*Tetrahedron* Vol. 40, No. 3, pp. 511 to 519, 1984
Printed in Great Britain.

0040-4020/84 $3.00 + .00
Pergamon Press Ltd

# AUTOCATALYTIC INACTIVATION OF CYTOCHROME P-450 AND CHLOROPEROXIDASE BY 1-AMINOBENZOTRIAZOLE AND OTHER ARYNE PRECURSORS

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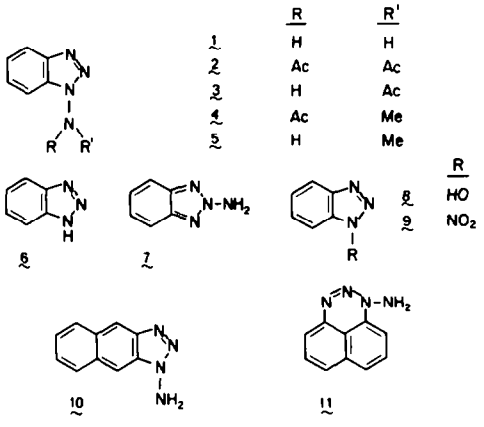
(*Received in U.S.A. October* 1982)

**Abstract**—Cytochrome P-450, as reported previously, is inactivated during catalytic turnover of 1-aminobenzotriazole due to alkylation of its prosthetic heme group. NMR analysis of the heme adduct after removal of the iron atom identifies it unequivocally as a derivative of protoporphyrin IX in which two of the nitrogens are bridged by a benzene ring. Cytochrome P-450 destructive activity is retained by analogues with Me or Ac substituents on the exocyclic N but is lost when the N itself is removed or is replaced by a hydroxyl or nitro function. Prosthetic heme alkylation also occurs with 1-amino-1H-naphtho(2,3-d)triazole, the analogue with one additional benzene ring. *In vivo* studies suggest that 1-aminobenzotriazole is relatively nontoxic. Catalytic turnover of 1-aminobenzotriazole by chloroperoxidase results in the formation of phenol and in inactivation of the enzyme. The phenol obtained in deuterated water incorporates one deuterium into the aromatic ring. The data indicate that benzyne, formed by enzymatic oxidation of 1-aminobenzotriazole, is responsible for inactivation of cytochrome P-450 and chloroperoxidase.

Cytochrome P-450 enzymes constitute a heterogeneous class of hemoproteins that reduce molecular oxygen to an Fe-coordinated species that is readily inserted into substrate molecules.[1,2] The finding that certain substrates long known to perturb heme biosynthesis reduce the hepatic level of cytochrome P-450 on administration to rodents[3,4] has resulted in the discovery that cytochrome P-450 is inactivated during catalytic turnover of terminal olefins,[5-7] acetylenes,[8-10] and allenes.[11] Destruction of the enzyme by these unsaturated substrates meets with the criteria that define the action of a suicide substrate: the process adheres to pseudo first order kinetics,[12,13] is attenuated by inhibitors of the enzyme,[5,12] requires catalytic turnover of the enzyme,[12-14] and is irreversible. Enzyme inactivation reflects N-alkylation of the prosthetic heme of the enzyme by the catalytically activated $\pi$-bond.[15-17] A number of the damaged heme moieties, isolated after removal of the iron atom and methylation of the carboxyl groups, have been unambiguously identified by spectroscopic methods.

The discovery that cytochrome P-450 is inactivated by terminal $\pi$-bonds instigated a search for other functionalities and structures that could be used in the construction of isozyme-specific suicide substrates. The first structure examined was 1-aminobenzotriazole, a compound shown by the chemical studies of Rees *et al.* to be oxidized by a variety of agents to a species that yields benzyne.[18,19] We have already reported that 1-aminobenzotriazole does, in fact, inactivate cytochrome P-450 by a mechanism that requires catalytic turnover of the enzyme.[14,20] A heme-derived porphyrin isolated from the livers of rats treated with the agent was tentatively identified as a derivative of protoporphyrin IX in which two carbons of a benzene moiety bridge two of the porphyrin nitrogens.[20] This structural postulate was based on a comparison of the electronic absorption spectrum of the isolated porphyrin with that of N,N-dimethylprotoporphyrin IX and on the molecular weight of the adduct determined by field desorption mass spectrometry. We report here (a) confirmation of the structure assigned to the adduct, (b) isolation of an additional minor product identified as N-phenylprotoporphyrin IX, (c) survey of the relationship between structure and activity for analogues of 1-aminobenzotriazole, (d) autocatalytic inactivation of chloroperoxidase by 1-aminobenzotriazole, and (e) evidence that benzyne is indeed produced by biological oxidation of 1-aminobenzotriazole.




## METHODS AND MATERIALS

Benzotriazole (**6**), 1-hydroxybenzotriazole (**8**), and 99.8% deuterated water were purchased from Aldrich Chemical Co. Literature procedures were used for the synthesis of the following compounds: 1-aminobenzotriazole (**1**),[18] 1-nitrobenzotriazole (**9**),[21] 2-aminobenzotriazole (**7**),[18] 1-aminonaphtho(1,8-de)triazine (**11**),[18,22] and 1-amino-1H-naphtho (2,3-d)triazole (**10**).[23] NADPH, tricaprylin, and serum transaminase (SGOT and SGPT) determination kits (catalogue No. 505) were purchased from Sigma Chemical Co. Serum transaminase levels were measured as previously described.[24]

