Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 2405–2419

BIOORGANIC & MEDICINAL CHEMISTRY

Syntheses of Amino Nitrones. Potential Intramolecular Traps for Radical Intermediates in Monoamine Oxidase-catalyzed Reactions

Boyu Zhong, Xingliang Lu and Richard B. Silverman*

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208-3113, USA

Received 8 May 1998; accepted 18 August 1998

Abstract—Monoamine oxidase (MAO) is a flavin-dependent enzyme that catalyzes the oxidative deamination of a variety of amine neurotransmitters and toxic amines. Although there have been several studies that support the intermediacy of an amine radical cation and an α-radical during enzyme catalysis, there is no direct, i.e. EPR, evidence for these species as they are formed. Amino nitrones have been designed which, upon radical formation would produce an intermediate that is a resonance structure of the corresponding nitroxyl radical, which should be observable by EPR spectroscopy. Syntheses of seven different amino nitrones, three acyclic, and four cyclic analogues were attempted. The protected amino nitrones were stable, but all three of the acyclic amino nitrones were unstable. One of the cyclic analogues was very stable (**39**), one was stable only in organic solvents (**40**), one was stable only in aqueous medium below pH 6.5 (**41**), and the other (**42**) was stable for just a short time at room temperature, decomposing to a stable free radical. None of these analogues produced a MAO-catalyzed radical, yet **41** is a poor substrate ($K_m = 0.2$ mM; $k_{cat} = 0.034\ min^{-1}$) and **39** is a mixed inhibitor ($K_i = 26.5$ mM). Although this approach does not appear to be applicable to amino nitrones, it should be a valuable approach for other enzymes where radical intermediates are suspected and nonamine nitrones can be utilized.

Introduction

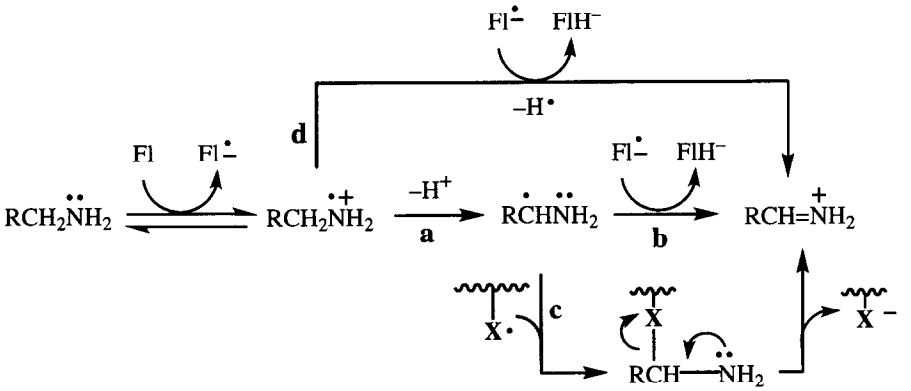
Monoamine oxidase (MAO, EC 1.4.3.4) is a flavin dependent enzyme that catalyzes the oxidative deamination of amine neurotransmitters such as 5-hydroxytryptamine and catecholamines and toxic amines in food.[1] The stoichiometric reaction of the enzyme is shown in Scheme 1. Although the reaction is usually represented as a primary amine, this enzyme also can efficiently oxidize many secondary and tertiary amines.[2,3] Over the years, a radical mechanism for MAO has emerged as the most likely pathway for this enzyme (Scheme 2).[4] Varieties of compounds were designed as alternate substrates to test a radical mechanism, and each one has provided indirect evidence to support a radical mechanism. Laser flash photolysis experiments of Maeda and Ingold[5] demonstrated that cyclopropylaminyl radicals were too unstable to be detected and cyclobutylaminyl radicals decomposed rapidly with homolytic cleavage of the ring. Based on these results, a series of *N*-cyclopropylamine analogues was designed and the MAO-catalyzed oxidation of each was studied.[6] All of these cyclopropyl substrate analogues undergo ring cleavage and attachment to the enzyme. The conclusion was that all of these results could be rationalized in terms of an initial single-electron transfer from the cyclopropyl amine to the flavin cofactor to give the cyclopropylamine radical cation and the flavin semiquinone. A second approach to demonstrate the intermediacy of an amine radical cation was the construction of an alternate substrate that would be expected to undergo a known one-electron rearrangement if the corresponding amine radical cation formed. 1-Phenylcyclobutylamine (**1**) was converted into 2-phenyl-1-pyrroline (**4**) by MAO in addition to inacti-

Key words: Monoamine oxidase; radical intermediate; amino nitrones; enzyme mechanism.
*Corresponding author. Tel: 847 491 5653; Fax: 847 491 7713; E-mail: agman@chem.nwu.edu

0968-0896/98/$ - see front matter
PII: S0968-0896(98)00191-6

$$RCH_2NH_2 + O_2 + H_2O \xrightarrow{MAO} RCHO + H_2O_2 + NH_3$$

Scheme 1.



Scheme 2.

vating the enzyme. Scheme 3 depicts a mechanism that is consistent with the initial formation of an amine radical cation that undergoes homolytic cleavage to radical **2** followed by *endo*-cyclization to radical **3** and second electron oxidation to **4**. This mechanistic pathway is known for a variety of chemical rearrangements[7] and was demonstrated for the enzyme cytochrome P-450,[8] a heme-dependent enzyme for which radical chemistry is well known. When this MAO-catalyzed reaction was carried out in the presence of a nitrone spin trap, the EPR spectrum of an organic radical was observed.[9] However, the identity of this radical was never elucidated, so it is not clear if it was derived directly from an intermediate.

Evidence for the α-radical comes from three sets of modified substrate/inactivator molecules.

(Aminomethyl)cubane (**5**) is a substrate and inactivator of MAO; oxidative turnover leads to destruction of the cubane ring, consistent with the formation of an intermediate cubylcarbinyl radical.[10] Cinnamylamine 2,3-oxide (**6**) is oxidized by MAO with cleavage of the C–C bond of the epoxide, supporting the α-radical.[11] Finally, ^{14}C was incorporated into the carbonyl carbon of *cis*- and *trans*-5-aminomethyl-3-(4-methoxyphenyl)dihydrofuran-2-(3*H*)-one (**7**) and it was shown that $^{14}CO_2$ is generated by incubation with MAO.[12] Loss of carbon dioxide can only be rationalized by the formation of an α-radical.

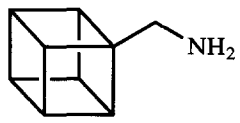
All of these modified substrate enzyme-catalyzed reactions provide indirect evidence for the intermediacy of either an amine radical cation or an α-radical in MAO-catalyzed oxidation reactions. Attempts to detect a radical intermediate by EPR spectroscopy, even in the presence of spin traps[13] or by stopped-flow spectrophotometry[14] failed, presumably because of the short lifetime of the radical species and the relatively slow reaction with spin traps. Even the very efficient nitrone spin traps have intermolecular rate constants of only $10^8\,M^{-1}s^{-1}$,[15] but spin trapping rates need to be larger than $10^{10}\,M^{-1}s^{-1}$ to compete with second electron transfer. Furthermore, since the radicals are generated inside the enzyme active site, the spin trap has to get into the active site and be oriented appropriately for reaction with the incipient radical.

We thought that a more efficient approach to 'spin trap' the amine radical cation or the α-radical was by incorporation of a spin trap into the substrate in such a way that formation of either of these radical species (**8** or **10**) would simply be resonance structures of the much more stable nitroxyl radical (**9** or **11**, Scheme 4). Consequently, the trapping rate should be comparable to the bond vibration frequency, i.e. $10^{12}\,s^{-1}$, which is definitely competitive with that of the second electron transfer. Furthermore, since the nitrone is part of the substrate structure, it will be brought into the active site. Since the spin density in the spin adduct is highly delocalized over almost the entire molecule, the EPR signals will be unique. Here we describe this approach, which, because of the hydrolytic reactivity of most of the amino nitrones in this instance, was unsuccessful.

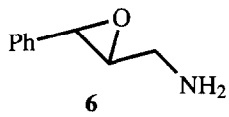
Results and Discussion

Model reactions for the amino nitrones

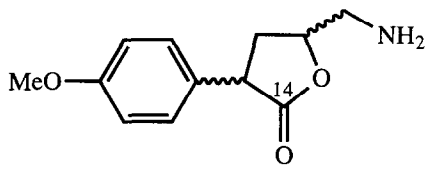
Because of the high reactivity of the nitrone group,[16] amino nitrones were expected to be synthetically



