conditions, such as the absence or presence of CO_2 and the atmospheric environment of air or argon, exerted little effect on these relative product yields.

Concurrent nitration, hydroxylation, and oxidative dimerization have recently been reported for the reaction of tyrosine (**7b**) and peroxynitrite.[10,26,27] Amino acid **7b** may be regarded as a side-chain modified *p*-cresol (**7a**), and therefore, would be expected to show behavior toward peroxynitrite similar to that of **7a**. Indeed, the ^{1}H NMR spectrum of the reaction mixture of **7b** and peroxynitrite showed a peak pattern very similar to that of the reaction product from **7a** (Fig. 2c). Thus, on the analogy of the spectral pattern, the major products were assigned as nitrotyrosine **8b** and two adducts **9b** and **10b**, which should correspond to the products **8a**–**10a**, respectively, derived from **7a**. Due to a low solubility of tyrosine **7b** and consequently a poor resolution of the ^{1}H NMR spectrum, we could not estimate the accurate yields of these products. We now report two additional types of new products **9b** and **10b**. The formation of several yet unidentified products was suggested from the ^{1}H NMR spectrum of the reaction product of **7b**, but their peaks were too weak to draw any positive evidence for chemical structures from them.

Table 2. Reaction of *p*-Cresol **7a** with Peroxynitrite

Procedure,	$ONOO^-$	Conversion	Conversion yield[a)]/%		
solvent	equiv	%	**8a**	**9a**	**10a**
A, D_2O	4	61	31	15	4
C, D_2O	4	< 5	—	—	—
A, CD_3CN	2	20	14	8	6
A[b)], CD_3CN	2	22	13	6	7
B, CD_3CN	2	30	11	6	4
C, CD_3CN	2	< 5	—	—	—

a) Conversion yield refers to the percent yield of each product against the total amount of **7a** consumed.
b) Reaction was carried out under an argon atmosphere.

The product from a similar reaction of 2,6-di-*t*-butylphenol (**12**) showed a simple ^{1}H NMR pattern consisting of four major singlet peaks (Fig. 3), three of which were readily attributed to 2,6-di-*t*-butyl-4-nitrophenol (**13**), 2,6-di-*t*-butyl-*p*-benzoquinone (**14**), and a 4,4′-dimeric bis-phenol (**15**), respectively. According to the procedure **C** as a control, no reaction was observed to take place. The formation of a dimer **15** suggested the radical nature of the present reaction. The phenoxyl radical generated from **12** is flanked and stabilized by two bulky *t*-butyl groups, so it in part dimerizes at *para* position to form a significant amount of bis-phenol **15**. Quinone **14** also has two *t*-butyl groups that would prevent the quinone framework from further degradation. This contrasts to *p*-benzoquinone **6**, which is susceptible to further transformation and can be de-

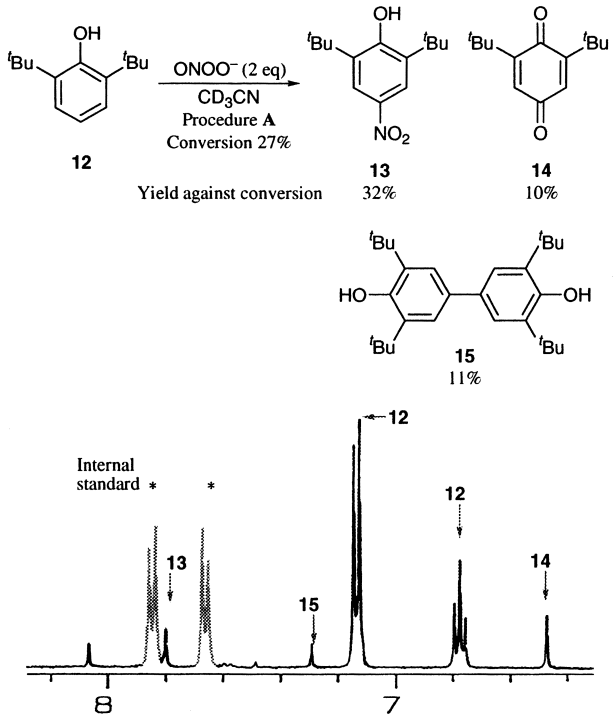


Fig. 3. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of 2,6-di-*t*-butylphenol **12** in CD_3CN according to procedure **A**.

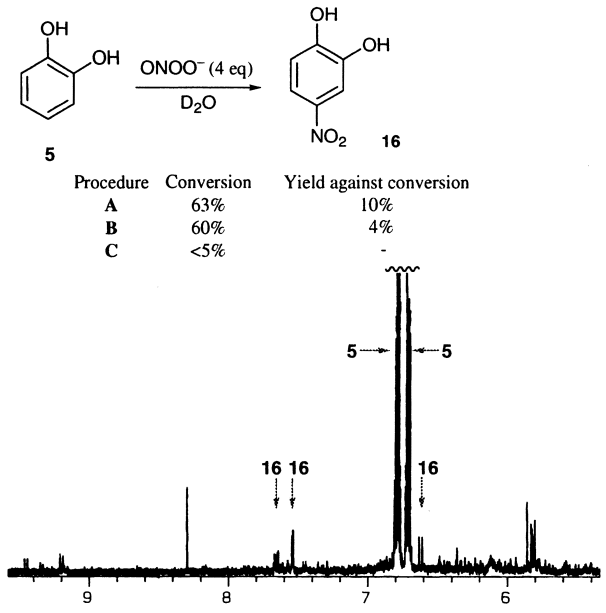
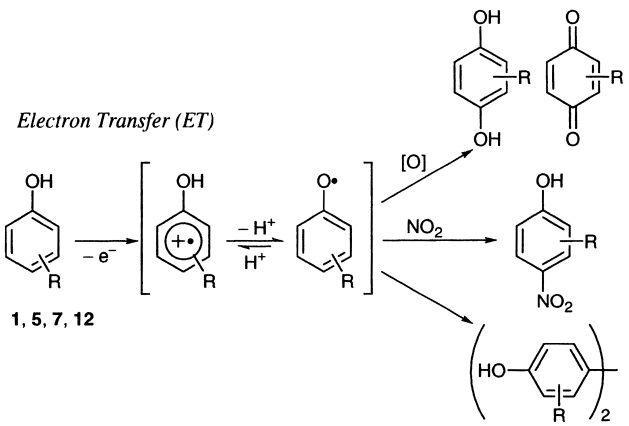


Fig. 4. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of catechol **5** in buffered D_2O according to procedure **A**.



Scheme 1.

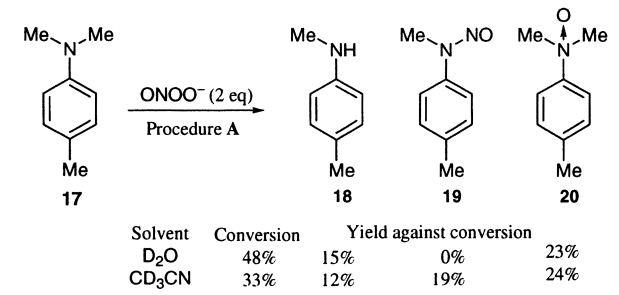
tected only in a trace amount in a similar reaction of **1**. This is the first example of the formation of significant amounts of a phenolic dimer and a quinone from the reaction of phenol and peroxynitrite.