N,N-*Diacetyl* 1-*aminobenzotriazole* (**2**). $Ac_2O$ (10.0 g) was

added to a stirred soln of 1-aminobenzotriazole (1.00 g, 7.46 mmol) and $K_2CO_3$ (3.0 g) and the mixture was stirred for approx. 12 hr at ambient temp. The mixture was then filtered, the solvent was removed on a rotary evaporator, and the crude residue was dissolved in 100 ml $CH_2Cl_2$. The soln, washed with three 100 ml aliquots water, dried over $MgSO_4$, and concentrated, gave N,N-diacetyl 1-aminobenzotriazole (1.51 g, 94%). An analytical sample was obtained by low pressure chromatography on a Merck silica gel 60 column eluted with 3:1 v/v $CHCl_3$–ethyl acetate: NMR ($C^2HCl_3$) 2.36 (6H, s, acetyl methyls), 7.29–7.65 (3H, m, aromatic protons), and 8.09–8.22 ppm (1H, m, proton at C-7); EIMS (*m/e*) 218 ($M^+$). (Found: C, 54.68; H, 4.97; N, 25.72. Calc. for $C_8H_8N_4O$: C, 55.04; H, 4.62; N, 25.67%.)

N-*Acetyl* 1-*aminobenzotriazole* (**3**). A suspension of N,N-diacetyl 1-aminobenzotriazole (1.00 g, 4.59 mmol) in 100 ml abs EtOH was refluxed for 8 hr. The soln, after it was cooled and concentrated, gave 799 mg (99%) of N-acetyl 1-aminobenzotriazole as white crystals, m.p. 159.5–161°. NMR ($CD_3OD$): 2.30 (3H, s, acetyl methyl), 7.30–7.59 (3H, m, aromatic protons), 7.90–8.04 (1H, m, proton at C-7), and 9.4 ppm (1H, s, NH, exchangeable, only present in $CDCl_3$). (Found: C, 54.87; H, 4.32; N, 31.66. Calc. for $C_8H_8N_4O$: C, 54.54; H, 4.58; N, 31.80%.)

N-*Acetyl*-N-*methyl* 1-*aminobenzotriazole* (**4**). N-acetyl 1-aminobenzotriazole (1.00 g, 5.68 mmol) was added to a suspension of $K_2CO_3$ (1.57 g) in 25 ml acetone. The soln was heated to reflux before MeI (1.00 g, 7.82 mmol) in 10 ml acetone was added in five equal portions over a period of 1 hr. After a further 2 hr the soln was cooled, filtered, and concentrated on a rotary evaporator. The residue, purified by low pressure chromatography on a Merck silica gel 60 column eluted with 1:1 v/v hexane:EtOAc, yielded 0.745 g (69% yield) N-methyl-N-acetyl 1-aminobenzotriazole as white crystals, m.p. 74.5–76.0°. NMR ($C^2HCl_3$): 1.81 (3H, s, acetyl Me), 3.57 (3H, s, N-methyl), 7.35–7.66 (3H, m, aromatic protons), and 8.05–8.20 ppm (1H, m, proton at C-7). EIMS (*m/e*): 190 ($M^+$). (Found: C, 56.81; H, 5.25; N, 29.36. Calc. for $C_9H_{10}N_4O$: C, 56.83; H, 5.30; N, 29.46%.)

N-*Methyl* 1-*aminobenzotriazole* (**5**). A soln of N-acetyl-N-methyl 1-aminobenzotriazole (510 mg, 2.68 mmol) in 50 ml 10 M aqueous KOH was vigorously stirred and refluxed for 2 hr. The cooled mixture was then extracted with five 25 ml aliquots $CH_2Cl_2$ and the combined extracts, dried over $Na_2SO_4$, were concentrated on a rotary evaporator. The yellow oily residue, purified by low pressure chromatography (1:1 v/v hexanes:THF), gave 309 mg (78% yield) N-methyl-1-aminobenzotriazole as white crystals, m.p. 69.5–71.0°. NMR ($C^2HCl_3$): 3.20 (3H, s, Me), 4.83 (1H, s, NH, exchangeable with $D_2O$), 7.31–7.65 (3H, m, aromatic protons), and 7.94–8.08 ppm (1H, m, proton at C-7). EIMS (*m/e*): 149 (M + 1). (Found: C, 56.75; H, 5.63; N, 37.62. Calc. for $C_7H_8N_4$: C, 56.74; H, 5.44; N, 37.81.

*Cytochrome P-450 inactivation.* Microsomes from phenobarbital-induced Sprague-Dawley male rats were obtained as previously described.[12,14] The *in vitro* assay of the ability of substrates to destroy cytochrome P-450 in microsomes from phenobarbital pretreated Sprague-Dawley male rats was performed as previously described.[12,20] The incubation mixture contained microsomal protein (1.0 mg/ml), NADPH (1.0 mM), EDTA (1.5 mM), KCl (150 mM), and the indicated amount of test substrate in 100 mM Na/K phosphate buffer (pH 7.4). Incubations were carried out at 37° for the indicated time. The loss of cytochrome P-450 was determined by difference spectroscopic analysis of the reduced, CO-saturated incubation mixture vs an unreduced CO-saturated reference sample as reported.[12,20] The efficiency of inactivation of cytochrome P-450 by ABT was estimated in incubations with a limiting concentration of the substrate and a known concentration of the enzyme as done previously with an olefinic substrate.[12]