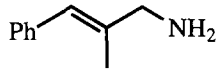
5



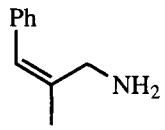
6



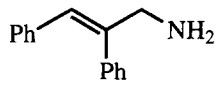
7



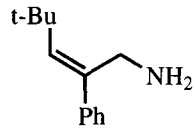
12



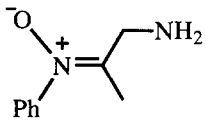
13



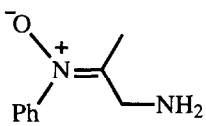
14



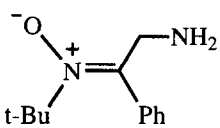
15



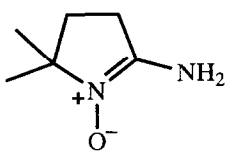
28a



28b

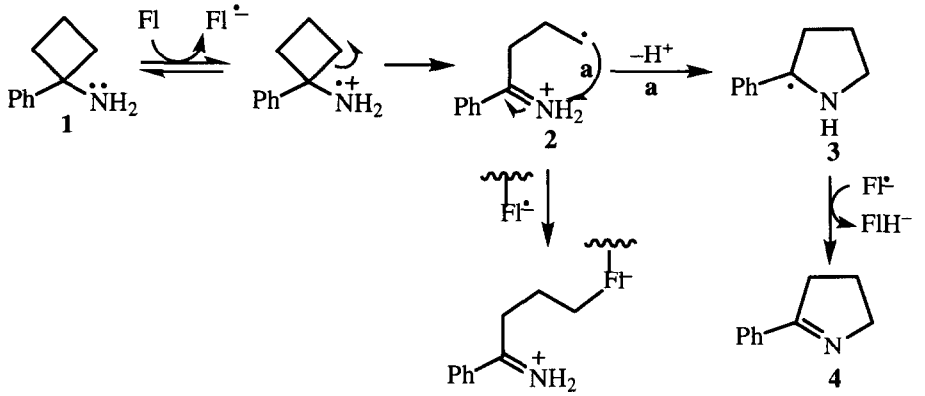


29

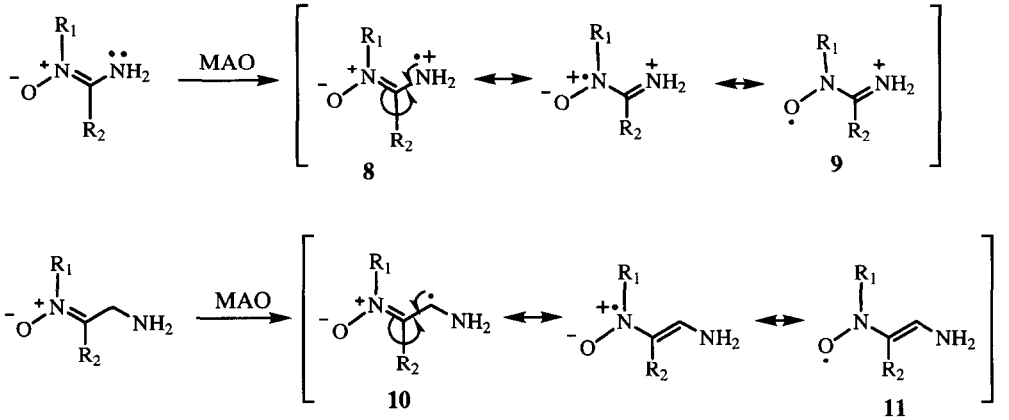


39

challenging. To avoid synthesizing amino nitrones, which were not substrates for MAO, model compounds that did not contain a nitrone were first synthesized and tested as substrates for MAO (**12–15**). In these model compounds a CH group replaces the NO group in the nitrone, aromatic functionalities are incorporated to improve their binding affinities with MAO, and sterically hindered groups, such as *tert*-butyl or phenyl, are used to stabilize the radical product which is expected to be formed in the enzymatic reaction of the corresponding amino nitrone.[17] The synthetic pathway for the model compounds is shown in Scheme 5. Only **15** was made by a slightly different route because treatment of **24** with trifluoroacetic anhydride did not produce the eliminated product (**27**); instead, **25** was obtained. Treatment of **25** with mesyl chloride in pyridine at −20 °C gave the mesylated analogue (**26**), which was converted to **27** with potassium *tert*-butoxide in refluxing THF. These aminomethyl alkenes exhibited different water solubilities because of the hydrophobic substituents. Compounds **12** and **13** can be dissolved in water even at pH 9.0. Compound **14** has two phenyl groups; therefore, its water solubility is so poor that it cannot be dissolved even in a mixed solvent containing



Scheme 3.



Scheme 4.

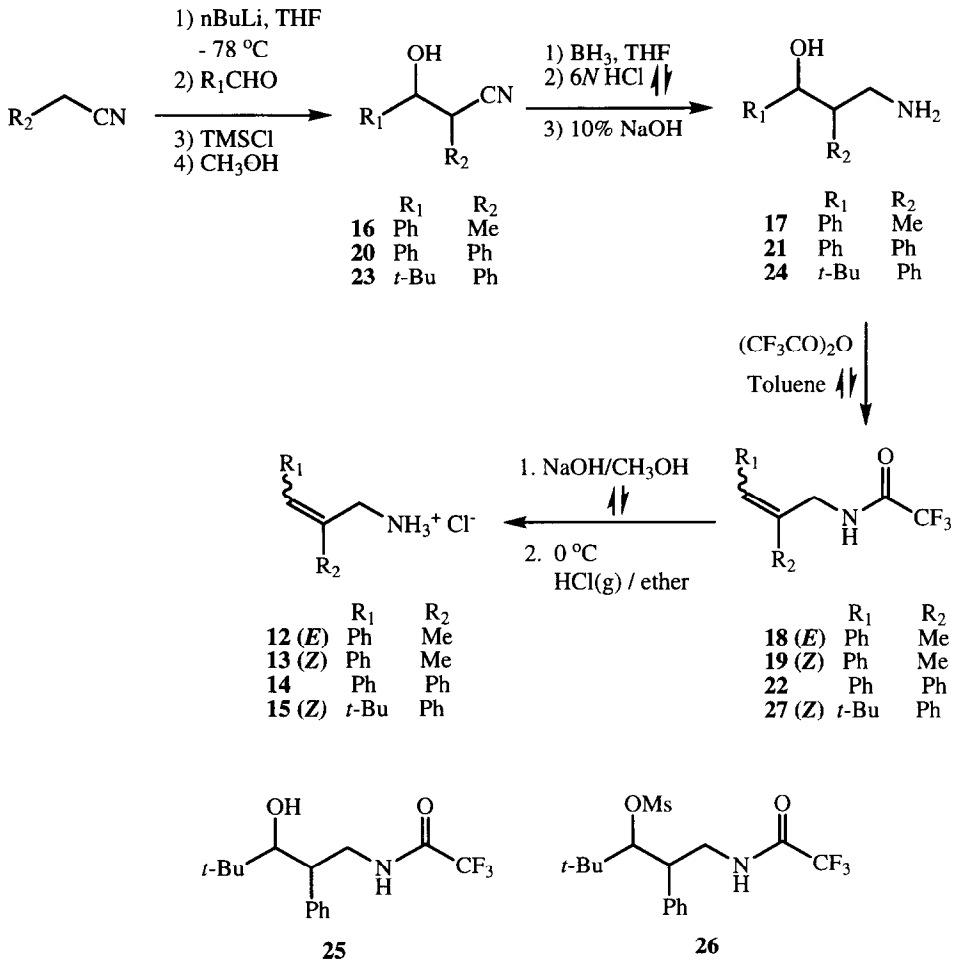
25% DMSO. Compound **15**, containing a *tert*-butyl group and a phenyl group, is soluble in a buffer solution at pH 7.2 but is insoluble at pH 9.0.

Kinetic data for the model compounds are summarized in Table 1. Compound **12** is an excellent substrate for MAO B. Its kinetic constants are comparable to those of benzylamine, an excellent substrate for MAO B. Compound **13** is a good substrate too. However, **15** is a poor substrate because its high K_m, and compound **14** could not be analyzed in aqueous solutions because of its poor solubility. Based on these results, amino nitrones **28** and **29** were synthesized first.

Syntheses of amino nitrones

Typical reactions of a nitrone include reduction, oxidation, nucleophilic addition, acid- and base-catalyzed enamine formation, electrophilic addition, *O*-alkylation, isomerization, alkene elimination, and 1,3-dipolar addition.[18] Considering that an amino group is reactive toward the nitrone group and that the nitrone group is very fragile, synthetic pathways must be carefully designed. Stimulated by the increasing importance of nitrones in organic synthesis, many new methodologies for the synthesis of an isolated nitrone group have been developed.[19] However, there is no protecting group for the nitrone functionality; on the other hand, general methods for forming an amino group are too harsh for the survival of a nitrone group; thus, the amino group should be made first and be protected before the incorporation of the nitrone group into the molecule. Additionally, the protecting group of the amine should be removable under mild conditions that do not destroy the nitrone. Of the known protecting methodologies for an amino group, Boc strategy appears to be compatible with the nitrone functionality.

The Boc protected *Z* and *E* isomers **28a** and **28b** were synthesized via the synthetic route in Scheme 6; however, neither compound is stable at room temperature, and they decompose during silica gel purification. When treated with an acid solution (either TFA in dichloromethane or



Scheme 5.

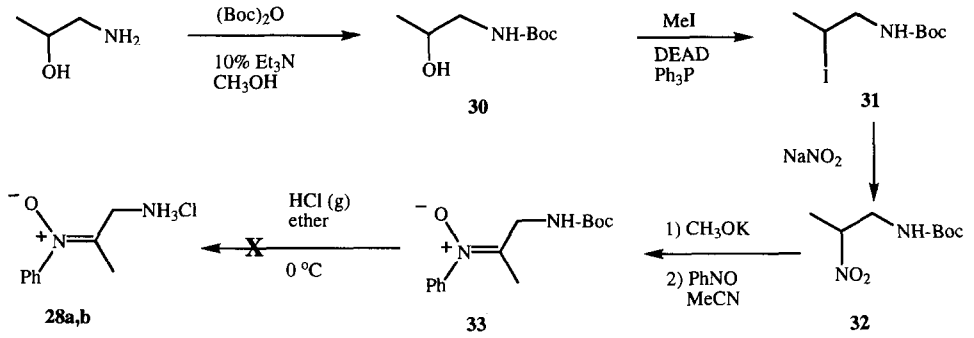
HCl in methanol), they are destroyed and turn into a black mixture.

A synthetic pathway to amino nitrone **29** is shown in Scheme 7. The protected amino nitrone was obtained as an apparently stable crude product; however, further purification on silica gel resulted in its decomposition.