Catechol type aromatic amino compounds, represented by dopamine and epinephrine, exist in neurons as neurotransmitters. The catechol framework in biomolecules is highly electron-rich and quite sensitive toward the action of electrophiles. Accordingly, this type of bio-substructure is of interest in terms of the reaction with peroxynitrite. The reaction of catechol with peroxynitrite has previously been reported to give melanin.[28] We carried out the reaction of catechol **5** with peroxynitrite under a variety of conditions and examined the products in detail. The reaction mixture showed a complex pattern of ^{1}H NMR (Fig. 4), part of which was attributed to 4-nitrocatechol (**16**) by comparison with the authentic specimen. This constitutes the first observation of a nitration product from the reaction of catechol and peroxynitrite anion, which suggests the possible formation of the nitro derivatives from bio-relevant catechol compounds in vivo.[29]

The proposed mechanism for the formation of nitration, oxidation, and dimerization products from phenolic substrates is depicted in Scheme 1. We postulate that a cation radical and a phenoxyl radical intermediate, which can interconvert through a protonation–deprotonation process, result from the one-electron oxidation of a phenolic substrate and act as a common precursor for a variety of descendent molecules. The CIDNP experiments[30] as well as a kinetic feature obtained for a series of 4-substituted phenols[25] also support the radical nature of the reaction. Homocoupling of the resulting radical species would lead to the dimerization products **10**, **11**, and **15**, while heterocoupling with nitrogen dioxide would form the nitration products **2**, **3**, **8**, **9**, **13**, and **16**. The attack of molecular oxygen or *O*-nucleophiles would give rise to the oxidation products **4**–**6** and **14**.

Reaction of *N*,*N*-Dimethyl-*p*-toluidine. The tertiary amino group constitutes one of the important functionalities found in biomolecules. The reaction with peroxynitrite was examined using *N*,*N*-dimethyl-*p*-toluidine **17** as a substrate due to the ease of detection and identification of the degradation products. The reaction was carried out in CD_3CN according to the procedure **A** and three major products were identified as *N*-methyl-*p*-toluidine (**18**), *N*-methyl-*N*-nitroso-*p*-toluidine (**19**), and *N*,*N*-dimethyl-*p*-toluidine *N*-oxide (**20**) by direct comparison with the authentic specimens (Fig. 5). A similar reaction in D_2O gave **18** and **20** in similar yields. Substrate **17** was inert in both solvents under a control condition according to the procedure **C**.

The dealkylation of tertiary amine **17** is supposed to occur via an electron transfer pathway,[31] as shown in Scheme 2,



Solvent	Conversion	Yield against conversion		
D_2O	48%	15%	0%	23%
CD_3CN	33%	12%	19%	24%

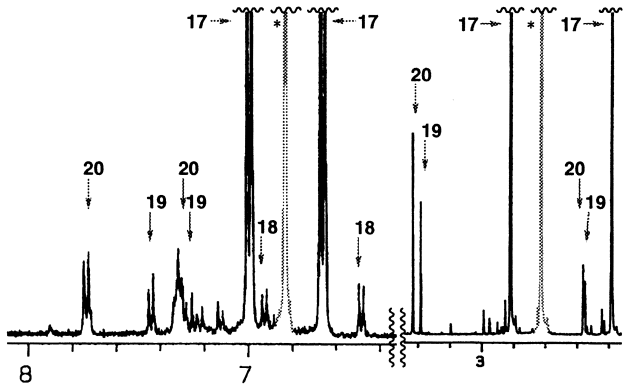
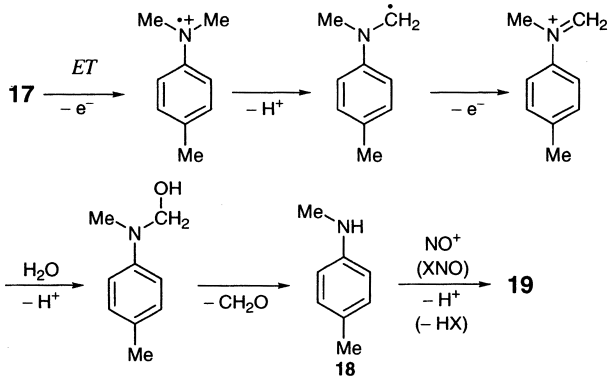


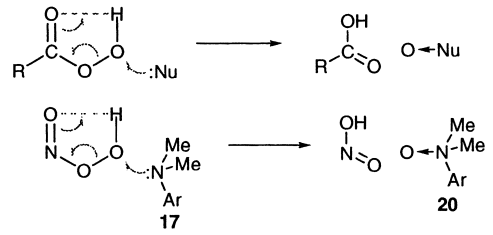
Fig. 5. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of *N*,*N*-dimethyl-*p*-toluidine **17** in CD_3CN according to procedure **A**.



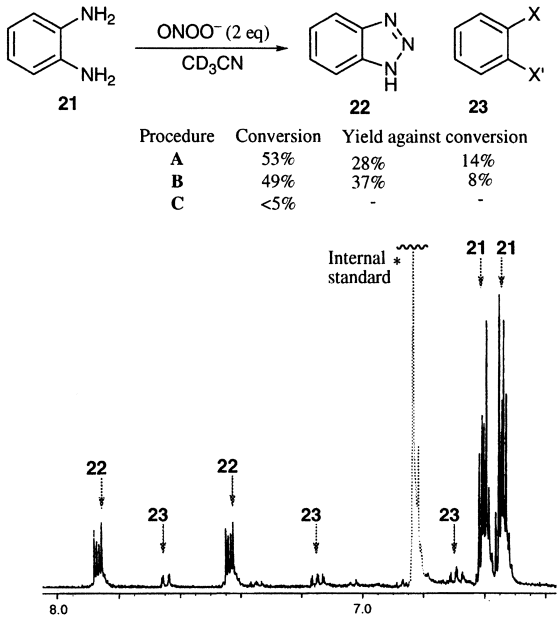
Scheme 2.

though no reports to date have mentioned the concurrent formation of an *N*-oxide **20**. Carboxylic peracids such as *m*-chloroperbenzoic acid and peracetic acid are known to oxidize tertiary amines to form the corresponding *N*-oxides.[32] Peroxynitrous acid (HOO–N=O) is isoelectronic in structure with carboxylic peracid (***H***OO–C(=O)R) and therefore could probably cause a similar type of oxidation (Scheme 3). By analogy with carboxylic peracid, the electrophilic hydroxy oxygen atom in peroxynitrous acid ONO***O***H would attack the nitrogen atom of amine **17** to form *N*-oxide **20**. Our observations have confirmed for the first time that two different types of reactions, i.e. electron transfer-induced dealkylation and electrophilic *N*-oxidation, proceed in parallel in the reaction of peroxynitrite with tertiary amine.