*Chloroperoxidase inactivation.* Chloroperoxidase ($4.77 \times 10^6$ units/mg protein, 2.0 mg protein/ml in 0.1 M $NaH_2PO_4$ buffer), glucose oxidase (18,000 units/g), and mono chlorodimedone were purchased from Sigma, glucose from Mallinckrodt, and ethylamine hydrochloride from Eastman Organic Chemicals. The inactivation of chloroperoxidase was assayed in incubation mixtures that contained the following: chloroperoxidase (43,300 units/ml), mono chlorodimedone (1 mM), 1-aminobenzotriazole (1 mM), glucose (15 mg/ml), and glucose oxidase (3.0 $\mu$g/ml). A number of control incubations, including one in which ethylamine was substituted for 1-aminobenzotriazole, were carried out. Individual incubations of 100 $\mu$l volume were initiated by addition of the glucose oxidase. The incubations were carried out at 25°. Aliquots of 10 $\mu$l were taken at various timepoints between 0 and 300 sec and were quenched by transfer into 2.50 ml of 0.1 M potassium phosphate buffer (pH 2.75) containing 0.1 mM mono chlorodimedone. The residual enzyme activity was assayed by the method of Hager *et al.*[25] with a Hewlett–Packard Model 8450A spectrophotometer. The change in absorbance at 278 nm with time was determined over the relatively linear period from 0 to 60 sec. Each time point was measured in three independent experiments. The cited values are averages of the three measurements, which differed from each other by less than 10%. The glucose oxidase–glucose generating system can be replaced by $H_2O_2$ but this results in some loss of activity even in the absence of 1-aminobenzotriazole.

*Metabolism of* 1-*aminobenzotriazole by chloroperoxidase.* The incubation mixtures contained chloroperoxidase (477,000 units), 1-aminobenzotriazole (9.26 mM), and $H_2O_2$ (7.96 mM) in a total volume of 1.35 ml of phosphate buffer (pH 2.75). The $H_2O_2$ was added in 17 aliquots at 15 sec intervals. Control incubations were carried out in the absence of $H_2O_2$, in the absence of chloroperoxidase, and with chloroperoxidase that was first inactivated by four cycles of freezing in a dry ice–acetone bath followed by heating 5 min at 90°. Five min after the final addition of $H_2O_2$ incubation mixtures were extracted with ether (the combined extracts were washed 3 times with 2–3 ml of buffer in the experiment with deuterated medium to insure removal of exchangeable protons) and the combined ether extracts were dried by passage through short columns of $Na_2SO_4$. The ether was removed under a stream of $N_2$ and the residues were analyzed by gas chromatography on a 6 ft. 3% OV 225 glass column maintained at 100° in a Varian Model 2100 instrument equipped with flame ionization detectors.

Analogous incubations of 1-aminobenzotriazole with chloroperoxidase were carried out to isolate and characterize the major metabolite. The incubation was carried out in normal buffer in one instance and in deuterated buffer in a second. The deuterated buffer was prepared by dissolving 136 mg of $KH_2PO_4$ and 25 $\mu$L of $H_3PO_4$ in 13 ml of 99.8% $^2H_2O$ (pD = 2.65). The concentrated ether extracts were chromatographed on a 5 in. × 0.25 in. 10% carbowax 20 M on 120/140 Chrom Q column maintained at 200° in a Varian Model 920 gas chromatograph fitted with a thermal conductivity detector. The fraction with the retention time of phenol was collected in a glass loop immersed in a dry ice–acetone bath. The collected material was washed out of the loop with ether and was analyzed by combined gas chromatography–mass spectrometry on a Kratos MS 25 under electron impact conditions (70 eV). The mass spectrometer gas chromatograph was fitted with a Supelco SP 2330 column.

*Isolation and characterization of the prosthetic heme adducts.* Phenobarbital pretreated (80 mg/kg daily for 4 days) Sprague-Dawley male rats were injected intraperitoneally with a 100 mg/kg dose of 1-aminobenzotriazole dissolved in 1 ml water. The rats were decapitated 4 hr later and their livers were perfused *in situ* with ice cold 0.9% NaCl aq before they were removed, homogenized, and suspended in 5% $H_2SO_4$ in MeOH soln (10 ml soln/g liver). The mixture, after standing overnight at 4°, was vacuum filtered and the filtrate was diluted with an equal volume of $CH_2Cl_2$. The organic layer was washed with several portions water until the aqueous phase was no longer acidic. The soln was then dried over $Na_2SO_4$ and the solvent was removed on a rotary evaporator at 30°. The viscous brown residue was dissolved in a small amount of $CH_2Cl_2$ and was chromatographed on

20 × 20 cm preparative plates with a 2 mm thick layer of silica gel G. The plates were developed with 5% v/v MeOH in $CH_2Cl_2$. A single red fluorescent band running just below the hemin fraction was observed after allowing continuous elution of the plate for 1.5 hr. The red fluorescent pigment was removed from the plate with the chromatography solvent and was rechromatographed twice more on 0.5 mm silica gel G plates developed with 5% MeOH in $CH_2Cl_2$. The electronic absorption spectrum of the product thus obtained was identical to that previously reported.[20] The NMR spectrum of the porphyrin was determined in $CDCl_3$ at 240 MHz.