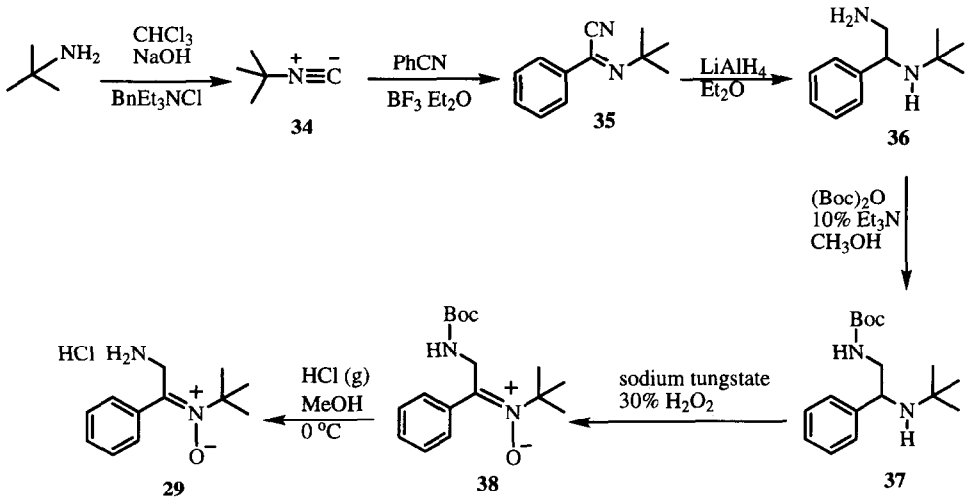
To increase the stability of the nitrone, cyclic analogues were made. Actually, amino nitrone **39** is a known compound, and it is stable in aqueous media.[20] Other analogues made include compounds **40–42** (Schemes 8–10). Compound **40** is stable in organic solvents but not in aqueous solution, whereas **41** is stable in aqueous medium at pH below 6.5 but decomposes in the free base form. Compound **42** is stable for only a short time at room temperature and turns bright pink in color, exhibiting a very complex EPR spectrum. Consequently, only **39–41** were at all suitable for enzymological study.

Table 1. Kinetic data for **12–15**

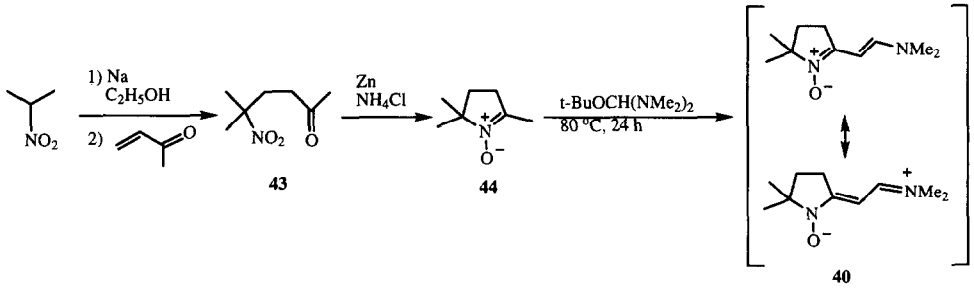
Compd	Buffer	pH	K_m (μm)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M\ min)^{-1}$
Benzylamine	Tris	9.0	340	270	0.79
12	Tris	9.0	380	243	0.64
13	Tris	9.0	700	20	0.03
14	Phosphate	7.2	—	—	—
15	Phosphate	7.2	30,000	178	0.006



Scheme 6.



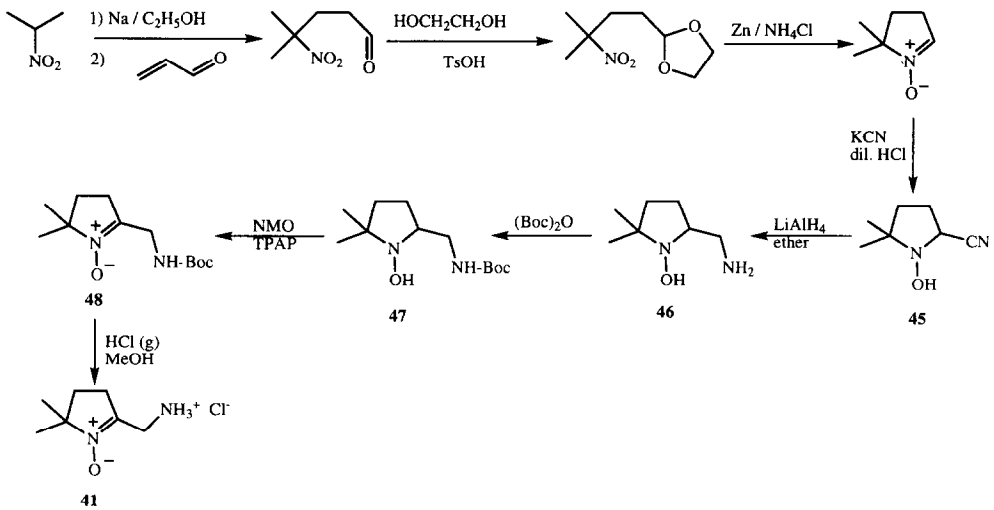
Scheme 7.



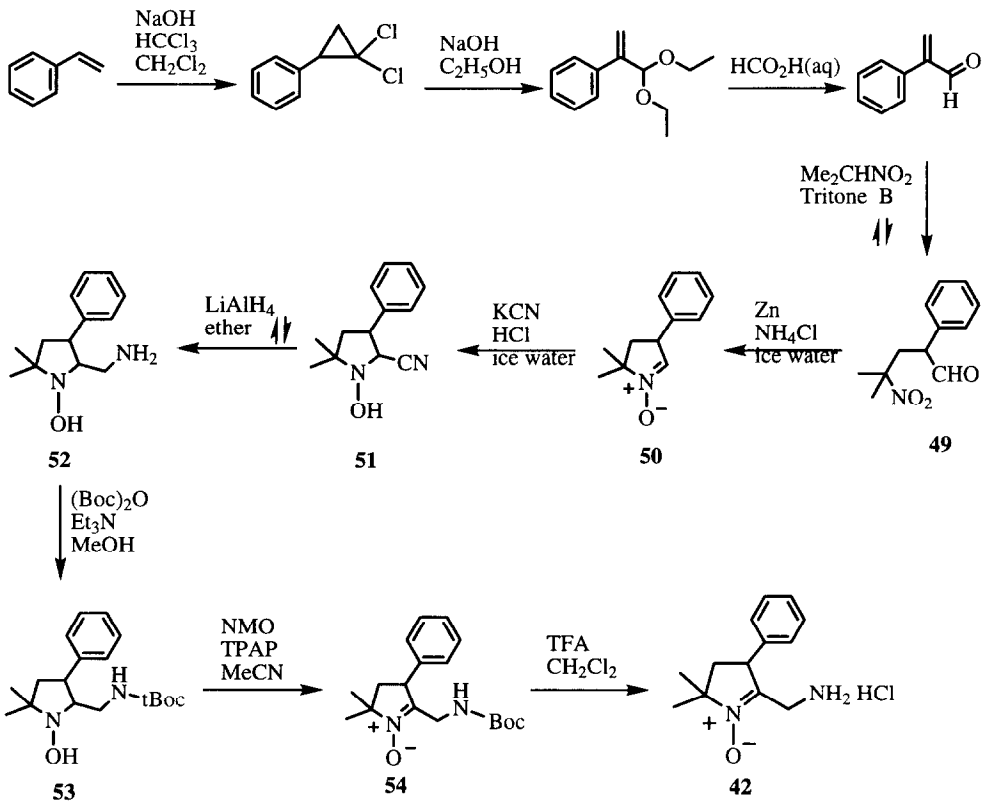
Scheme 8.

Enzymology with amino nitrones

MAO catalyzed deamination requires the free amine form; therefore, effective oxidation generally occurs under neutral or basic conditions. The pH dependence of the activity and stability of MAO B was studied, and the results are shown in Figure 1. The enzyme is stable when the pH is higher than 5.0. The optimum pH value for the enzymatic reaction, however, is about 9.0, which corresponds to a large proportion of the free amine form. The activity of the enzyme decreases dramatically as the pH decreases. Below pH 5, the enzyme loses the activity irreversibly. On the other hand, the stability of the amino nitrones largely depends on the solvents and the pH values of the solutions.



Scheme 9.



Scheme 10.

The stability of the amino nitrones and the activity of MAO B in different solvents and different solutions are summarized in Table 2. The conditions used for the enzymology studies are marked with circles. Nitrones **39** and **40** were incubated with MAO B for 2 h or overnight (18 h), and then samples were prepared for the EPR experiment. No signals were observed in their EPR spectra. This is, most likely, because MAO B catalyzes

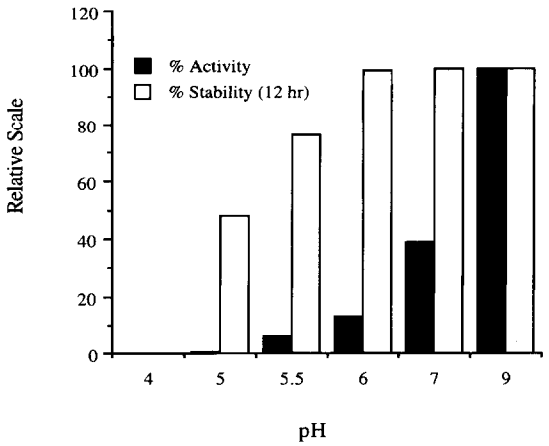


Figure 1. The pH dependence of activity and stability of bovine liver MAO-B.

the oxidation of amines, and the amines in **39** and **40** are more like amide groups; the oxidation potentials may be too high for MAO.

The incubation solution of amino nitrone **41** with MAO gave a triplet EPR signal at $g = 2.0050$. However, the same signal occurs in the EPR spectrum of the amino nitrone control sample. Several possible reasons may contribute to the failure to observe the expected EPR signal: the nitrone group is not properly shielded so that it is difficult for the probe to enter the active site of MAO; the binding ability with the enzyme is poor because of the lack of an aromatic functional group; or the poor substrate and the low activity of the enzyme under the incubation conditions result in an undetectably slow enzymatic reaction.