Reaction of 1,2-Phenylenediamine. Aromatic 1,2-diamine is a good chemical probe for in situ-generated nitrosyl

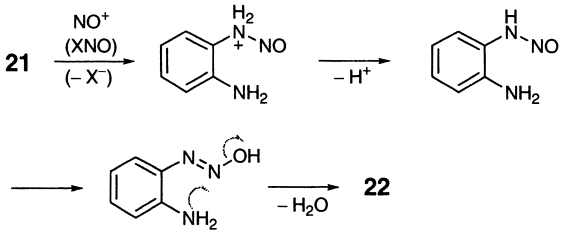


Scheme 3.



Procedure	Conversion	Yield against conversion	
A	53%	28%	14%
B	49%	37%	8%
C	<5%	-	-

Fig. 6. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of 1,2-phenylenediamine **21** in CD_3CN according to procedure **A**.



Scheme 4.

ion (NO^+) and has been used for the quantification of nitrite ion under acidic conditions.[33] The reaction of 1,2-phenylenediamine (**21**) in CD_3CN according to the procedures **A** and **B** gave benzotriazole (**22**) and an unidentified aromatic product (**23**) (Fig. 6). A similar reaction in D_2O also formed **22** as a precipitate in undetermined yield. Aromatic 1,2-diamines are inactive to nitrite ion under neutral aqueous conditions. So the triazole formation can be interpreted in terms of the initial electrophilic attack by nitrosyl ion or its equivalent (XNO) on the nitrogen atom of 1,2-diamine and subsequent ring closure of the resulting diazotized species leading to triazole **22** (Scheme 4). An alternative free radical mechanism has also been presented.[34]

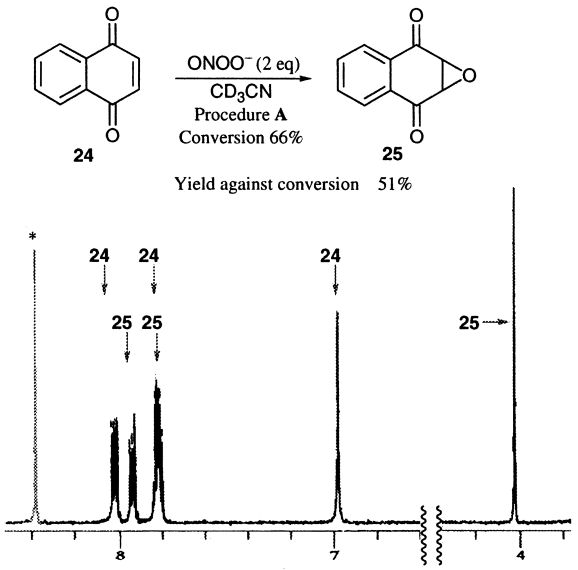
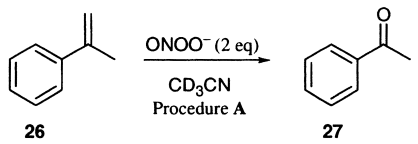


Fig. 7. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of 1,4-naphthoquinone **24** in CD_3CN according to procedure **A**.

Reaction of 1,4-Naphthoquinone. Many polycyclic quinones are of biological importance and this type of structural unit is widely distributed in natural products. As a simplified model compound, we chose 1,4-naphthoquinone (**24**) and investigated the reactivity of its electron deficient C–C double bond, doubly conjugated with two carbonyl functions. The reaction of **24** with peroxynitrite gave a product of simple ^{1}H NMR spectral pattern as the sole product, which was readily identified as 2,3-epoxy-2,3-dihydro-1,4-naphthoquinone (**25**) (Fig. 7). No reaction took place in a control experiment according to the procedure **C**.

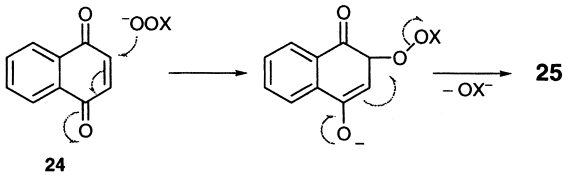
A plausible mechanism for the epoxidation of an electron deficient C–C double bond in **24** is the Michael-type addition of peroxynitrite or its equivalent (XOO^-) and subsequent ring closing, as shown in Scheme 5.[35]

Reactions of α-Methylstyrene, *trans*-Stilbene, and Diphenylacetylene. In order to know the behavior of the electronically neutral C–C double bond toward peroxynitrite, we have monitored the reaction of α-methylstyrene (**26**) by means of ^{1}H NMR and GC-MS. The conversion was fair to moderate, and the major product was readily identified as acetophenone (**27**) by direct comparison with the authentic specimen (Eq. 2).

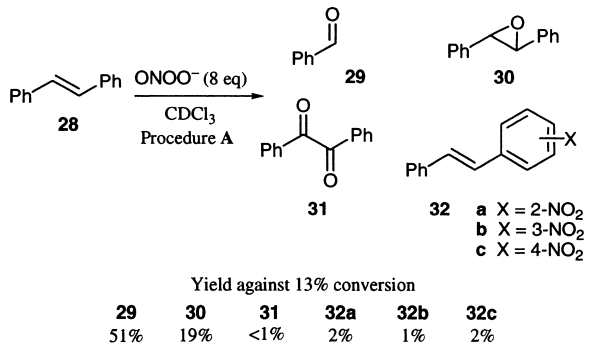


(2)

The intermediate formation of an epoxidation product could not be observed, which contrasts to the behavior of the electron-deficient C–C double bond in quinone **24**. A control experiment according to the procedure **C** confirmed that the substrate **26** remained intact under the conditions employed.



Scheme 5.



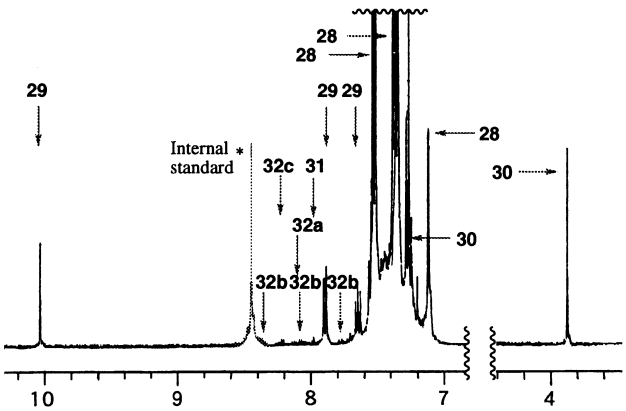


Fig. 8. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of *trans*-stilbene **28** in $CDCl_3$ according to procedure **A**.

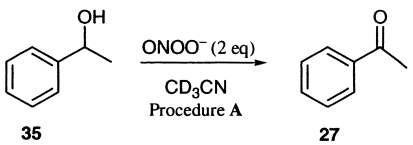
The activated olefinic bond in *trans*-stilbene (**28**) reacted with peroxynitrite anion with comparative ease, giving benzaldehyde (**29**) and *trans*-stilbene oxide (**30**) as the major products, together with a trace amount of benzil (**31**). Concurrent ring nitration was observed to a small extent, giving a mixture of *trans*-nitrostilbenes (**32a–c**). Noteworthy is a finding that the epoxide **30** and diketone **31** are not the precursors to aldehyde **29**; competitive oxidation of **28** and **30** (or **31**) by peroxynitrite has been found to result in the preferential consumption of the former, leading to the formation of **29** and **30**. Compounds **30** and **31** remained almost intact under the conditions employed (Fig. 8). On a similar treatment, *trans,trans*-1,4-diphenyl-1,3-butadiene readily gave a complicated mixture of products, the only identified product being aldehyde **29** (conversion yield, $< 10\%$). The conjugated diene system is quite susceptible to the action of peroxynitrite.