A second (minor) red fluorescent band is observed if the crude pigment extracted from the livers of rats treated with 1-aminobenzotriazole is chromatographed as previously reported.[20] This minor fraction, not obtained in sufficient quantity for NMR analysis, was isolated from the thin layer plates and its chromatographic properties and electronic absorption spectrum were compared with those of an authentic sample of the dimethyl ester of N-phenylprotoporphyrin IX.[26]

## RESULTS

*Structure of the porphyrin adducts.* Preliminary work on the structure of the major abnormal porphyrin isolated from the livers of rats treated with 1-aminobenzotriazole established that its molecular weight was equal to that of the dimethylester of protoporphyrin IX (the dimethyl ester because of the workup procedure) plus the equivalent of benzyne.[20] The electronic absorption spectrum[20] was furthermore very similar to that of the dimethylester of N,N-dimethylprotoporphyrin IX alkylated on adjacent rather than trans nitrogens.[27] Purification of the porphyrin to the point where NMR studies could be undertaken, however, has proven much more difficult than the purification of adducts obtained with olefins and acetylenes because, due to the high basicity of N,N-dialkylated porphyrins,[28] the 1-aminobenzotriazole adduct is obtained in the protonated rather than free base form. Chromatographic procedures developed for other porphyrin adducts therefore have been ineffective. We have nevertheless succeeded in purifying the porphyrin even though we have not been able to separate the isomers due to alkylation of the different pairs of nitrogens in the parent porphyrin. The NMR spectrum of the isomer mixture (Fig. 1) confirms the postulate that two vicinal nitrogens of the porphyrin are bound to vicinal carbons of a benzene ring, a structure consistent with the addition of benzyne (or its equivalent) across two nitrogens of the prosthetic heme group. The signal assignments for protons of the protoporphyrin skeleton are the following: 10.0–11.3 (4 meso H), 7.9–8.2 (2 internal vinyl group H), 6.2–6.6 (4 terminal vinyl group H), 4.55 (4 sidechain methylene H adjacent to the porphyrin ring), 3.5–4.0 (18 methyl H), and 3.2–3.5 ppm (4 side chain methylene H adjacent to the carboxyl groups). These signal assignments, consistent with the number of protons determined by integration, have been made by analogy with the unambiguously assigned signals of mono N-alkylated porphyrins.[15,16,29] The side chain proton assignments are confirmed by the observation that irradiation of the methylene proton multiplet at approx. 3.3 ppm results in decoupling of the signal at 4.55 attributed to the second set of methylene protons on the propionic acid side chains. Irradiation of the protons at 4.55 ppm, the reverse decoupling experiment, likewise results in collapse of the broad triplet at 3.3 to a singlet (Fig. 1). The highly shielded protons of the bridging benzene group are located at 1.9 ppm (*ortho* protons) and 4.2 ppm. As required, irradiation of the multiplet at 1.9 ppm results in collapse of the multiplet at 4.2 ppm to a broad singlet (Fig. 1). The NMR spectrum of the adduct thus confirms the assigned structure but clearly is not that of a single isomer. The meso proton region, for example, is populated by two major sets of four singlets, as expected if two major isomers are present.

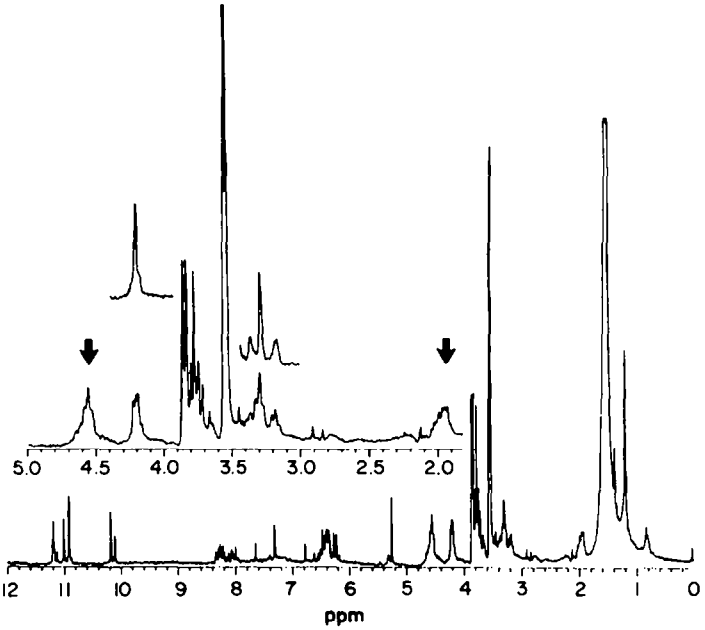



Fig. 1. NMR spectrum (360 MHz) of the dimethyl esterified hepatic porphyrin isolated from rats treated with 1-aminobenzotriazole. The peaks between 1 and 1.8 ppm are due to water and impurities. The chloroform peak at 7.2 ppm has been deleted from the spectrum. The region from 2 to 5 ppm is shown in expanded scale in the inset. The change in the spectrum observed upon irradiation at the position indicated by each of the arrows is shown in the inset.

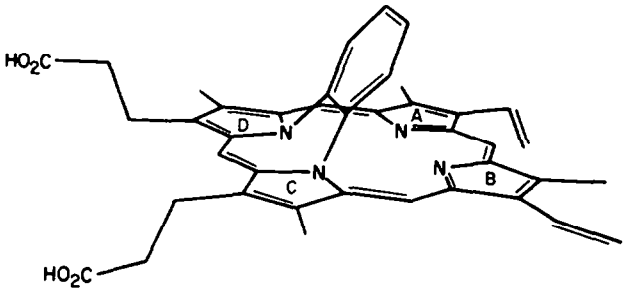



Fig. 2. Structure of the dimethylesterified porphyrin obtained with 1-aminobenzotriazole. One of the four possible regioisomers is arbitrarily shown although two major (still unassigned) isomers are actually formed.

We cannot determine at this time which two of the four possible isomers ($N_AN_B$, $N_BN_C$, $N_CN_D$, or $N_DN_A$) are involved. The $N_CN_D$-bridged structure is arbitrarily given in Fig. 2.

