Reduction of 1,3-Diphenyl-1-triazene by Rat Hepatic Microsomes, by Cecal Microflora, and in Rats Generates the Phenyl Radical Metabolite: An ESR Spin-Trapping Investigation

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · DECEMBER 2000

Impact Factor: 3.53 · DOI: 10.1021/tx000098v · Source: PubMed

4 AUTHORS, INCLUDING:

Maria Kadiiska
National Institute of Environmental Health S…
120 PUBLICATIONS **4,183** CITATIONS
SEE PROFILE

Ronald P Mason
National Institute of Environmental Health S…
565 PUBLICATIONS **21,077** CITATIONS
SEE PROFILE

Reduction of 1,3-Diphenyl-1-triazene by Rat Hepatic Microsomes, by Cecal Microflora, and in Rats Generates the Phenyl Radical Metabolite: An ESR Spin-Trapping Investigation

Maria B. Kadiiska,*,† Kristi S. De Costa,‡ Ronald P. Mason,† and James M. Mathews‡

Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, National Institutes of Health, and Center for Bioorganic Chemistry, Research Triangle Institute, Research Triangle Park, North Carolina 27709

Received April 28, 2000

An ESR spin-trapping technique was used to determine whether free radical metabolites are formed as a result of the reduction of 1,3-diphenyl-1-triazene (DPT) in vivo and in vitro by components of the cytochrome P450 (P450) mixed-function oxidase system in microsomes or by gut microflora in anaerobic cecal incubations. The ESR spectrum of the DMPO−phenyl radical adduct was detected in a microsomal incubation containing DPT, DMPO, and NADPH with the following hyperfine coupling constants: $a^{N} = 15.95$ G and $a_{\beta}^{H} = 24.37$ G. The amplitude of the spectrum from the phenyl radical adduct generated in microsomal incubations of DPT with DMPO and NADPH was not attenuated by the P450 inhibitor 1-aminobenzotriazole (ABT) or by carbon monoxide, indicating that P450 is not significantly involved in phenyl radical formation. The formation of a DMPO−phenyl radical adduct was also catalyzed by recombinant human cytochrome P450 reductase. Addition of anti-rat P450 reductase antibody led to an attenuation of the signal in incubations containing either microsomes or reductase. Low concentrations of DMPO−phenyl radical adducts were detected by ESR in the toluene extract of cecal contents containing DPT and the spin trap. In the in vivo experiments with rats treated with DPT and the spin trap DMPO, the six-line ESR signal of the DMPO−phenyl radical adduct was readily detected in bile 40−60 min after rats were treated with DPT and DMPO. The results show for the first time that the phenyl radical is formed by the reduction of DPT and may indicate a toxic potential for this chemical.

Introduction

1,3-Diphenyl-1-triazene (diazoaminobenzene, DPT) is used in the synthesis of polymers and dyes, and has been found as an impurity in colorants used in food products (*1*), in lipsticks, and in ingested and externally applied drugs (*2*). Exposure of humans to DPT induces many toxic effects, including eye and skin irritation, nausea, vomiting, and methemoglobinemia (*3*). We have recently reported that DPT is metabolized in vivo to metabolites mainly excreted in the urine, but also in feces and in the expired breath of rodents (*4*). Benzene was exhaled in the breath following oral administration of DPT to male rats and mice, and the profile of metabolites detected in the urine was common to those of benzene (hydroquinone glucuronide, muconic acid, prephenylmercapturic acid, phenol glucuronide, and phenyl sulfate) and aniline (4-acetamidophenyl sulfate). Benzene and aniline were also detected in the blood of rats, and their metabolites were found in human liver slices after incubation with DPT. These observations are consistent with DPT reduction, which initially forms the phenyl diazenyl radical and aniline. The phenyl diazenyl radical is known to decompose to form the phenyl radical (and ultimately benzene) and nitrogen gas (*5*, *6*). Reductions can be catalyzed by hepatic microsomal NADPH-cytochrome P450 reductase (*7*) and by gut microflora (*8*). The gut microflora are an important agent of reductive metabolism because of their anaerobic environment and high nitroreductase activity (*8*−*12*). In the study presented here, we used the ESR spin-trapping technique to determine whether phenyl radicals are formed as a result of the reduction of DPT by components of the cytochrome P450 (P450) mixed-function oxidase system in microsomes or by gut microflora in anaerobic cecal incubations.

Materials and Methods

Chemicals. DPT (95%) was purchased from Acros Organics (Pittsburgh, PA) or Aldrich Chemical Co. (Milwaukee, WI). The identity of DPT was confirmed by nuclear magnetic resonance spectrometry (NMR) and by mass spectrometric analysis.

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Fluka Chemical Corp. (Ronkonkoma, NY). DMPO was vacuum-distilled twice and stored at −70 °C.

[$^{13}C_2$]Dimethyl sulfoxide (DMSO) (minimum of 99% ^{13}C) was from Isotec (Miamisburg, OH). Deferoxamine mesylate, 2,2-dipyridyl (DP), superoxide dismutase, and β-NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Recom-

* To whom correspondence should be addressed: National Institute of Environmental Health Sciences, National Institutes of Health, P.O. Box 12233, Research Triangle Park, NC 27709. Phone: (919) 541-0201. Fax: (919) 541-1043. E-mail: Kadiiska@niehs.nih.gov.

† National Institutes of Health.

‡ Research Triangle Institute.