The present observation constitutes the first example of the oxidative cleavage of the C–C double bond caused by peroxynitrite. This is of interest in view of the metabolic degradation of olefinic and polyenic compounds in biological tissues.

The mechanism for the oxidative cleavage of the electron-rich C–C double bond would probably involve initial attack by an electrophilic *O*-atom of peroxynitrous acid ONO*O*H, as suggested in Scheme 3. However, the mode of subsequent C–C bond cleavage is not clear at the present stage.

The acetylenic bond is uncommon among organic compounds of biological origin, but its behavior toward peroxynitrite is of interest from a mechanistic point of view. The triple bond in diphenylacetylene (tolan, **33**) was found to undergo oxygenation with peroxynitrite anion to afford benzil **31** as the sole major product. No cleavage of the C–C bond was observed. Concurrent ring nitration took place to a small extent, but strangely enough, the ortho and meta nitro derivatives (**34a**,**b**) were the only nitration products observed (Fig. 9). This contrasts to the reaction of *trans*-stilbene **28**, where all three possible nitro isomers were formed. The formation of 1,2-diketone **31** suggests a sequence involving the nucleophilic attack of peroxynitrite anion onto the acetylenic carbon, followed by the ring closure to a 1,2-dioxetene intermediate which collapses to diketone **31**. The relative reactivity of the double bond against the triple bond, as determined by the competitive reaction of **28** and **33** toward peroxynitrite anion, was around 4.4. This aspect of peroxynitrite chemistry is under investigation.

Reaction of 1-Phenylethanol. To investigate the reactivity of activated secondary alcohols toward peroxynitrite, we examined the reaction of 1-phenylethanol (**35**) according to the procedure **A**. Ketone **27** was the sole major product obtained, though the conversion yield was quite low (Eq. 3).



(3)

Yield 62% against 7% conversion

We may conclude that the major reaction mode of secondary alcohols toward peroxynitrite is the oxidation to carbonyl compounds. Even the activated alcoholic function in **35** was much less reactive as compared with the other functional groups examined above. Therefore, this mode of reaction would be unimportant for peroxynitrite under in vivo conditions, in view of the presence of a variety of functional groups of higher reactivity in complex biomolecules. This information should be important for understanding the behavior of multi-functionalized biomolecules toward peroxynitrite.

Reaction of Azacyclic Compounds. The reaction with peroxynitrite has been examined for several bio-relevant azacyclic compounds. Cytosine and uracil were completely unreactive under the conditions of the procedure **A**. Imidazole (**29**), 1-methylimidazole, 2′-deoxyadenosine, and 2′-deoxyguanosine were all quite reluctant to react with peroxynitrite in CD_3CN to form trace amounts of yet unidentified products. The low reactivity of these azacyclic compounds suggests the insignificant contribution of peroxynitrite to the in vivo degradation of these heterocyclic systems.

Possible Active Species Involved in the Reaction of Peroxynitrite. What is the actual species that is responsible for the peroxynitrite-induced transformation of biomolecules? As described above, the peroxynitrite has been found to exhibit various types of reactions depending on the type of substrates employed. Thus it hardly seems possible to account for such multiplicity of the reaction modes demonstrated herein by assuming only a single attacking species as the actual reactant. As many control experiments according to the procedure **C** showed, no reaction took place after the complete disappearance of peroxynitrite anion in both aqueous and acetonitrile solutions. This finding confirms the view that any active species derived from peroxynitrite is short-lived under neutral conditions.

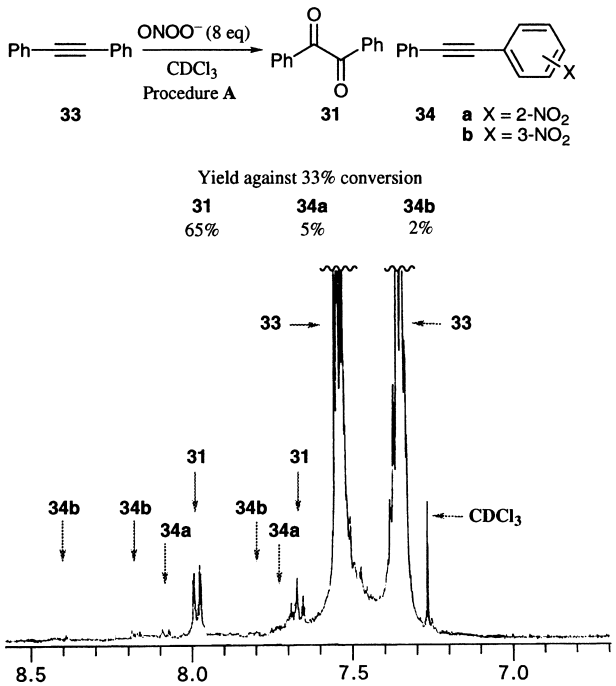


Fig. 9. ^{1}H NMR (δ/ppm, 400 MHz) spectrum from the reaction of diphenylacetylene **33** in $CDCl_3$ according to procedure **A**.

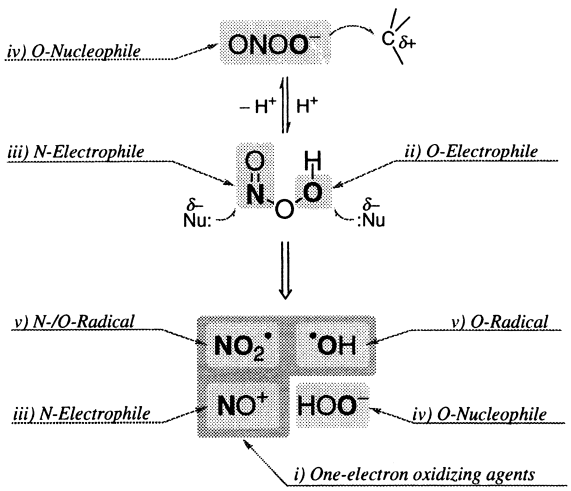
All reactions so far observed for peroxynitrite may be classified into five categories according to their types, as summarized in Table 3. The first category includes the nitration, dimerization, and hydroxylation of phenols, quinone formation, and dealkylation of tertiary amine. The products from this category of reactions presumably arise from the electron-transfer type reaction, as shown in Schemes 1 and 2. The second category includes the *N*-oxide formation from tertiary amine, oxidative cleavage of olefinic bond, and oxidation of alcohols to carbonyl compounds, which resemble the reactions caused by peracid reagent (Scheme 3). The third category includes the formation of triazole and *S*-nitrosation of thiols,[12,13] that should result from the initial attack of an *N*-electrophile, as shown in Scheme 4. The fourth category includes the epoxidation of electron-deficient C–C double bond, in which the reaction is highly likely to involve an *O*-nucleophile (Scheme 5). The oxidation of aldehyde is assumed to proceed via nucleophilic attack of peroxynitrite at the carbonyl carbon, and therefore, it may be better included in this category.[21,22] The