A minor red fluorescent porphyrin, in addition to that described above, has been isolated from the livers of rats treated with 1-aminobenzotriazole. This porphyrin is shown by the identity of both its chromatographic properties and characteristic electronic absorption spectrum (not shown) with those of an authentic sample[26] to be the dimethylester of N-phenylprotoporphyrin IX.

*Analogues of 1-aminobenzotriazole.* A number of derivatives of 1-aminobenzotriazole in which the exocyclic N is derivatized or replaced have been synthesized and examined to explore the relationship between structure and destructive activity. The results of these experiments (Table 1) indicate that the exocyclic amino group is critical for enzyme destruction. Its removal (as in benzotriazole, **6**), or its replacement with a hydroxyl function (as in 1-hydroxybenzotriazole, **8**) suppresses NADPH-dependent destructive activity, although high concentrations of benzotriazole mediate NADPH-independent conversion of cytochrome P-450 to cytochrome P-420 (Table 1). The nitro analogue (**9**), the nitro group of which can, in principle, be reduced to the hydroxylamine or amine,[30] could not be evaluated because it rapidly oxidized cytochrome P-450 during the spectrophotometric assay and thus interfered with quantitation of the reduced-CO bound form. Substitution of Me or Ac groups on the exocyclic N, in contrast to replacement of the N, gives derivatives that retain cytochrome P-450 destructive activity. The N,N-diacetyl (**2**), N-acetyl (**3**), N-acetyl-N-methyl (**4**), and N-methyl (**5**) derivatives of 1-aminobenzotriazole, administered to phenobarbital induced rats as already described for the parent compound, in all cases gave rise to abnormal porphyrins with the same chromatographic properties and electronic absorption spectra (not shown) as the porphyrin (Fig. 2) obtained with the parent compound. The isomer of aminobenzotriazole with the amino group at the 2-position (**7**) also destroys cytochrome P-450 but it is not known whether it causes the formation of an abnormal porphyrin.

Two structures have been synthesized to determine if the destructive activity associated with the aminotriazole moiety is specifically a property of 1-aminobenzotriazole or is expressed in other systems. Both of the structures examined are known to be chemically oxidized to the corresponding benzyne or diradical intermediates.[22,23] The linear naphthalene analogue, 1-amino-1H-naphtho(2,3-d)triazole (**10**), inactivates cytochrome P-450 and gives an abnormal hepatic porphyrin *in vivo*, whereas the derivative with the aminotriazole bridging the two naphthalene rings (**11**) destroys the enzyme but does not give rise to a hepatic pigment detectable by our normal procedure.

*Efficiency of cytochrome P-450 destruction by 1-aminobenzotriazole.* In order to estimate the statistical number of substrates turned over per cytochrome P-450 inactivation event, incubations were carried out with a known but limiting amount of 1-aminobenzotriazole. A loss of 15.4 nmol of cytochrome P-450 was observed in an incubation of 55.0 nmol of microsomal cytochrome P-450 with 187 nmol of 1-aminobenzotriazole at the point where destruction of the enzyme had ceased (Table 2). If the assumption is made that all of the substrate had been exhausted, the data indicate that approximately 12 molecules of **1** were metabolically eliminated per cytochrome P-450 enzyme inactivated. This metabolic ratio is not necessarily equal to the number of molecules turned over by cytochrome P-450 before it is inactivated because it has not been demonstrated that the substrate is only metabolized by this enzyme. The ratio of metabolism to inactivation for cytochrome P-450, however, must be equal to or smaller than 12. A similar approach has been used previously to determine the inactivation ratio for an olefin.[12]

*Toxicity of 1-aminobenzotriazole and its analogues.* The toxicity of the derivatives of 1-aminobenzotriazole prepared in this study has been briefly examined to evaluate their relative utility for the inactivation of cytochrome P-450 *in vivo*. The N-nitro derivative, in accordance with its ability to oxidize heme ($Fe^{+2}$ to $Fe^{+3}$), causes marked methemoglobinemia when administered to Sprague-Dawley rats. A 40 mg/kg dose of this compound is lethal. The N,N-diacetyl derivative of 1-aminobenzotriazole is also acutely toxic. Four out of six phenobarbital pretreated Charles River male rats injected intraperitoneally with a 300 mg/kg dose of this agent (injection in 1 ml of DMSO) died within 24 hr. At the lower dose of 100 mg/kg, the diacetyl derivative caused a doubling of serum transaminase levels within 24 hr (Table 3). In contrast, a dose of 1 mmol/kg of 1-aminobenzotriazole or of its N-acetyl, N-methyl, or N-acetyl-N-methyl derivatives

Table 1. Destruction of hepatic microsomal cytochrome P-450 by analogues of 1-aminobenzotriazole

| Substrate (mM) | | Cytochrome P-450 loss (%)[a] | Porphyrin adduct formed in vivo[b] |
|---|---|---|---|
| 1 | (10.0) | 86 | yes |
| | ( 1.0) | 77 | |
| | ( 0.1) | 59 | |
| 2 | (10.0) | 32 | yes |
| 3 | (10.0) | 29 | yes |
| | ( 1.0) | 20 | |
| 4 | ( 1.0) | 10 | yes |
| 5 | ( 1.0) | 43 | yes |
| 6 | ( 1.0) | c | - |
| 7 | ( 1.0) | 34 | - |
| 8 | ( 1.0) | N.D. | - |
| 9 | | d | N.D. |
| 10 | ( 5.0) | 65 | yes |
| 11 | ( 5.0) | 67[e] | N.D. |

[a]Loss of cytochrome P-450 after 30 min incubation. The substrate concentrations are nominal because some of the compounds are insoluble in water. The standard deviations for the cited values are less than ±2%.