A kinetic analysis of the activity of **39**–**41** as substrates and/or inhibitors of MAO B was carried out to determine

Table 2. Summary of stability of MAO-B and amino nitrones

Solvent	39	40	41	MAO-B
H_2O (acidic)	S	D	(S)	pH > 5
			pH = < 5.5	
H_2O (neutral)	(S)	D	D	S
H_2O (basic)	D	D	D	S
MeOH	S	S	S	X
C_6H_6	Insol.	(S)	Insol.	0.5% H_2O

D: decomposed; S: soluble or stable; X: no activity; Insol: insoluble; encircled S: conditions used in the enzymology studies.

if these possibilities are real. In fact, only **41** is a substrate, having K_m and k_{cat} values of 0.2 mM and 0.034 min^{-1}, respectively. The k_{cat}/K_m for **41**, therefore, is 0.17 $min^{-1} mM^{-1}$ but that for the substrate benzylamine is 800 $min^{-1} mM^{-1}$. This 4700-fold difference may account for the inability to observe an EPR signal with **41**. Compound **39** is an inhibitor, but only a weak mixed inhibitor ($K_i = 26.5$ mM), not a competitive inhibitor. This also is consistent with the lack of an EPR signal for **39**.

In conclusion, the approach described here for trapping radical intermediates in enzyme-catalyzed reactions has potential. However, amino nitrones appear to be too unstable to exemplify this approach. This methodology could be applied to other enzyme systems that do not catalyze reactions of amines, such as cytochrome P-450.

Experimental

General methods

Optical spectra and MAO assays were recorded on a Perkin–Elmer Lambda 1, a Perkin–Elmer Lambda 10, or a Beckman DU-40 UV/Vis spectrophotometer. EPR spectra were recorded on a modified Varian E4 EPR spectrometer with a Varian TE_{102} sample cavity. NMR spectra were recorded on a Varian Gemini 300 MHz, a Varian VXR 300 MHz, or a Varian Unity plus 400 spectrometer. Chemical shifts are reported as δ values in parts per million down field from Me_4Si as the internal standard in $CDCl_3$. An Orion Research Model 701 pH meter with a general combination electrode was used for pH measurements. Mass spectra were obtained on a VG Instrument VG70-250SE high-resolution spectrometer with Maspec Data System. Elemental analyses were performed by either Oneida Research Service Inc. (Whitesboro, NY) or the Department of Geological Sciences at Northwestern University. Melting points were taken on a Fisher–Johns melting point apparatus and are uncorrected. The flash column chromatography was carried out with Merck silica gel 60 (230–400 mesh ASTM). TLC was run with EM Science silica gel 60 F_{254} precoated glass plates.

Reagents

Deuterium oxide, chloroform-*d*, and dimethyl sulfoxide-d_6 (99% atom% D) were purchased from either Aldrich or Cambridge Isotope Laboratories. Other reagents were purchased from chemical companies without further purification except for the following: anhydrous ether and tetrahydrofuran (THF) were distilled over sodium metal under nitrogen; anhydrous dichloromethane was distilled over calcium hydride.

2-Cyano-1-phenylpropanol (16). To a cooled (−78 °C) solution of propionitrile (1.65 g, 30 mmol) in dried THF (30 mL) was added 2.5 M *n*-butyllithium solution in hexane (10 mL, 25 mmol). The reaction mixture was stirred for 2 h at −78 °C. A solution of benzaldehyde (2.12 g, 20 mmol) in dried THF (15 mL) was slowly added over 20 min, and then chlorotrimethylsilane (8 mL) was added. The resulting solution was stirred at −78 °C for another 20 min. After addition of methanol (10 mL), the reaction solution was allowed to warm up to room temperature. Removal of the solvent gave a viscous oil, which was then purified by flash chromatography (4% ethyl acetate/hexanes, R_f 0.20) to give two colorless liquids (3.70 g, 91%, diastereomer ratio A:B = 3:1). The diastereomeric mixture was used in the next step.

A: ^{1}H NMR ($CDCl_3$) δ 1.28 (d, 3H), 2.40 (d, 1H), 3.02 (m, 1H), 4.83 (q, 1H), 7.35–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.5, 34.0, 74.1, 121.2, 126.5, 128.7, 128.7, 139.8; HRMS calcd for $C_{10}H_{11}NO$ 161.0841, found 161.0834. Anal. calcd for $C_{10}H_{11}NO$: C 74.51, H 6.88, N 8.69. Found: C 73.94, H 6.99, N 8.67.

B: ^{1}H NMR ($CDCl_3$) δ 1.25 (d, 3H), 2.30 (d, 1H), 2.96 (m, 1H), 4.74 (q, 1H), 7.35–7.45 (m, 5H). ^{13}C NMR ($CDCl_3$) δ 14.8, 34.6, 74.2, 121.1, 126.4, 128.9, 128.9, 140.1; HRMS calcd for $C_{10}H_{12}NO$ (M + 1) 162.0919. Found 162.0915.

3-Amino-2-methyl-1-phenylpropanol (17). To a cooled (0 °C) solution of 1.0 M borane in THF (30 mL, 30 mmol) was added **16** (2.1 g, 13 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent on a rotary evaporator, 6.0 N aqueous hydrochloric acid solution (20 mL) was added. The mixture was heated to reflux for 2 h, and then was cooled in an ice-water bath, sodium hydroxide pellets (7.2 g) were added, and after filtration the solution was extracted with ether and dried (Na_2SO_4). Removal of the solvent led to a brown oil, which was used directly in the next step without further purification.

***Z*/*E*-β-Methyl-β-(*N*-trifluoroacetyl-aminomethyl)styrene (18, 19).** A mixture of crude 3-amino-2-methyl-1-phenylpropanol (2.5 g), trifluoroacetic anhydride (10 mL), and toluene (200 mL) was heated to reflux for 2 h. Then the solvent was distilled under vacuum to yield a brown oil. This liquid was purified over silica gel (10% ethyl acetate in hexanes) to give two products; major product (0.63 g, R_f 0.25); minor product (0.12 g, R_f 0.34).

***E*-β-Aminomethyl-β-methylstyrene hydrochloride (12).** The major product obtained in the previous step was dissolved in methanol (20 mL) and THF (10 mL), and then sodium hydroxide (0.40 g) was added. The reaction mixture was heated to reflux for 2 h. After removal of the solvent, water (10 mL) was added. The resulting mixture was extracted three times with ether (10 mL each), dried with anhydrous potassium carbonate, and cooled in an ice-water bath. Anhydrous hydrogen chloride gas was bubbled through the solution to give a white precipitate (0.60 g, 100%). The stereochemistry of this product was confirmed by its NOE NMR spectra. ^{1}H NMR (D_2O) δ 1.89 (s, 3H), 3.64 (s, 2H), 6.68 (s, 1H), 7.25–7.40 (m, 5H). Anal. calcd for $C_{10}H_{11}NCl$: C 65.39, H 7.68, N 7.63. Found: C 65.51, H 7.60, N 7.76.

***Z*-β-Aminomethyl-β-methylstyrene (13).** The minor product was processed through the same procedure as that above to give a white precipitate (0.10 g, 100%). ^{1}H NMR (D_2O) δ 1.92 (s, 3H), 3.73 (s, 2H), 6.69 (s, 1H), 7.15–7.40 (m, 5H).

α-Cyano-α′-hydroxy-bibenzyl (20). To a cooled (−78 °C) solution of benzyl cyanide (3.51 g, 30 mmol) in dried THF (30 mL) was added 2.5 M *n*-butyllithium solution in hexane (10 mL, 25 mmol). The reaction mixture was stirred for 2 h at −78 °C. A solution of benzaldehyde (2.12 g, 20 mmol) in dried THF (15 mL) was slowly added over 20 min. Then chlorotrimethylsilane (8 mL) was added. The resulting solution was stirred at −78 °C for another 20 min. After addition of methanol (10 mL), the reaction solution was allowed to warm up to room temperature. Removal of the solvent gave a viscous oil, which was then flash chromatographed (20% ethyl acetate-hexanes, R_f 0.20) to give a white crystalline solid (4.69 g, 97%, diastereomer ratio 4:1). Major product: mp 101–102 °C; ^{1}H NMR ($CDCl_3$) δ 2.68 (d, 1H), 4.05 (d, 1H), 4.96 (q, 1H), 7.20–7.40 (m, 10H); ^{13}C NMR ($CDCl_3$) δ 47.4 (d), 76.3, 118.9, 126.3, 128.5, 128.6, 128.8, 129.0, 132.5, 139.5; HRMS calcd for $C_{15}H_{13}NO$ 223.0997, found 223.0974. Anal. calcd for $C_{15}H_{13}NO$: C 80.69, H 5.87, N 6.27. Found C 80.77, H 5.91, N 6.41.

α-Aminomethyl-α′-hydroxy-bibenzyl (21). To a stirring solution of 1.0 M borane in THF (33 mL, 33 mmol) was added α-cyano-α′-hydroxybibenzyl (2.2 g, 10 mmol). The reaction mixture was stirred at room temperature for 20 h. After removal of the solvent on a rotary evaporator, 6.0 N aqueous hydrochloric acid solution (20 mL) was added. The mixture was heated to reflux for 2 h and then was cooled in an ice-water bath, sodium hydroxide pellets (7.2 g) were added, and after filtration the solution was extracted four times with ether (10 mL each), and dried (Na_2SO_4). Removal of the solvent led to a brown oil that was used in the next step without further purification.

α-(*N*-Trifluoroacetyl-aminomethyl)stilbene (22). A mixture of crude α-aminomethyl-α′-hydroxy-bibenzyl (2.5 g),

trifluoroacetic anhydride (4.5 mL), and toluene (200 mL) was heated to reflux for 2 h. The solvent was distilled under vacuum to yield a brown oil. This liquid was purified over silica gel (10% ethyl acetate in hexanes, R_f 0.39) to give a white crystalline solid (1.71 g, 56% for two steps).