Table 3. Classification of Peroxynitrite-induced Reactions According to Their Types

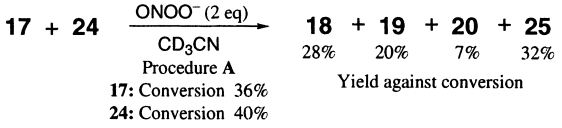
Reaction mode	Reaction type	Probable active species
· Nitration of phenols · Hydroxylation of phenols · Dimerization of phenols · Oxidation of phenol to quinone · Dealkylation of amines	Electron transfer	NO^+ NO_2 $OH^\bullet$
· *N*-oxide formation from amine · Oxidative cleavage of olefinic bond · Oxidation of alcohol to ketone	*O*-Electrophile	ONOOH
· Triazole formation · *S*-Nitrosation	*N*-Electrophile	ONOOH NO^+
· Epoxidation of quinone · Oxidation of aldehyde	*O*-Nucleophile	$ONOO^-$ HOO^-
· Lipid oxidation · Oxidation of alcohol to ketone · Hydroxylation of electron poor aromatic compound	Radical	NO_2 $OH^\bullet$

last category is the radical type reaction that includes the oxidation of lipid[14] and hydroxylation of electron-deficient aromatic compounds.[9] Reactions of the last type have not yet been elucidated in detail.

Possible candidates for the active species that should arise from peroxynitrite are illustrated in Scheme 6. Peroxynitrite anion itself can work as an *O*-nucleophile, which is protonated to form peroxynitrous acid ONOOH ($pK_a = 6.8$) under neutral conditions. Peroxynitrous acid is susceptible to the attack of nucleophile at both nitrogen and hydroxylic oxygen atoms. In the former type of reactions, peroxynitrous acid undergoes heterolytic cleavage to transfer the NO^+ unit to a given substrate. In the latter case, this acid transfers an electrophilic oxygen



Scheme 6.



Scheme 7.

atom to a substrate, as has been observed in the *N*-oxide formation from tertiary amine. Therefore, both *N*- and *O*-atoms, as indicated in the formula O*NO**O*H, are capable of reacting as the electrophile depending on substrates. The dissociation mode of peroxynitrous acid is still controversial,[36,37] but we now propose that there could arise four types of reactive species from peroxynitrite: NO_2, $OH^\bullet$, NO^+, and OOH^-. The former three species are capable of inducing the one-electron transfer oxidation. Two radical species, NO_2 and $OH^\bullet$, may cause the radical addition and hydrogen abstraction, while two ionic species NO^+ and OOH^- work as an *N*-electrophile and an *O*-nucleophile, respectively. In the presence of CO_2, an additional active species is likely to be involved.[30,38,39]

The following experiment has disclosed that the above-mentioned active species can be generated in situ and concurrently under certain conditions. An equimolar solution of **17** and **24** was treated with peroxynitrite and the conversion of these substrates to the respective products was monitored by ^{1}H NMR (Scheme 7). Both substrates were converted with similar ease to give a similar yield of the expected products in parallel, each yield being close to that obtained from a separate reaction of the individual compounds. This example clearly shows the multiple reactions to run abreast, leading to the formation of products **18** and **19** via the electron-transfer process, of **20** via the attack of an *O*-electrophile, and of **25** via the attack of an *O*-nucleophile.

Conclusion

The multiplicity in reaction mode of the peroxynitrite-induced transformation of bio-related organic molecules has been established. New reaction modes observed in the present work include the dealkylation of tertiary amine, triazole formation, epoxidation of quinone, oxidation of alcohol to carbonyl compound, oxidative cleavage of olefinic bond, and oxygenation of acetylenic bond. The versatility of the reaction mode is suggestive of the involvement of several different active species in the reaction of peroxynitrite. The reactions exhibited by peroxynitrite are classified into five categories according to their types, and the relationships between the reaction modes and the likely active species involved therein are summarized in Table 3. There are many yet unidentified products, the identification of which may provide additional new reaction modes. These reactions would probably be involved in vivo and contribute to the degradation of biological systems, eventually leading to the pathogenic process of diseases. Better understanding about the behavior of peroxynitrite toward a wide variety of bio-relevant molecules should enable us to predict the role of peroxynitrite in vivo and should provide invaluable information on its physiological significance.

Experimental

All chemicals used as substrates were purchased from Aldrich,

TCI, Wako, Ishizu, and Acros. Authentic samples used for identifying the products were synthesized by the following procedures. *N*-methyl-*p*-toluidine (**18**) was obtained from *p*-toluidine and methyl iodide. *N*-methyl-*N*-nitroso-*p*-toluidine (**19**) was synthesized by the *N*-nitrosation of *N*-methyl-*p*-toluidine with sodium nitrite in sulfuric acid.[40] *N*,*N*-dimethyl-*p*-toluidine *N*-oxide (**20**) was obtained by the oxidation of *N*,*N*-dimethyl-*p*-toluidine with *m*-chloroperbenzoic acid.[32] Authentic nitrostilbenes were synthesized by the Wittig reaction of benzaldehyde with isomeric nitrobenzylidene(triphenyl)phosphoranes, while nitrodiphenylacetylenes were obtained by the Sonogashira coupling of phenylacetylene with isomeric iodonitrobenzenes.

^{1}H NMR spectra were obtained with a JEOL 400 MHz spectrometer for solutions in D_2O, CD_3CN, or $CDCl_3$. Chemical ionization mass spectra (CI-MS) were obtained on a Shimadzu GC-MS QP-5000 instrument using isobutane as an ionizing gas. An apparatus from Nippon Ozone Co. Ltd., type ON-1-2, was used for the generation of ozone, the efficiency of which was monitored by a MODEL-1700 ozone monitor from Dylec, Inc. The machine produced ozone at a rate of 12 mmol h^{-1} under the conditions of an oxygen flow rate of 10 dm^3 h^{-1} and an applied voltage of 100 V. Products were identified by ^{1}H NMR and GC-MS analyses or by direct comparison with the authentic samples.

Preparation of Peroxynitrite. Peroxynitrite was prepared according to the Pryor method, by passing ozone at a rate of 12 mmol h^{-1} for 1.2 h into a solution of sodium azide (10 mmol) in alkaline D_2O (5 mL, 0.01 M NaOH solution).[41] The concentration of peroxynitrite (0.78–0.96 M) was determined on the basis of a characteristic absorption at 302 nm (ε = 1670 M^{-1} cm^{-1}).