[b]N.D., not detected: -, experiment not done

[c]Benzotriazole causes NADPH-independent conversion of cytochrome P-450 to cytochrome P-420 (9% at 1 mM and 55% at 10 mM).

[d]The N-nitro analogue interferes with the cytochrome P-450 assay.

[e]A drift of the baseline in the assay system caused by this highly insoluble compound reduces the accuracy of the reported value.

Table 2. Destruction of cytochrome P-450 by a limiting concentration of 1-aminobenzotriazole

| Incubation time (min) | P-450 loss (%) | P-450 remaining (nmol) |
|---|---|---|
| 0 | - | 55.0 |
| 30 | 20.0±0.5 | 44.0 |
| 60 | 27.7±1.2 | 39.8 |
| 90 | 27.3±1.5 | 40.0 |
| 120 | 28.0±1.0 | 39.6 |

1-Aminobenzotriazole/P-450 = 187 nmol/15.4 nmol = 12.1

Table 3. Serum transaminase levels in phenobarbital induced rats treated with 1-aminobenzotriazole and its analogues

| Agent administered | SGOT (I.U./L) | SGPT (I.U./L) |
|---|---|---|
| None (tricaprylin) | 88 | 22 |
| None (saline) | 60±8 | 19±1 |
| None (DMSO) | 64±8 | 21±2 |
| 2[a] | 164±12 | 41±5 |
| 1[b] | 53±8 | 21±4 |
| 3[c] | 57±6 | 22±3 |
| 4[c] | 62±4 | 20±3 |
| 5[c] | 58±3 | 20±2 |

[a]Administered in tricaprylin (0.5 mmol/Kg in 1 ml solvent).

[b]Administered in isotonic saline (1 mmol/Kg in 1 ml solvent).

[c]Administered in DMSO (1 mmol/Kg in 1 ml solvent).

did not significantly alter serum transaminase levels (Table 3).

*Inactivation of chloroperoxidase.* Chloroperoxidase is unusual among the peroxidases in that it appears to have a thiolate heme iron ligand,[31] as does cytochrome P-450, and catalyzes reactions similar to those identified with turnover of the latter enzyme.[32,33] These include the hydroxylation and dealkylation of nitrogen. Incubation of chloroperoxidase with 1-aminobenzotriazole, in further analogy with cytochrome P-450, results in time dependent inactivation of the enzyme (Fig. 3). Inactivation requires the presence of $H_2O_2$ but, under our conditions, is not observed in the absence of 1-aminobenzotriazole even when a simple amine is present (Fig. 3). In order to prevent inactivation of the enzyme by $H_2O_2$ alone[34] it is necessary to maintain a relatively high concentration (1 mM) of mono chlorodimedone, a substrate for the enzyme, during the entire incubation. The catalytic turnover of 1-aminobenzotriazole that results in enzyme inactivation thus competes with the metabolism of mono chlorodimedone. The inactivation of chloroperoxidase by 1-aminobenzotriazole, as predicted by this, is much slower when the concentration of mono chlorodimedone is raised to 10 mM while that of 1-aminobenzotriazole is held at 1 mM

*Metabolism of 1-aminobenzotriazole.* Catalytic turnover of 1-aminobenzotriazole by chloroperoxidase produces one major and two (detectable) minor metabolites (Fig. 4). The metabolites are not

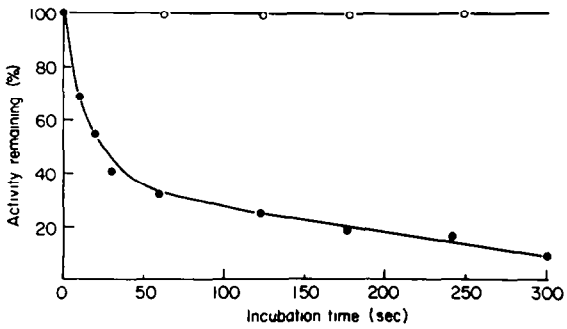



Fig. 3. Inactivation of chloroperoxidase by 1-aminobenzotriazole. The reaction conditions are given in Materials and Methods: (a) incubation in the presence of 1 mM 1-aminobenzotriazole (●), or (b) in the presence of 1 mM ethyl amine (○).

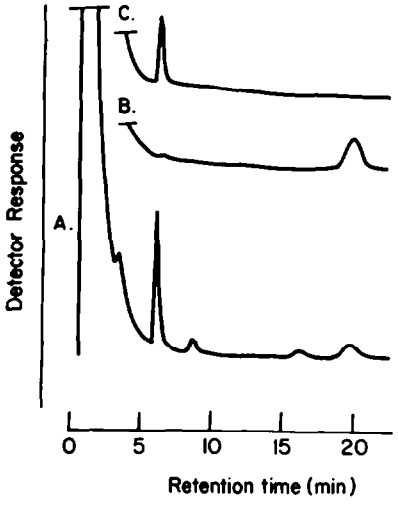


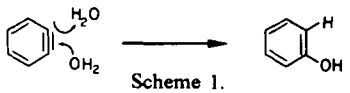

Fig. 4. Gas–liquid chromatographic analysis of the 1-aminobenzotriazole metabolites produced by chloroperoxidase: (a) analysis of the organic extract from a complete incubation, (b) analysis of the extract from an incubation without $H_2O_2$ (the same chromatogram is obtained from incubations with boiled chloroperoxidase), and (c) authentic phenol. The peak with highest retention time is due to a trace impurity in the substrate.

observed when boiled chloroperoxidase is used, or when either $H_2O_2$ or 1-aminobenzotriazole is excluded from the incubation. No effort has been made to identify the minor metabolites. The major metabolite, however, is identified by its retention time (Fig. 4) and mass spectrum (not shown) as phenol. In order to elucidate the mechanism by which phenol is formed, the principal metabolite was isolated from an incubation carried out in buffer prepared with 99.8% $^2H_2O$. The phenol thus obtained, after washing with buffer (pH 2.75) to remove exchangeable deuterium, is shown by the ratio of the molecular ions at *m/e* 94 and 95 to be approx. 60% monodeuterated. No evidence was found for the incorporation of two D atoms, as might be expected if phenol itself underwent a secondary reaction with the deuterated medium.