Z-α-Aminomethyl-stilbene hydrochloride (14). A mixture of *N*-trifluoroacetyl-α-aminomethylstilbene (0.21 g, 0.7 mmol), methanol (10 mL), THF (20 mL), and sodium hydroxide (1.0 g) was heated to reflux for 0.5 h. After removal of the solvent, water (10 mL) was added. The resulting mixture was extracted three times with ether (10 mL each), dried with anhydrous potassium carbonate, and cooled in an ice-water bath. Then anhydrous hydrogen chloride gas was bubbled through the cooled solution to give a white precipitate (0.15 g, 87%). The stereochemistry was confirmed by its NOE NMR spectra. ^{1}H NMR (DMSO-d_6) δ 3.80 (s, 2H), 6.80 (s, 1H), 6.90–7.40 (m, 10H), 8.45 (s, 3H). Anal calcd for $C_{15}H_{16}NCl$: C 73.31, H 6.56, N 5.70. Found: C 73.80, H 6.72, N 6.00.

3-Hydroxy-4,4-dimethyl-2-phenylvaleronitrile (23). To a cooled (−78 °C) solution of benzyl cyanide (3.51 g, 30 mmol) in dried THF (30 mL) was added 2.5 M *n*-butyllithium solution in hexane (10 mL, 25 mmol). The reaction mixture was stirred at −78 °C for 2 h. A solution of pivalaldehyde (1.72 g, 20 mmol) in dried THF (15 mL) was slowly added over 30 min, then chlorotrimethylsilane (8 mL) was added. The resulting solution was stirred at −78 °C for 5 min. After addition of methanol (10 mL), the reaction mixture was allowed to warm up to room temperature. Removal of the solvent gave a viscous oil (diastereomer ratio 4:1). The diastereomeric mixture was used in the next step, which was purified by flash chromatography (40% ethyl acetate/hexanes, R_f 0.10) to give a white crystalline solid (2.94 g, 91%); mp 67.5–68 °C; ^{1}H NMR ($CDCl_3$), δ 1.11 (s, 9H), 2.05 (d, 1H), 3.42 (d, 1H), 4.05 (d, 1H), 7.21–7.45 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 26.2, 36.0, 39.9 (d), 82.3, 119.1, 127.9, 128.2, 129.2, 135.9. Anal. calcd for $C_{13}H_{17}NO$: C 76.81, H 8.43, N 6.89. Found: C 76.62, H 8.50, N 6.95.

3-Hydroxy-4,4-dimethyl-2-phenylpentamine (24). To a stirring solution of 1.0 M borane in THF (50 mL, 50 mmol) was added all of the above 3-hydroxy-4,4-dimethyl-2-phenylvaleronitrile. The reaction mixture was stirred at room temperature for 14 h. After removal of the solvent on a rotary evaporator, 6.0 N aqueous hydrochloric acid solution (32 mL) was added. The mixture was heated to reflux for 2 h and then was cooled in an ice-water bath, sodium hydroxide pellets (11.2 g) were added, and after filtration the solution was extracted three times with ether (30 mL each) and dried (Na_2SO_4). Removal of the solvent led to a brown oil that was used in the next step without further purification. ^{1}H NMR ($CDCl_3$) δ 0.80 (s, 9H), 2.80 (q, 1H), 3.00–3.15 (m, 2H), 3.78 (d, 1H), 7.15–7.30 (m, 5H).

5-(*N*-Trifluoroacetylamino)-2,2-dimethyl-4-phenyl-3-pentanol (25). A mixture of crude 3-hydroxy-4,4-dimethyl-2-phenylpentamine (3.6 g), triethylamine (4.3 g), trifluoroacetic anhydride (4.40 g), and toluene (50 mL) was heated to reflux overnight. The solvent was distilled under vacuum to yield a brown oil. This liquid was purified over silica gel (dichloromethane R_f 0.23) to give a white crystalline solid (2.48 g). ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 9H), 2.35 (d, 1H), 3.05 (q, 1H), 3.66 (m, 2H), 3.80 (td, 1H), 7.15 (s, 1H), 7.20–7.40 (m, 5H).

5-(*N*-Trifluoroacetylamino)-2,2-dimethyl-4-phenyl-3-pentyl mesylate (26). A mixture of 5-(*N*-trifluoroacetylamino)-2,2-dimethyl-4-phenyl-3-pentanol (0.9 g, 3 mmol), pyridine (7.0 mL), and mesyl chloride (2 mL) was cooled below −20 °C in an ice-sodium chloride bath and was stirred for 3 h. Then, a chilled saturated sodium chloride solution (10 mL) was added, extracted twice with dichloromethane (10 mL each), washed three times with water (10 mL each), and dried (Na_2SO_4). Removal of the solvent led to a yellow solid (0.85 g, 75%), which was used directly in the next step. ^{1}H NMR ($CDCl_3$) δ 0.94 (s, 9H), 3.19 (s, 3H), 3.40 (m, 1H), 3.51 (m, 1H), 3.87 (m, 1H), 4.97 (d, 1H), 6.65 (s, 1H), 7.20–7.40 (m, 5H).

β-*tert*-Butyl-α-(*N*-trifluoroacetylaminomethyl)-styrene (27). A mixture of 5-(*N*-trifluoroacetylamino)-2,2-dimethyl-4-phenyl-3-pentyl mesylate (0.85 g, 2.2 mmol) in dried THF (25 mL) and potassium *tert*-butoxide (1.7 g) was refluxed for 2 h. After removal of the solvent, the residue was extracted three times with ether (25 mL each) and dried ($MgSO_4$). Ether was evaporated to give a brown oil which was purified by flash chromatography (5% ethyl acetate in hexanes, R_f 0.14) to give a white solid (0.20 g, 25%). ^{1}H NMR ($CDCl_3$) δ 4.60 (d, 2H), 5.97 (s, 1H), 6.02 (s, 1H), 7.25–7.40 (m, 5H).

α-Aminomethyl-β-*tert*-butyl-styrene (15). A mixture of β-*tert*-butyl-α-(*N*-trifluoroacetylaminomethyl)-styrene (0.2 g, 0.7 mmol), methanol (10 mL), THF (10 mL), and sodium hydroxide (0.2 g) was heated to reflux for 0.5 h. After removal of the solvent, water (10 mL) was added. The resulting mixture was extracted three times with ether (10 mL each), dried with anhydrous potassium carbonate, and cooled in an ice-water bath. Anhydrous hydrogen chloride gas was bubbled through the cooled solution to give a white precipitate (0.08 g, 51%); ^{1}H NMR (DMSO-d_6) δ 1.20 (s, 9H), 4.02 (s, 2H), 5.82 (s, 1H), 7.20–7.40 (m, 5H), 8.15 (s, 3H). The stereochemistry was confirmed by its NOE NMR spectrum.

1-(*tert*-Butoxycarbonylamino)-2-propanol (30). A reported procedure was followed.[21] To a stirred solution of 1-amino-2-propanol (1.88 g, 25 mmol) in 10% triethylamine-methanol (80 mL) was added di-*tert*-butyl dicarbonate (8.18 g, 37.5 mmol). The reaction mixture was stirred at room temperature for 6 h. Evaporation of the solvent on a rotary evaporator gave a colorless oil. This liquid was chromatographed on silica gel (hexanes/ethyl acetate (1/1); R_f 0.20) to afford a colorless oil (4.1 g, 93%): ^{1}H NMR ($CDCl_3$) δ 1.12 (d, 3H), 1.40 (s, 9H), 2.90 (m, 1H), 3.20 (m, 1H), 3.38 (b, 1H), 3.82, (m, 1H), 5.22 (s, 1H); HRMS calcd for $C_8H_{17}NO_3$ 175.1209. Found 175.1205.

1-(*tert*-Butoxycarbonylamino)-2-iodopropane (31). A reported procedure was followed.[22] To cold (−10 °C), dried tetrahydrofuran (THF, 80 mL) were added triphenylphosphine (10.0 g, 38 mmol) and diethyl azodicarboxylate (DEAD, 6.46 g, 37 mmol). After the reaction mixture was stirred at this temperature for 15 min, a solution of 1-(*tert*-butoxycarbonylamino)-2-propanol (6.12 g, 35 mmol) and iodomethane (5.03 g, 35.4 mmol) in dried THF (40 mL) was added. The reaction mixture was stirred at −20 °C for 12 h, then the solvent was evaporated under reduced pressure. The crude product was submitted to flash chromatography (hexanes-ethyl acetate (4:1); R_f 0.88) to give the product as a white solid (8.96 g, 90%): ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.87 (d, 3H), 3.36 (m, 1H), 4.25 (m, 1H), 5.00 (s, 1H); HRMS calcd for $C_8H_{16}INO_2$ 285.0226. Found 285.0236.

1-(*tert*-Butoxycarbonylamino)-2-nitropropane (32). A reported procedure was followed.[23] Into a 100-mL flask were added 1-(*tert*-butoxycarbonylamino)-2-iodopropane (6.0 g, 21 mmol), sodium nitrite (3.0 g, 43.5 mmol), 1,3,5-trihydroxybenzene dihydrate (4.0 g, 24.7 mmol), and dimethyl sulfoxide (DMSO, 20 mL). The reaction mixture was stirred overnight and then was quenched with water (100 mL), extracted six times with hexanes/ethyl acetate (2/1, 100 mL each), washed with water, and dried (Na_2SO_4). After removal of the solvent and purification over silica gel (hexanes/ethyl acetate (6/1); R_f 0.13), a white solid (1.23 g, 60% conversion, 52% yield) was obtained; mp 64.5–66.0 °C; ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.52 (d, 3H), 3.51 (ABX, 2H), 4.74 (m, 1H), 5.02 (s, 1H); HRMS calcd for $C_7H_{13}N_2O_4$ (M-CH_3) 189.0880. Found 189.0863.