Synthesis of Authentic Specimens. *trans*-4-Nitrostilbene (32c). A mixture of 4-nitrobenzyl bromide (3.0 g, 14 mmol), triphenylphosphine (3.7 g, 14 mmol), and *m*-xylene (35 mL) was heated under reflux for 12 h to afford (4-nitrobenzyl)triphenylphosphonium bromide as crystalline solid (5.3 g, 79%). To an equimolar solution of phosphonium salt (5.3 g, 11 mmol) and benzaldehyde (1.2 g, 11 mmol) in dichloromethane (100 mL) was added dropwise aqueous NaOH (0.5 g, 13 mmol) over 10 min and the resulting mixture was heated with stirring at 50 °C for 30 min. The organic layer was separated, washed successively with saturated aqueous $NaHSO_3$ (20 mL) and water (30 mL × 3), and dried over $MgSO_4$. The solvent was removed and the solid residue was recrystallized from ethanol to give **32c** as pale yellow needles (1.3 g, 51%). Mp 155–157 °C (Ref. 42 155–157 °C). ^{1}H NMR ($CDCl_3$) δ 8.22 (2H, d, J = 8.1 Hz), 7.64 (2H, d, J = 8.1 Hz), 7.56 (2H, d, J = 8.1 Hz), 7.41 (2H, t, J = 8.1 Hz), 7.3–7.4 (2H, m), 7.14 (1H, d, J = 8.1 Hz).

Other isomeric *trans*-nitrostilbenes were similarly obtained as pale yellow needles.

***trans*-2-Nitrostilbene (32a):**[43] mp 71–73 °C; ^{1}H NMR ($CDCl_3$) δ 8.13 (1H, dd, J = 8.1, 1.1 Hz), 7.42 (8H, m), 7.12 (2H, m).

***trans*-3-Nitrostilbene (32b):**[42] mp 113–115 °C; ^{1}H NMR ($CDCl_3$) δ 8.36 (1H, s), 8.08 (1H, dd, J = 8.7, 1.2 Hz), 7.78 (1H, dd, J = 8.7, 1.2 Hz), 7.54 (1H, t, J = 8.7 Hz), 7.3–7.4 (5H, m), 7.1–7.2 (2H, m).

3-Nitrodiphenylacetylene (34b).[44] To a solution of phenylacetylene (0.9 g, 9 mmol) and 4-iodonitrobenzene (2.2 g, 9 mmol) in THF (50 mL) was added successively tetrakis(triphenylphosphine)palladium (10 mol%), CuI (10 mol%), and triethylamine (30 mol%) and the resulting mixture was stirred for 3 h at room temperature. The solvent was removed under reduced pressure to leave a solid residue, which was washed thoroughly with water and chromatographed over silica gel using a mixture of ethyl acetate and hexane (1:5) as the solvent to give pure **34b** as pale yellow crystals (0.8 g, 42%). Mp 67–69 °C; ^{1}H NMR ($CDCl_3$) δ 8.38 (1H, s), 8.1–8.2 (2H, m), 7.82 (1H, dd, J = 5.1, 2.7 Hz), 7.5–7.6 (2H, m), 7.3–7.4 (3H, m). Anal. Calcd for $C_{14}H_9NO_2$: C, 75.33; H, 4.06; N, 6.27%. Found: C, 75.58; H, 4.17; N, 6.45%.

Other isomeric nitrodiphenylacetylenes were synthesized using the same procedure.

2-Nitrodiphenylacetylene (34a):[45] oil; ^{1}H NMR ($CDCl_3$) δ 8.07 (1H, dd, J = 6.3, 1.1 Hz), 7.71 (1H, dd, J = 6.3, 1.1 Hz), 7.5–7.7 (3H, m), 7.45 (1H, t, J = 6.3 Hz), 7.3–7.4 (3H, m).

4-Nitrodiphenylacetylene (34c):[45] mp 113–115 °C; ^{1}H NMR ($CDCl_3$) δ 8.22 (2H, d, J = 8.7 Hz), 7.67 (2H, d, J = 8.7 Hz), 7.5–7.6 (2H, m), 7.4–3.5 (3H, m).

NMR Experiments for the Reaction of Peroxynitrite with Organic Substrates. All reactions were performed at room temperature in an NMR tube containing a buffered deuterium oxide (D_2O), acetonitrile-d_3 (CD_3CN), or chloroform-d ($CDCl_3$) solution according to the following three different procedures (denoted hereafter as procedures **A**–**C**). A buffered D_2O solution (0.25 M, pH 7.0) was prepared by dissolving sodium dihydrogenphosphate and disodium hydrogenphosphate in a 4:6 molar ratio in D_2O. For the reactions in CD_3CN and $CDCl_3$, sodium dihydrogen phosphate (2 mg) was added and dispersed by sonication. The ^{1}H NMR analyses were carried out before and after the addition of peroxynitrite and the conversion and product yield were estimated on the basis of ^{1}H NMR peak area of each compound. *p*-Dichlorobenzene, *p*-dinitrobenzene, *p*-dimethoxybenzene, *p*-nitrotoluene, and 1,3,5-trinitrobenzene were employed as the internal standard for the reactions in organic solvents, while 3,3-dimethylglutaric acid and 2,2-dimethylmalonic acid were used for the reactions in aqueous systems. All major products were identified by direct comparison with the authentic specimens.

Procedure A: To a solution of a substrate (10 μmol) and an internal standard in a buffered solvent (0.6 mL) was added a solution of peroxynitrite (20–40 μmol) all at once under air. Immediately after the addition, the mixture was shaken vigorously and, after being kept for 10 min, it was analyzed by ^{1}H NMR and GC-MS.

Procedure B: All reactions were carried out in a manner similar to procedure A, excepting the additional presence of sodium hydrogencarbonate (10 μmol).

Procedure C (Control experiment): A solution of peroxynitrite (20–40 μmol) was added to a buffered solvent (0.2 mL) and allowed to stand for 10 min, during which time the peroxynitrite decomposed completely. Then, a solution (0.4 mL) of a given substrate (10 μmol) and an internal standard was added to this solution; after it was kept for 10 min, the reaction mixture was analyzed by ^{1}H NMR.

Spectral Data of Reaction Products. 4-Nitrophenol (2): ^{1}H NMR (D_2O) δ 8.02 (2H, d, J = 9.2 Hz), 6.71 (2H, d, J = 9.2 Hz).

2-Nitrophenol (3): ^{1}H NMR (D_2O) δ 7.93 (1H, dd, J = 8.8, 1.9 Hz), 7.45 (1H, t, J = 8.8 Hz), 6.95 (1H, d, J = 8.4 Hz), 6.75 (1H, t, J = 8.4 Hz).

Hydroquinone (4): ^{1}H NMR (D_2O) δ 6.65 (4H, s).

4-Methyl-2-nitrophenol (8a): ^{1}H NMR (D_2O) δ 7.80 (1H, s), 7.35 (1H, d, J = 8.4 Hz), 6.92 (1H, d, J = 8.4 Hz), 2.17 (3H, s); ^{1}H NMR (CD_3CN) δ 7.85 (1H, s), 7.43 (1H, d, J = 8.6 Hz), 6.97 (1H, d, J = 8.6 Hz), 2.29 (3H, s); CI-MS *m*/*z* (rel intensity) 154 (M+1, 100).