Incubations of 1-aminobenzotriazole with hepatic microsomes from phenobarbital pretreated rats have been extracted and the extracts have been analyzed for the presence of metabolites. Neither phenol nor any other metabolite was detected in these extracts.

## DISCUSSION

The structure established by NMR analysis (Fig. 1) for the porphyrin derived from the reaction of 1-aminobenzotriazole with prosthetic heme (Fig. 2) supports the postulate that benzyne, liberated by the catalytic action of cytochrome P-450, reacts with the prosthetic heme group. Benzyne formation is, of course, predicted by the known chemistry of 1-aminobenzotriazole.[18,19] Independent evidence for benzyne as a distinct intermediate in the biological oxidation of 1-aminobenzotriazole is provided by the metabolites formed from this substrate. Although no metabolites have been isolated from incubations with hepatic microsomes, presumably because of the low number of substrate molecules turned over per inactivation event (Table 2) and the possible interception of benzyne by microsomal constituents rather than water, solid evidence for the formation of benzyne in the oxidation of 1-aminobenzotriazole by chloroperoxidase has been obtained. Phenol, the principal metabolite produced by the catalytic action of chloroperoxidase on 1-aminobenzotriazole, has been shown to result from incorporation of a OH group and a proton into the substrate-derived benzene ring. Benzyne is known to react with water to give phenol (Scheme 1).[35] The formation of detectable amounts of



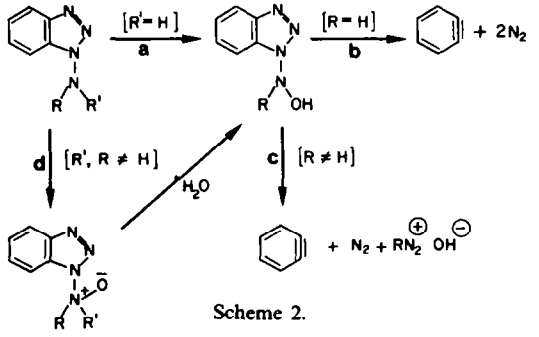

Scheme 1.

phenol with chloroperoxidase requires both a relatively low destructive efficiency (very preliminary data suggest a value of several hundred molecules of substrate turned over per enzyme molecule destroyed) and reasonable exposure of the reactive intermediate to the aqueous medium. Phenol formation has not been quantitated because chloroperoxidase catalyzes the further oxidation of phenol to colored polymeric products.

Attempts to isolate a prosthetic heme adduct from the reaction of chloroperoxidase with 1-aminobenzotriazole have not been successful. The large amounts of colored products formed in the reaction interfere with detection of the adduct if it is formed. On the other hand, the inactivation of chloroperoxidase by this substrate may involve reaction of benzyne with the protein rather than the prosthetic heme group or the prosthetic heme adduct may be rapidly degraded in the peroxidative incubation medium.

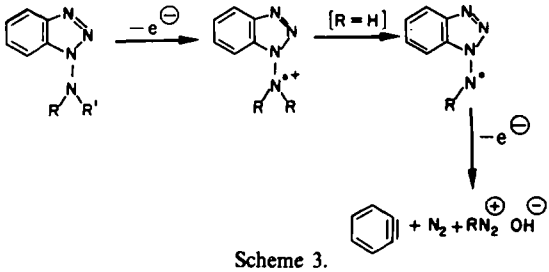
Loss of destructive activity upon replacement of the exocyclic amino function by other moieties is not surprising because it is the trigger that releases benzyne from 1-aminobenzotriazole. The oxidation of 1-hydroxybenzotriazole, for example, gives a stabilized radical but does not yield benzyne.[36] The N-nitro derivative accepts electrons from the reduced Fe of heme, as shown by the efficiency with which it oxidizes hemoglobin and cytochrome P-450, but it apparently is not reduced to a species that merges into the pathway leading to benzyne. Studies with aromatic nitro compounds have shown that nitro radical anions obtained by electron transfer to the nitro function are discharged in the presence of oxygen by transfer of the electron to oxygen with concomitant generation of superoxide.[30] It is possible that the N-nitro derivative is not reduced to a destructive species due to the intervention of such a redox cycle, although this has not been explicitly demonstrated.