α-(*tert*-Butoxycarbonylaminomethyl)-α-methyl-*N*-phenylnitrone (33). A reported procedure was followed.[24] A reaction mixture of 1-(*tert*-butoxycarbonylamino)-2-nitropropane (0.20 g, 1.06 mmol) and 1.0 M potassium hydroxide solution (1 mL, 1.0 mmol) was stirred at room temperature to homogeneity. After the solvent was removed, acetonitrile (2.0 mL) was added to redissolve the residue. To this solution was added nitrosobenzene powder (0.11 g, 1.0 mmol). The reaction mixture was stirred for 1.5 h at −10 °C. Solvent was evaporated under reduced pressure to afford yellow oil. Two isomers (3/1) were distinguishable in the NMR spectrum of the crude product. They could not be purified because of their instability at room temperature. Major product: ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 9H), 2.00 (s, 3H), 4.30 (d, 2H), 5.80 (s, 1H), 7.30–7.50 (m, 5H); minor product: ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 2.30 (s, 3H), 3.80 (d, 2H), 5.10 (s, 1H), 7.30–7.50 (m, 5H).

α-(Aminomethyl)-α-methyl-*N*-phenylnitrone (28a,b). The crude product mixture obtained from the preceding step was dissolved in methanol or dichloromethane. Then hydrochloric acid or trifluoroacetic acid was added, respectively. A dark brown to black mixture was always obtained. The NMR spectra of the resulting residues indicated a messy mixture.

α-*tert*-Butoxycarbonylaminomethyl-*N*-*tert*-butylbenzylamine (37). The starting material, α-(*N*-*tert*-butylamino) phenylamine, was synthesized as reported.[25] The yellow residue obtained (**36**) was dissolved in a solution of 20% triethylamine in methanol (150 mL) and then di-*tert*-butyl dicarbonate (21.06 g, 96 mmol) was added. The reaction mixture was stirred at room temperature for 4 h. Removal of the solvent and precipitation with hexanes gave a white solid (8.8 g, 21% for two steps); mp 95.5–96.0 °C; ^{1}H NMR δ 1.02 (s, 9H), 1.45 (s, 9H), 3.70 (m, 2H), 3.88 (t, 1H), 4.85 (s, 1H), 7.20–7.35 (m, 5H); HRMS calcd for $C_{17}H_{29}N_2O_2$ (M + 1) 293.2229. Found 293.2232.

α-*tert*-Butoxycarbonylaminomethyl-*N*-*tert*-butyl-α-phenylnitrone (38). A reported procedure was followed.[26] Into a 10-mL flask were added α-*tert*-butoxycarbonylaminomethyl-*N*-*tert*-butylbenzylamine (500 mg, 1.7 mmol), sodium tungstate dihydrate (13 mg, 0.04 mmol), and methanol (2 mL). Then 30% hydroperoxide aqueous solution (0.29 g, 2.6 mmol) was added slowly. After addition, the reaction mixture was stirred at room temperature for 18 h. Solvent was evaporated under reduced pressure, and the residue was redissolved in dichloromethane (10 mL). The resulting solution was washed with brine (4 mL) and dried (Na_2SO_4). Removal of the solvent gave a yellow oil. Further purification attempts failed because the product decomposes on silica gel (5% MeOH in CH_2Cl_2; R_f 0.21); ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 9H), 1.31 (s, 9H), 4.24 (d, 2H), 5.72 (b, 1H), 7.20–7.40 (m, 5H).

α-Aminomethyl-*N*-*tert*-butyl-α-phenylnitrone (29). The above residue was dissolved in methanol (25 mL). Anhydrous hydrogen chloride gas was bubbled through the solution at 0 °C for 0.5 h. Solvent was evaporated on

a rotary evaporator, and the residue was pumped to dryness. The NMR spectrum of the crude product indicated that most of the product decomposed. No further purification was successful because of its instability.

5,5-Dimethyl-2-(*N*,*N*-dimethylaminovinyl)-1-pyrroline-*N*-oxide (40). A reported procedure was followed.[27] 2,5,5-Trimethyl-1-pyrroline-*N*-oxide (synthesized as reported,[45–47] 1.27 g, 10 mmol) was added to *tert*-butoxybis(dimethylamino)methane (2.37 g, 13.6 mmol). The reaction mixture was stirred at 60 °C for 24 h and then was cooled to room temperature. Purification by flash column chromatography gave a highly hygroscopic white solid (0.4 g, 22%); mp 102.5–104.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.37 (s, 6H), 1.94 (t, 2H), 2.60 (t, 2H), 2.87 (s, 6H), 5.27 (s, 1H), 7.14 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 25.4, 25.6, 32.8, 71.3, 87.0, 142.6, 147.2, 147.2; HRMS calcd for $C_{10}H_{18}N_2O$ 182.1419, found 182.1415. Anal. calcd for $C_{10}H_{18}N_2O$ C 65.90, H 9.95, N 15.37. Found C 65.78, H 9.85, N 15.42.

2-Aminomethyl-5,5-dimethyl-1-hydroxylpyrrolidine (46). Into a 250-mL flask were added 2-cyano-5,5-dimethyl-1-hydroxylpyrrolidine (synthesized as reported,[28] 6.5 g, 46.4 mmol) and anhydrous ether (150 mL). After the solution was cooled with an ice-water bath, lithium aluminum hydride (3.25 g, 86 mmol) was added slowly. The mixture was refluxed for 4 h and then was cooled to 0 °C, quenched with an aqueous saturated potassium carbonate solution, and extracted three times with ether (200 mL). The extracts were combined and dried with anhydrous potassium carbonate. Removal of the solvent gave a white crystalline solid (6.5 g, 98%), which quickly turned yellow in air; ^{1}H NMR ($CDCl_3$) δ 0.96 (s, 3H), 1.10 (s, 3H), 1.30 (m, 1H), 1.50 (m, 1H), 1.75 (m, 2H), 2.94 (m, 1H), 2.76 (m, 2H), 3.05 (b, 1H), 1.55 (b, 2H). This compound was used directly in the next reaction.

2-(*tert*-Butoxycarbonylaminomethyl)-5,5-dimethyl-1-hydroxypyrrolidine (47). The crude product obtained in the preceding step was treated with di-*tert*-butyl dicarbonate (25 g, 115 mmol) in 15% triethylamine-methanol (200 mL). The reaction mixture was stirred at room temperature overnight. Solvent was evaporated on a rotary evaporator to give a viscous oil. This liquid was chromatographed over silica gel. Elution with hexanes/ethyl acetate (4/1; R_f 0.10) gave a white powder (7.0 g, 62% for two steps); mp 72.5–74.0 °C; ^{1}H NMR ($CDCl_3$) δ 1.03 (s, 3H), 1.18 (s, 3H), 1.45 (s, 9H), 1.40–1.60 (m, 3H), 1.80 (m, 1H), 3.08 (m, 1H), 3.21 (m, 1H), 3.38 (m, 1H), 5.15 (b, 1H), 5.40 (b, 1H); HRMS calcd for $C_{12}H_{24}N_2O_3$ 244.1787. Found 244.1791.

2-(*tert*-Butoxycarbonylaminomethyl)-5,5-dimethyl-1-pyrroline-*N*-oxide (48). A reported procedure was followed.[29] To a solution of 2-(*tert*-butoxycarbonylaminomethyl)-5,5-dimethyl-1-hydroxypyrrolidine (3 g, 12.3 mmol) in acetonitrile (50 mL) were added *N*-methylmorpholine-*N*-oxide (NMO, 2.2 g, 18.8 mmol), tetra-*n*-propylammonium perruthenate (TPAP, 0.1 g, 0.3 mmol), and dried 4 Å molecular sieves (5.0 g). The reaction mixture was stirred at room temperature for 0.5–1 h and then was filtered through a pad of Celite and silica gel. The cake was washed with methanol. Solvent was evaporated under reduced pressure, and the residue was purified on a silica gel column (5% methanol in dichloromethane; R_f 0.15), affording a white solid (1.45 g, 49%); mp 135.0–136.0 °C; ^{1}H NMR ($CDCl_3$) δ 2.02 (t, 2H), 2.68 (t, 2H), 4.09 (d, 2H), 5.64 (b, 1H), 1.40 (s, 6H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 25.3, 26.8, 28.4, 32.5, 37.3, 74.2, 79.8, 142.4, 156.2; HRMS calcd for $C_{12}H_{23}N_2O_3$ (M+1) 243.1708. Found 243.1711. Anal. calcd for $C_{12}H_{22}N_2O_3$: C 59.48, H 9.15, N 11.56. Found: C 59.53, H 9.09, N 11.40.

2-Aminomethyl-5,5-dimethyl-1-pyrroline-*N*-oxide hydrochloride (41). To a solution of 2-(*tert*-butoxycarbonylaminomethyl)-5,5-dimethyl-1-pyrroline-*N*-oxide (100 mg, 0.4 mmol) in methanol (10 mL) at 0 °C was bubbled anhydrous hydrochloride gas for 30 min. After the solvent was evaporated on a rotary evaporator, the residue was pumped to dryness. No further purification was successful because of its instability in non-acidic aqueous medium. ^{1}H NMR (D_2O) δ 1.34 (s, 3H), 2.13 (t, 2H), 2.80 (t, 2H), 3.97 (s, 2H); ^{13}C NMR (D_2O) δ 24.1, 27.0, 31.8, 35.7, 75.9, 145.8.

4-Methyl-4-nitro-2-phenylvaleraldehyde (49). To a solution of 2-phenylacrolein (synthesized as reported,[30] 8.0 g, 60 mmol) in ether (40 mL) were added Triton B (500 μL) and 2-nitropropane (6 g, 67 mmol). The reaction mixture was refluxed overnight and then was cooled to room temperature, quenched with water (50 mL), washed three times with an aqueous saturated sodium chloride solution (50 mL each), and dried ($MgSO_4$). The solvent was evaporated on a rotary evaporator. The crude product was submitted to flash chromatography (hexanes–ethyl acetate (19:5; R_f 0.20)) to give a colorless oil (10.8 g, 81%); ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 3H), 1.58 (s, 3H), 2.30 (dd, 1H), 2.95 (dd, 1H), 3.61 (t, 1H), 7.48 (m, 3H), 7.14 (d, 2H), 9.60 (s, 1H); HRMS calcd for $C_{12}H_{15}NO_3$ 221.1052. Found 221.1042.