Nitrotyrosine (8b): ^{1}H NMR (D_2O) δ 7.7 (1H, s), 7.29 (1H,

d, J = 8.4 Hz), 6.88 (1H, d, J = 8.8 Hz), 3.7 (1H, m), 3.0 (2H, m).

4-Methyl-4-nitro-2,5-cyclohexadienone (9a):[46] ^{1}H NMR (D_2O) δ 7.22 (2H, d, J = 9.8 Hz), 6.31 (2H, d, J = 9.8 Hz), 1.82 (3H, s); ^{1}H NMR (CD_3CN) δ 7.16 (2H, d, J = 9.6 Hz), 6.33 (2H, d, J = 9.6 Hz), 1.88 (3H, s).

1,1′-Dimethyl-4,4′-dioxo-1,1′,4,4′-tetrahydrobiphenyl (10a): ^{1}H NMR (D_2O) δ 6.96 (2H, d, J = 9.6 Hz), 6.07 (2H, d, J = 9.6 Hz), 1.34 (3H, s); ^{1}H NMR (CD_3CN) δ 6.92 (2H, d, J = 9.6 Hz), 6.02 (2H, d, J = 9.6 Hz), 1.36 (3H, s).

2,2′-Dihydroxy-5,5′-dimethylbiphenyl (11a):[47] ^{1}H NMR ($CDCl_3$) δ 7.11 (2H, d, J = 8.4 Hz), 7.07 (2H, s), 6.93 (2H, d, J = 8.4 Hz), 2.30 (6H, s); CI-MS *m/z* (rel intensity) 215 (M+1, 100).

2,6-Di-*t*-butyl-4-nitrophenol (13): ^{1}H NMR (CD_3CN) δ 7.79 (2H, s), 1.31 (18H, s); CI-MS *m/z* (rel intensity) 252 (M+1, 100), 236 (9), 220 (5).

2,6-Di-*t*-butyl-1,4-benzoquinone (14):[48] ^{1}H NMR (CD_3CN) δ 6.47 (2H, s), 1.24 (18H, s); CI-MS *m/z* (rel intensity) 221 (M+1, 100).

4,4′-Dihydroxy-3,3′,5,5′-tetra-*t*-butylbiphenyl (15):[49] ^{1}H NMR (CD_3CN) δ 7.29 (4H, s), 1.42 (36H, s); CI-MS *m/z* (rel intensity) 410 (M^+, 100), 299 (52), 243 (44).

4-Nitrocatechol (16): ^{1}H NMR (D_2O) δ 7.65 (1H, d, J = 8.8 Hz), 7.55 (1H, s), 6.60 (1H, d, J = 8.8 Hz).

***N*-Methyl-*p*-toluidine (18):**[50] ^{1}H NMR (D_2O) δ 7.00 (2H, d, J = 8.4 Hz), 6.68 (2H, d, J = 8.4 Hz), 2.59 (3H, s), 2.09 (3H, s); ^{1}H NMR (CD_3CN) δ 6.93 (2H, d, J = 8.4 Hz), 6.49 (2H, d, J = 8.4 Hz), 2.70 (3H, s), 2.18 (3H, s); CI-MS *m/z* (rel intensity) 120 (M−1, 100), 91 (70), 77 (60).

***N*-Methyl-*N*-nitroso-*p*-toluidine (19):**[51] ^{1}H NMR (CD_3CN) δ 7.44 (2H, d, J = 8.4 Hz), 7.30 (2H, d, J = 8.4 Hz), 3.38 (3H, s), 2.31 (3H, s); CI-MS *m/z* (rel intensity) 151 (M+1, 57), 122 (100).

***N*,*N*-Dimethyl-*p*-toluidine-*N*-oxide (20):**[52] ^{1}H NMR (D_2O) δ 7.55 (2H, d, J = 8.4 Hz), 7.23 (2H, d, J = 8.4 Hz), 3.43 (6H, s), 2.22 (3H, s); ^{1}H NMR (CD_3CN) δ 7.74 (2H, d, J = 8.4 Hz), 7.27 (2H, d, J = 8.4 Hz), 3.60 (6H, s), 2.36 (3H, s); CI-MS *m/z* (rel intensity) 150 (M−1, 100).

Benzotriazole (22): ^{1}H NMR (CD_3CN) δ 7.82 (2H, dd, J = 6.2, 3.2 Hz), 7.44 (2H, dd, J = 6.2, 3.2 Hz); MS *m/z* (rel intensity) 120 (M+1, 100).

Unidentified product (**23**): ^{1}H NMR (CD_3CN) δ 7.65 (1H, d, J = 8.4 Hz), 7.15 (1H, t, J = 8.4 Hz), 6.82 (1H, d, J = 8.4 Hz), 6.69 (1H, t, J = 8.4 Hz).

2,3-Epoxy-2,3-dihydro-1,4-naphthoquinone (25):[53] ^{1}H NMR (CD_3CN) δ 7.94 (2H, dd, J = 5.6, 3.2 Hz), 7.82 (2H, dd, J = 5.6, 3.2 Hz), 4.02 (2H, s); CI-MS *m/z* (rel intensity) 175 (M+1, 100%), 146 (23), 105 (35).

Acetophenone (27): ^{1}H NMR (CD_3CN) δ 7.95 (2H, d, J = 8.4 Hz), 7.5–7.6 (1H, m), 7.49 (2H, t, J = 8.4 Hz), 2.55 (3H, s).

***trans*-Stilbene Oxide (30):** ^{1}H NMR ($CDCl_3$) δ 7.3–7.4 (10H, m), 3.87 (2H, s).

Benzil (31): ^{1}H NMR ($CDCl_3$) δ 7.98 (4H, dd, J = 7.2, 1.5 Hz), 7.67 (2H, t, J = 7.2 Hz), 7.51 (4H, t, J =7.2 Hz).

This work was financially supported by a grant for Specially Promoted Scientific Research (No. 08101003) and a grant (No. 12640576) from the Ministry of Education, Science, Sports and Culture. N.N. thanks the Japan Society for the Promotion of Science for a Fellowship (No. 9191).

References

1 R. E. Huie and S. Padmaja, *Free Rad. Res. Commun.*, **18**, 195 (1993).

2 J. S. Beckman, T. W. Beckman, J. Chen, P. A. Marshall, and B. A. Freeman, *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620 (1990).

3 C. Szabo and H. Ohshima, *Nitric Oxide: Biol. Chem.*, **1**, 373 (1997).

4 L. J. Kennedy, K. Moore, J. L. Caulfield, S. R. Tannenbaum, and P. C. Dedon, *Chem. Res. Toxicol.*, **10**, 1118 (1997).