The ability of all the derivatives with substituents on the exocyclic amino function to inactivate cytochrome P-450 is surprising. The N,N-diacetyl, N-acetyl, N-acetyl-N-methyl, and N-methyl derivatives not only destroy the enzyme *in vitro* but also give prosthetic heme adducts *in vivo* (Table 1). It is conceivable that 1-aminobenzotriazole is liberated metabolically from each of the derivatives, although it would be highly surprising if all the analogues are converted to the parent compound, particularly *in vitro*. The alternative is that cytochrome P-450 oxidizes the exocyclic nitrogen, even when substituted, to a species that unmasks benzyne or a benzyne equivalent. It is of interest that, if benzyne is liberated from the substituted analogues, the reaction must be accompanied not by the liberation of two molecules of nitrogen but rather of one molecule of nitrogen and of a diazoalkane or diazoacetyl moiety. Although oxidation of 1-aminobenzotriazole and its N-monosubstituted derivatives could proceed by way of hydroxylamine derivatives (Scheme 2), oxidation of the disubstituted species necessarily is more complex. Two electron oxidation to the N-oxide and subsequent hydrolysis (or rearrangement of the amide function to the N-hydroxyacetate) is one possibility (Scheme 2). On the other hand, we have




Scheme 2.

recently provided strong evidence that the oxidation of nitrogen by cytochrome P-450 can proceed in one-electron steps.[37] It is therefore possible that not only the disubstituted derivatives but, indeed, 1-aminobenzotriazole itself, are activated by a one electron oxidation (Scheme 3).

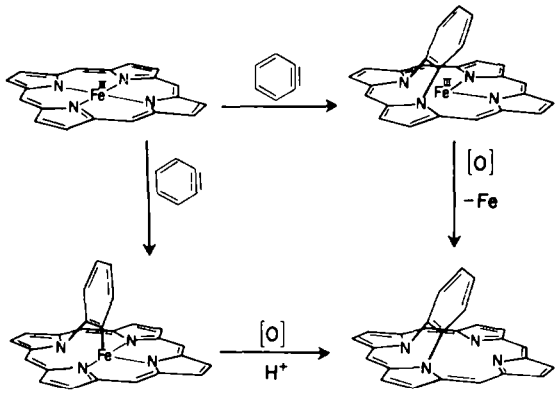



Scheme 3.

The ability of 1-amino-1H-naphtho(2,3-d)triazole (**10**), the "stretched-out" homologue of 1-aminobenzotriazole, to destroy cytochrome P-450 by prosthetic heme alkylation suggests that other analogues of the polycyclic aromatic hydrocarbons in which a carbon ring is replaced by the aminotriazole moiety will retain destructive potential. The failure of 1-aminonaphtho(1,8-de)triazine (**11**) to give an adduct, however, indicates that the carbon ring that is replaced may not bridge two other rings. The clues provided by these results should facilitate the design of suicide substrates that selectively or specifically inactivate forms of cytochrome P-450 involved in the oxidative metabolism of polycyclic aromatic hydrocarbons. In the context of isozyme selectivity, 1-aminobenzotriazole has recently been found in plants to be a selective suicide substrate for cinnamic acid 4-hydroxylase, a cytochrome P-450 enzyme that specifically carries out the title reaction.[38]

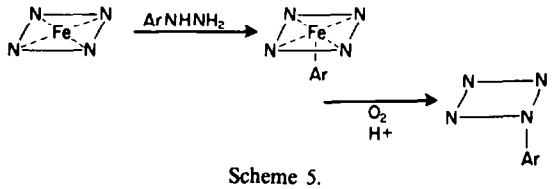
We have reported that 1-aminobenzotriazole does not decrease cytochrome $b_5$ levels and does not stimulate lipid peroxidation.[20] The results in Table 3 establish that 1-aminobenzotriazole also does not detectably elevate serum transaminase levels, a result that precludes hepatic damage severe enough to result in leakage of cytosolic enzymes. The absence of evidence for acute toxicity suggests that 1-aminobenzotriazole can be used heuristically to suppress the activity of hepatic cytochrome P-450 enzymes. The relatively broad range of isozymes susceptible to the action of this substrate makes it a useful complement to more selective agents like 2-isopropyl-4-pentenamide.[13,14] The N,N-diacetyl and the nitro analogues of 1-aminobenzotriazole, however, are highly toxic. Toxicity thus appears to be associated with factors other than the production of benzyne, a result which argues that little benzyne escapes from the cytochrome P-450 active site. The relatively low ratio of substrates turned over per enzyme inactivation event and the failure to detect metabolites of 1-aminobenzotriazole in microsomal incubations are consistent with this observation.

The mechanism of the reaction of the cytochrome P-450 prosthetic heme with benzyne (or its equivalent) remains to be defined. Benzyne may react in a concerted fashion with two nitrogens of the heme moiety (Scheme 4), a reaction that would give rise to a reduced porphyrin. Autoxidation of the dihydroporphyrin at a point prior to its isolation would yield




Scheme 4.

the observed adduct (Fig. 2). An alternative is that the benzyne triple bond reacts, either simultaneously or in a nonconcerted sequential fashion, with a nitrogen and with the Fe atom (Scheme 4). Recent work from this laboratory has demonstrated that the reaction of hemoglobin with phenylhydrazine yields a stable iron-phenyl intermediate that oxidatively rearranges, in the presence of air, to the N-phenyl derivative (Scheme 5).[39,40] Thus, if a species with the




Scheme 5.

benzene ring bridging the Fe and one of the porphyrin nitrogens is formed, a shift analogous to that in the phenylhydrazine–hemoglobin system would account for the observed N,N-bridged product. The isolation in low yield of N-phenylprotoporphyrin IX from rats treated with 1-aminobenzotriazole is pertinent in this regard because non-oxidative solvolysis of the iron–nitrogen bridged species would be expected, again in analogy with the hemoglobin system,[39,40] to yield N-phenyl heme.

*Acknowledgments*—We are greatly indebted to Dr. Kent L. Kunze for the NMR spectrum of the 1-aminobenzotriazole adduct. This research was supported by National Institutes of Health Grant GM 25515 and by an allocation from the University of California Cancer Research Coordinating Committee.

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