5,5-Dimethyl-3-phenyl-1-pyrroline-*N*-oxide (50). A mixture of 4-methyl-4-nitro-2-phenylvaleraldehyde (1.0 g, 4.5 mmol) and water (15 mL) was cooled with an ice-water bath. Then ammonium chloride (2.5 g, 46.7 mmol) and zinc dust (10.0 g, 154 mmol) were added slowly. The slurry was stirred for 1.5 h and then was filtered. The cake was washed three times with hot water (70 °C, 10 mL each). The filtrate was saturated with sodium chloride, extracted five times with ether (10 mL each),

and dried ($MgSO_4$). Removal of the solvent and purification on silica gel (hexanes–ethyl acetate (1/4); R_f 0.15) led to a colorless oil (0.60 g, 70%), which solidified upon standing; mp 93.5–95.0 °C; ^1H NMR ($CDCl_3$) δ 1.50 (s, 3H), 1.51 (s, 3H), 2.00 (dd, 1H), 2.65 (dd, 1H), 4.11 (dt, 1H), 6.91 (d, 1H), 7.15–7.40 (m 5H); HRMS calcd for $C_{12}H_{15}NO$ 189.1154. Found 189.1156.

2-Cyano-1-hydroxy-5,5-dimethyl-4-phenylpyrrolidine (51). A mixture of 5,5-dimethyl-3-phenyl-1-pyrroline-*N*-oxide (1.0 g, 5.3 mmol), water (4 mL), and potassium cyanide (0.8 g, 12.3 mmol) was cooled to 4 °C with an ice-water bath. Then 2.0 N hydrochloride solution (5.0 mL) was slowly added. The reaction mixture was stirred for 4 h and then was saturated with sodium chloride, extracted four times with ether (10 mL each), and dried ($MgSO_4$). Removal of the solvent gave a yellow oil. This liquid was chromatographed on silica gel (hexanes/ethyl acetate (4/1), R_f 0.25) to give a white solid (0.65 g, 66% conversion, 85% yield). Two diastereomers (2/1) could be distinguished in the NMR spectrum of the product. mp 75.0–77.0 °C; HRMS calcd for $C_{13}H_{16}N_2O$ 216.1263. Found 216.1264.

Major compound: ^1H NMR ($CDCl_3$) δ 1.30 (s, 3H), 1.39 (s, 3H), 1.98 (m, 1H), 2.25 (m, 1H), 3.61 (m, 1H), 4.05 (d, 1H), 6.38 (s, 1H), 7.25–7.40 (m, 5H).

Minor compound: ^1H NMR ($CDCl_3$) δ 1.20 (s, 3H), 1.39 (s, 5H), 1.98 (m, 1H), 2.25 (m, 1H), 3.61 (m, 1H), 4.31 (d, 1H), 6.04 (s, 1H), 7.25–7.40 (m, 5H).

2-Aminomethyl-1-hydroxy-5,5-dimethyl-4-phenylpyrrolidine (52). To a solution of 2-cyano-1-hydroxy-5,5-dimethyl-4-phenylpyrrolidine (0.65 g, 3 mmol) in anhydrous ether (25 mL) at 0 °C was added lithium aluminum hydride (500 mg, 13 mmol). The slurry was refluxed overnight and then was quenched with 5% sodium hydroxide aqueous solution (10 mL) at 0 °C. The ether layer was decanted and dried with anhydrous potassium carbonate. Removal of the solvent gave a yellowish oil. This liquid became darker quickly in air. No further purification was possible, and it was used directly in the next step.

2-(*tert*-Butoxycarbonylaminomethyl)-1-hydroxyl-5,5-dimethyl-4-phenylpyrrolidine (53). The residue obtained in the above reaction was dissolved in 10% triethylamine/methanol (100 mL), and then di-*tert*-butyl dicarbonate was added (3 g, 13.7 mmol). The reaction mixture was stirred at room temperature for 3 h. After removal of the solvent, a yellow viscous liquid was obtained. This liquid was purified by flash chromatography (ethyl acetate; R_f 0.20) to give a white solid (0.30 g, 31% yield for the two steps); mp 98.0–100.5 °C. Two diastereomers were found in the NMR spectrum; HRMS calcd for $C_{18}H_{28}N_2O_3$ 320.2100. Found 320.2101.

2-(*tert*-Butoxycarbonylaminomethyl)-5,5-dimethyl-4-phenyl-1-pyrroline-*N*-oxide (54). Into a 10-mL flask were added 2-(*tert*-butoxycarbonylaminomethyl)-1-hydroxyl-5,5-dimethyl-4-phenylpyrrolidine (1.0 g, 3.1 mmol), acetonitrile (10 mL), dried 4 Å molecular sieve powder (4 g), *N*-methylmorpholine-*N*-oxide (600 mg, 5.2 mmol), and TPAP (60 mg, 0.17 mmol). The reaction mixture was stirred at room temperature for 0.5 h and then was filtered. Removal of the solvent gave a dark oil. The crude product was triturated with petroleum ether (5 mL) to yield a white solid. This solid was chromatographed on silica gel (ethyl acetate; R_f 0.10) to afford a crystalline solid (0.67 g, 68%); mp 103.0–104.5 °C; ^1H NMR ($CDCl_3$) δ 1.44 (s, 3H), 1.54 (s, 3H), 1.95 (dd, 1H), 2.60 (dd, 1H), 3.88 (dd, 1H), 3.95 (d, 1H), 4.19 (t, 1H), 1.39 (s, 9H), 5.75 (b, 1H), 7.25–7.40 (m, 3H), 7.14 (d, 2H); HRMS calcd for $C_{18}H_{26}N_2O_3$ 319.2021. Found 319.2021. Anal. calcd for $C_{18}H_{26}N_2O_3$: C 67.90, H 8.23, N 8.80. Found: C 67.68, H 8.23, N 8.92.

2-Aminomethyl-5,5-dimethyl-4-phenyl-1-pyrroline-*N*-oxide (42). About 100 mg of the above white solid was dissolved in 10 mL of 50% TFA solution in dichloromethane. The mixture was stirred for 5 h at room temperature. The solvent was evaporated on a rotary evaporator to give yellowish oil. Upon standing at room temperature, it turns quickly into a bright pink liquid. No further purification was possible. ^1H NMR ($CDCl_3$) δ 1.49 (s, 3H), 1.55 (s, 3H), 2.10 (dd, 1H), 3.68 (d, 1H), 3.95 (d, 1H), 4.49 (t, 1H), 7.15–7.45 (m, 5H), 8.50–9.00 (b, 1H).

Enzyme and assay

Beef liver MAO-B was isolated as described previously[31] and stored as a concentrated solution (15–25 mg/mL) in sodium phosphate buffer (50 mM, pH 7.2) at 4 °C. The specific activity is in the range of 4.0 to 7.0 units/mg, where a unit of activity is the conversion of 1 μmol of benzylamine to benzaldehyde per min at pH 9.0 and 30 °C.

pH dependence of enzyme activity and stability

A concentrated MAO-B solution (150 μM, 5 μL) was diluted with sodium phosphate buffer (50 mM, 95 μL). An aliquot of the enzyme solution (5 μL) was added to a 1.01 mM benzylamine solution in 50 mM buffers (495 μL) at different pH values: 4.0 (sodium citrate), 5.0 (sodium citrate), 5.5 (sodium citrate), 6.0 (sodium phosphate), 7.0 (sodium phosphate), and 9.0 (Tris, tris(hydroxymethyl)aminomethane). The reaction rates were obtained by monitoring the formation of benzaldehyde at 250 nm. To determine the enzyme stability the assay was repeated every hour during the first 4 h and every 2 h thereafter up to 12 h.

Substrate activity of 39–41

The general method of Makinen and Tenovuo[32] for the assay of hydrogen peroxide production was used. The amino nitrones (**39–41**) (990 μL, 20 mM) were incubated with MAO-B (10 μL, 46.8 μM) using 0.18 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt (ABTS) in 0.5 M citric-phosphate buffer (pH 6.0) containing horseradish peroxidase (1 unit/mL) as a dye and monitoring the absorption at 414 nm. Hydroperoxide was only generated from the incubation solution of **41**. Solutions of **41** (2.0, 1.0, 0.5 and 0.3 mM) were prepared in 100 mM potassium phosphate buffer (pH 6.0) containing 20 μM NaN_3 and stored at 4 °C. The solution of ABTS (0.18 mM) in 0.5 M citric-phosphate buffer (pH 6.0) containing horseradish peroxidase (1 unit/mL) was stored in an ice bath. Aliquots (10 μL) of the MAO-B stock solution (46.8 μM) were added to each of the solutions containing **41** and a control solution without MAO-B, then 10 μL aliquots of the reaction solutions were periodically added to 490 μL of the ABTS solution and monitored at 414 nm. The concentration of the hydrogen peroxide was calculated from a calibration curve and was placed on a plot of hydrogen peroxide concentration versus time. The Michaelis constant was determined by the method of Lineweaver–Burk.[33]

Inhibitory activity of 39

The reversible inhibition of MAO-B by **39** (0, 1.25, 2.50, 5.00, 7.50, and 10 mM) was determined in sodium phosphate buffer (100 mM, pH 7.4) under initial rate conditions with the MAO-B assay using cinnamyl amine (0.05, 0.10, 0.20, 0.30, and 0.50 mM) as the substrate, monitored at 290 nm (**39** has an absorbance that interferes with oxidation of benzyl amine at 250 nm). The amount of inhibition was determined by adding 10 μL of MAO-B solution (5 μM) to 490 μL of an inhibitor–substrate solution at 25 °C and monitoring the increase in absorbance at 290 nm. The data were analyzed by the method of Dixon[34] and Cornish–Bowden.[35]

Stability of amino nitrones 39, 40, and 41 in water

A solution of the amino nitrone **39** or **40** (10 mM) in deuterium sodium phosphate buffers (measured pD 6.0, 7.0, and 8.0) was monitored by NMR at 8 and 18 h after preparation at room temperature. A solution of amino nitrone **41** (10 mM) in 0.5% deuterium oxide-chloroform-*d* solution was monitored by NMR.