5 D. M. Kuhn and T. J. Geddes, *J. Biol. Chem.*, **274**, 29726 (1999).

6 K. Takakura, J. S. Beckman, L. A. MacMillan-Crow, and J. P. Crow, *Arch. Biochem. Biophys.*, **369**, 197 (1999).

7 L. A. MacMillan-Crow and J. A. Thompson, *Arch. Biochem. Biophys.*, **366**, 82 (1999).

8 A. van der Vliet, C. A. O'Neill, B. Halliwell, C. E. Cross, and H. Kaur, *FEBS Lett.*, **339**, 89 (1994).

9 A. Daiber, M. Mehl, and V. Ullrich, *Nitric oxide: Biol. Chem.*, **2**, 259 (1998).

10 M. S. Ramazanian, S. Padmaja, and W. H. Koppenol, *Chem. Res. Toxicol.*, **9**, 232 (1996).

11 J-N. Lemercier, S. Padmaja, R. Cueto, G. L. Squadrito, R. M. Uppu, and W. A. Pryor, *Arch. Biochem. Biophys.*, **345**, 160 (1997).

12 M. Balazy, P. M. Kaminski, K. Mao, J. Tan, and M. S. Wolin, *J. Biol. Chem.*, **273**, 32009 (1998).

13 A. van der Vliet, P. A. C. Hoen, P. S.-Y. Wong, A. Bast, and C. E. Cross, *J. Biol. Chem.*, **273**, 30255 (1998).

14 V. B. O'Donnell, J. P. Eiserich, P. H. Chumley, M. J. Jablonsky, N. R. Krishna, M. Kirk, S. Barnes, V. M. Darley-Usmar, and B. A. Freeman, *Chem. Res. Toxicol.*, **12**, 83 (1999).

15 R. Radi, J. S. Beckman, K. M. Bush, and B. A. Freeman, *Arch. Biochem. Biophys.*, **288**, 481 (1991).

16 B. Alvarez, H. Rubbo, M. Kirk, S. Barnes, B. A. Freeman, and R. Radi, *Chem. Res. Toxicol.*, **9**, 390 (1996).

17 Y. Kato, S. Kawakishi, T. Aoki, K. Itakura, and T. Osawa, *Biochem. Biophys. Res. Commun.*, **234**, 82 (1997).

18 H. Zhang, G. L. Squadrito, and W. A. Pryor, *Biochem. Biophys. Res. Commun.*, **251**, 83 (1998).

19 J. C. Niles, S. Burney, S. P. Singh, J. S. Wishnok, and S. R. Tannenbaum, *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 288 (1999).

20 K. A. Skinner, C. R. White, R. Patel, S. Tan, S. Barnes, M. Kirk, V. Darley-Usmar, and D. A. Parks, *J. Biol. Chem.*, **273**, 24491 (1998).

21 L. S. Nakao, D. Ouchi, and O. Augusto, *Chem. Res. Toxicol.*, **12**, 1010 (1999).

22 R. M. Uppu, G. W. Winston, and W. A. Pryor, *Chem. Res. Toxicol.*, **10**, 1331 (1997).

23 S. Goldstein and G. Czapski, *Nitric Oxide: Biol. Chem.*, **1**, 417 (1997).

24 N. Nonoyama, K. Hisatome, C. Shoda, and H. Suzuki, *Tetrahedron Lett.*, **40**, 6939 (1999).

25 N. Nonoyama, K. Chiba, K. Hisatome, H. Suzuki, and F. Shintani, *Tetrahedron Lett.*, **40**, 6933 (1999).

26 H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J. C. Martin, C. D. Smith, and J. S. Beckman, *Arch. Biochem. Biophys.*, **298**, 431 (1992).

27 A. van der Vliet, J. P. Eiserich, C. A. O'Neill, B. Halliwell, and C. E. Cross, *Arch. Biochem. Biophys.*, **319**, 341 (1995).

28 C. Daveu, C. Servy, M. Dendane, P. Marin, and C. Ducrocq, *Nitric Oxide: Biol. Chem.*, **1**, 234 (1997).

29 F. Shintani, T. Kinoshita, S. Kanba, T. Ishikawa, E. Suzuki, N. Sasakawa, R. Kato, M. Asai, and T. Nakaki, *J. Biol. Chem.*, **271**, 13561 (1996).

30 M. Lehnig, *Arch. Biochem. Biophys.*, **368**, 303 (1999).
31 R. N. Loeppky, S. P. Singh, S. Elomari, R. Hastings, and T. E. Theiss, *J. Am. Chem. Soc.*, **120**, 5193 (1998).
32 C. J. Craig and K. K. Purushothaman, *J. Org. Chem.*, **35**, 1721 (1970).
33 T. P. Misko, R. J. Schilling, D. Salvemini, W. M. Moore, and M. G. Currie, *Anal. Biochem.*, **214**, 11 (1993).
34 R. M. Uppu and W. A. Pryor, *J. Am. Chem. Soc.*, **121**, 9738 (1999).
35 S. Patai, "The Chemistry of Alkenes," Wiley, New York (1964), Vol. 1, pp. 512–517.
36 G. Merényi, J. Lind, S. Goldstein, and G. Czapski, *J. Phys. Chem. A.*, **103**, 5685 (1999).
37 J. W. Coddington, J. K. Hurst, and S. V. Lymar, *J. Am. Chem. Soc.*, **121**, 2438 (1999).
38 G. R. Hodges and K. U. Ingold, *J. Am. Chem. Soc.*, **121**, 10695 (1999).
39 S. Goldstein and G. Czapski, *J. Am. Chem. Soc.*, **121**, 2444 (1999).
40 W. W. Hartman and L. J. Roll, *Org. Synth.*, II, 460 (1943).
41 R. M. Uppu, G. L. Squadrito, R. Cueto, and W. A. Pryor, *Methods Enzymol.*, **269**, 311 (1996).
42 G. Maerkl and A. Merz, *Synthesis*, **1973**, 295.
43 J. M. Bakke, *Acta Chem. Scand. Ser.* B, **29**, 667 (1975).
44 N. A. Bumagin, L. I. Sukhomlinova, T. P. Tolstaya, and I. P. Beletskaya, *Tetrahedron Lett.*, **37**, 897 (1996).
45 K. Okubo, M. Furuume, M. Enna, M. Miura, and M. Nomura, *J. Org. Chem.*, **58**, 4716 (1993).
46 J. H. Ridd, S. Trevellick, and J. P. B. Sandall, *J. Chem. Soc., Perkin Trans. 2*, **1992**, 573.
47 J. Marquet, L. Refecas, A. Cantos, M. Moreno-Manas, and M. Cervera, *Tetrahedron*, **49**, 1297 (1993).
48 I. Pallagi, A. Toro, and O. Farkas, *J. Org. Chem.*, **59**, 6543 (1994).
49 K. Omura, *J. Org. Chem.*, **63**, 10031 (1998).
50 R. C. Cambie, G. R. Clark, S. L. Coombe, S. A. Coulson, P. S. Rutledge, and P. D. Woodgate, *J. Organomet. Chem.*, **507**, 1 (1996).
51 J. Calvert, L. Eberson, M. P. Hartshorn, and J. O. Svensson, *J. Chem. Soc., Perkin Trans. 2*, **1995**, 645.
52 J. R. L. Smith, J. M. Linford, L. C. McKeer, and P. M. Morris, *J. Chem. Soc., Perkin Trans. 2*, **1984**, 1099.
53 R. A. Flowers, S. Naganathan, P. Dowd, E. M. Arnett, and S. W. Ham, *J. Am. Chem. Soc.*, **115**, 9409 (1993).