Sample preparation for the EPR study of the amino nitrone 39 and 41

A mixture of MAO-B (150 μM, 30 μL for **39**; 20 μL for **41**), sodium phosphate buffer (pH 7.2, 100 mM, 86 μL), and amino nitrone **39** (100 mM, 6 μL) or **41** (200 mM, 12 μL) was incubated at room temperature in the dark for 18 h. Either the aqueous solution or a THF extract of the lyophilized incubation mixture was put in a quartz sample tube and was subjected to the EPR experiment described below.

Sample preparation for the EPR study of the amino nitrone 40

MAO-B (150 μM, 50 μL) was lyophilized for 2 h, and the powder enzyme was put into a quartz EPR sample tube. Then water (1.5 μL) and a solution of amino nitrone **40** in benzene (2wasmM, 150 μL) were mixed. The mixture was incubated at room temperature in the dark for 8 h and then was subjected to the EPR experiment described below.

EPR experiment

Aqueous samples were cooled with liquid nitrogen in a quartz Dewar during data collection. The organic extracts or solutions were directly used at room temperature. The microwave frequency was set at 9.5 GHz and the magnetic field was set at 3400 gauss. Other parameters were optimized for each individual sample. The spectrum was scanned over a range of 100 or 2000 gauss.

Acknowledgements

The authors are grateful to the National Institutes of Health (GM32634) for financial support of this research.

References

1. *Methods in Enzymology*; Kaufman, S., Ed.; Academic: New York, 1987; Vol. 142, Section III, p 617.

2. Rimoldi, J. M.; Wang. Y.-X.; Nimkar, S. K.; Kuttab, S. H.; Anderson, A. H.; Burch, H.; Castagnoli, Jr. N. *Chem. Res. Toxicol.* **1995**, *8*, 703.

3. Silverman, R. B.; Lu, X. *J. Am. Chem. Soc.* **1994**, *116*, 4129.

4. Silverman, R. B. *Acc. Chem. Res.* **1995**, *28*, 335.

5. Maeda, Y.; Ingold, K. U. *J. Am. Chem. Soc.* **1980**, *102*, 328.

6. (a) Silverman, R. B.; Hoffman, S. J. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 1396. (b) Silverman, R. B. *J. Biol. Chem.* **1983**, *258*, 14766. (c) Silverman, R. B.; Yamasaki, R. B. *Biochemistry* **1984**, *23*, 1322. (d) Silverman, R. B. *Biochemistry* **1984**, *23*, 5206. (e) Silverman, R. B.; Zieske, P. A. *Biochemistry* **1985**, *24*, 2128. (f) Silverman, R. B.; Zieske, P. A. *J. Med. Chem.* **1985**, *28*, 1953. (g) Vazquez, M. L.; Silverman, R. B. *Biochemistry* **1985**, *24*, 6538. (h) Yamasaki, R. B.; Silverman, R. B. *Biochemistry* **1985**, *24*, 6543. (i) Silverman, R. B.; Zieske, P. A. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 154. (j) Silverman, R. B.; Cesarone, J. M.; Lu, X. *J. Am. Chem. Soc.* **1993**, *115*, 4955. (k) Lu, X.; Yang, S.; Silverman, R. B. *J. Org. Chem.* **1996**, *61*, 8961. (l) Silverman, R. B.; Lu, X.; Blomquist, G.;

Ding, C. Z.; Yang, S. *Bioorg. Med. Chem.* **1997**, *5*, 297. (m) Zhong, B.; Silverman, R. B. *J. Am. Chem. Soc.* **1997**, *119*, 6690.

7. (a) Wilt, J. W.; Maravetz, L. L.; Zawadzki, J. F. *J. Org. Chem.* **1966**, *31*, 3018. (b) Menapace, L. W.; Kuivila, H. G. *J. Am. Chem. Soc.* **1964**, *86*, 3047. (c) Tanner, D. D.; Rahimi, R. M. *J. Org. Chem.* **1979**, *44*, 1674.

8. Bondon, A.; Macdonald, T. L.; Harris, T. M.; Guengerich, F. P. *J. Biol. Chem.* **1989**, *264*, 1988.

9. Yelekci, K.; Lu, X.; Silverman, R. B. *J. Am. Chem. Soc.* **1989**, *111*, 1138.

10. Silverman, R. B.; Zhou, J. P.; Eaton, P. E. *J. Am. Chem. Soc.* **1993**, *115*, 8841.

11. Silverman, R. B.; Lu, X.; Zhou, J. J. P.; Swihart, A. *J. Am. Chem. Soc.* **1994**, *116*, 11590.

12. Silverman, R. B.; Zhou, J. J. P.; Ding, C. Z.; Lu, X. *J. Am. Chem. Soc.* **1995**, *117*, 12895.

13. Tan, A.; Glantz, M. D.; Piette, L. H.; Yasunobu, K. T. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 517.

14. Husain, M.; Edmondson, D. E.; Singer, T. P. *Biochemistry* **1982**, *21*, 595.

15. (a) Janzen, E. G.; Evans, C. A.; Nishi, Y. *J. Am. Chem. Soc.* **1972**, *94*, 8236. (b) Janzen, E. G.; Evans, C. A. *J. Am. Chem. Soc.* **1973**, *95*, 8205.

16. (a) Aurich, H. G. In *The Chemistry of Function Group, Supplement F: the Chemistry of Amino, Nitroso, and Nitro Compounds and Their Derivatives*; Patai, S. Ed.; John Wiley & Sons: New York, 1982; Chapter 14. (b) Torssel, K. B. G. *Nitrile Oxides, Nitrones, and Nitronates in Organic Synthesis*; VCH: New York, 1988; Chapter 3. (c) Hamer, J.; Macaluso, A. *Chem. Rev.* **1964**, *64*, 473.

17. (a) Dostert, P.; Strolin Benedetti, M. *Biochem. Soc. Trans.* **1991**, *19*, 207. (b) Palmer, S. L.; Mabic, S.; Castagnoli, Jr. N. *J. Med. Chem.* **1997**, *40*, 1982.

18. (a) Torssel, K. B. G. *Nitrile Oxides, Nitrones, and Nitronates in Organic Synthesis*; VCH: New York, 1988; Chapter 3. (b) Hamer, J.; Macaluso A. *Chem. Rev.* **1964**, *64*, 473.

19. (a) Sandler, S. R.; Karo, W. *Organic Functional Group Preparations*, 2nd ed.; Academic: New York, 1989; Vol. III, Chapter 9. (b) Sakaue, S.; Sakata, Y.; Nishiyama, Y.; Ishii, Y. *Chem. Lett.* **1992**, 289. (c) Murray, R. W.; Singh, M. *J. Org. Chem.* **1990**, *55*, 2954. (d) Boyd, D. R.; Coulter, P. B.; McGuckin, M. R.; Sharma, N. D. *J. Chem. Soc., Perkin Trans. 1*, **1990**, 301. (d) Bartoli, G.; Marcantoni, E.; Petrini, M. *J. Org. Chem.* **1990**, *55*, 4456. (e) Bartoli, G.; Marcantoni, E.; Petrini, M. *J. Org. Chem.* **1992**, *57*, 5834.

20. Buckley, G. D.; Elliott, T. J. *J. Chem. Soc.* **1947**, 1508.

21. Ponnusamy, E.; Fotadar, U.; Spisni, A.; Fiat, D. *Synthesis* **1986**, 48.

22. Hughes, D. L. *Org. Reactions* **1992**, *42*, 335.

23. Kornblum, N. *Org. Reactions* **1962**, *12*, 101.

24. Lyapkalo, I. M.; Loffe, S. L.; Strelenko, Y. A.; Tartakovskii, V. A. *Mendeleev Commun.* **1994**, (2), 51.

25. (a) Ugi, I.; Fetzer, U.; Eholzer, U.; Knupfer, H.; Offermann K. *Angew. Chem. Int. Ed. Engl.* **1965**, *4*, 472. (b) Saegusa, T.; Takaishi, N.; Ito, Y. *J. Org. Chem.* **1969**, *34*, 4040.

26. Murahashi, S.; Mitsui, H.; Shiota, T.; Tsuda, T.; Watanabe, S. *J. Org. Chem.* **1990**, *55*, 1736.

27. Black, D. S. C.; Strauch, R. J. *Aust. J. Chem.* **1991**, *44*, 1217.

28. (a) Warner, D. T.; Moe, O. A. *J. Am. Chem. Soc.* **1952**, *74*, 1064. (b) Bonnett, R.; Clark, V. M.; Giddey, A.; Todd, A. *J. Chem. Soc.* **1959**, 2087.

29. Goti, A.; De Sarlo, F.; Romani, M. *Tetrahedron Lett.* **1994**, *35*, 6571.

30. Crossland, I. *Org. Synth.* **1981**, *60*, 6.

31. Salach, J. I. *Arch. Biochem. Biophys.* **1979**, *192*, 128.

32. Makinen, K. K.; Tenovuo, J. *Anal. Biochem.* **1982**, *126*, 100.

33. Segel, I. H. *Enzyme Kinetics*; Wiley: New York; 1975.

34. Dixon, M. *Biochem. J.* **1953**, *55*, 170.

35. Cornish-Bowden, A. *Biochem. J.* **1974**, *137*, 143.