10.1021/tx000098v CCC: $19.00
Published on Web 10/10/2000

binant human NADPH-P450 oxidoreductase was purchased from Panvera Corp. (Madison, WI). Anti-rat NADPH cytochrome P450 reductase serum containing a polyclonal antibody produced by immunizing a goat was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Catalase free of superoxide dismutase was from Boehringer Mannheim (Indianapolis, IN). Toluene was obtained from J. T. Baker, Inc. (Phillipsburg, NJ). The reagents and buffer were prepared using deionized water and were treated with Chelex 100 (Bio-Rad, Hercules, CA).

Animals and Treatments. Adult male Fischer 344 (F-344) rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC), and they were furnished Purina Rodent Chow (#5002) and water ad libitum. The intragastric dose formulation contained DPT in a water/Emulphor EL-620 mixture (8:2, v/v) to allow delivery of the target dose in a total of 5 mL/kg of body weight.

Microsome Preparation. Liver samples were homogenized with a Potter-Elvehjem homogenizer in 3 volumes of ice-cold 2.5 mM HEPES buffer (pH 7.4) containing 0.15 M KCl. The homogenates were first centrifuged at 9000*g* for 20 min, and then the supernatant was centrifuged at 100000*g* for 50 min. The microsomal pellets were removed, resuspended, and resedimented at 100000*g* for 30 min. The resulting microsomal pellets were resuspended in buffer, flash-frozen in liquid nitrogen, and stored at −70 °C until they were used.

In Vitro Incubations. In vitro experiments were performed with cecal contents, microsomes, and recombinant human NADPH-P450 oxidoreductase. Incubations of the cecal contents [ca. 100 mg/mL in 100 mM phosphate buffer (pH 7.4)] with DPT (ca. 20 mM final concentration) were carried out in a glovebag saturated with nitrogen gas. An extraction of the incubation mixture with nitrogen-sparged toluene was required for detection of the radical adducts. Incubation of DPT (ca. 20 mM final concentration) with microsomes (1 mg of protein/mL) or reductase (2.4 pmol/mL) in 100 mM phosphate buffer (pH 7.4) containing 50 μM deferoxamine mesylate and 1 mM NADPH was performed under ambient conditions.

In Vivo Studies. In vivo experiments were conducted with anesthetized (sodium pentobarbital, 50 mg/kg ip) and bile duct-cannulated rats. DPT was administered directly into the stomach, and DMPO was administered ip. Bile samples were collected for 2 h post dosing at 20 min intervals after administration of DMPO (1 g/kg) and DPT (16 mg/kg) in four rats. An aliquot (50 μL) of the iron-chelating agent 2,2-dipyridyl (30 mM) was added to bile collections to inhibit the formation of iron radical adducts generated ex vivo.

ESR Measurement and Computer Simulations. ESR spectra were recorded from a Varian E-109 spectrometer equipped with a TM_{110} cavity operating at 9.33 GHz, a power of 20 mW, and a modulation frequency of 100 kHz.

The ESR spectral simulations were performed using an automatic optimization procedure (*13*). The parameters that were used for simulating each species are described in the figure legends.

Results

The ESR spectrum of the DMPO radical adduct detected in a microsomal incubation containing DPT, DMPO, and NADPH is shown in Figure 1A. The ESR spectrum of this radical adduct could be detected when scanned immediately or 30 min later. The computer simulation of this radical adduct was calculated using the following hyperfine coupling constants: $a^N = 15.95$ G and $a_\beta^H = 24.37$ G (Figure 1B). This radical adduct was assigned to the DMPO−phenyl free radical adduct on the basis of comparison of these splitting constants and line widths with those reported in the literature (*6*, *14*, *15*). The full complement of microsomes, NADPH, DMPO, and DPT were required for optimal radical detection (Figure 1C−F). The addition of superoxide

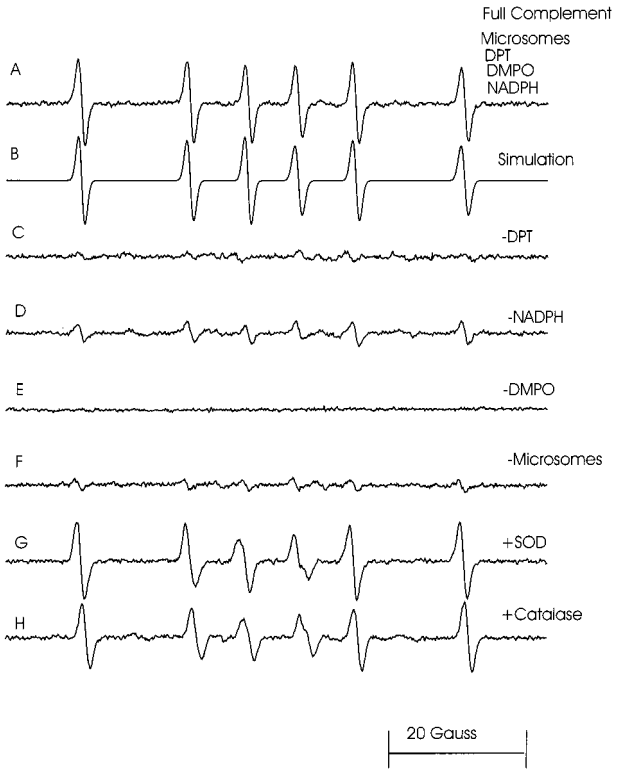


Figure 1. ESR spectra of the DMPO−phenyl adduct detected from microsomal incubations of DPT, DMPO, and NADPH. (A) DMPO−phenyl radical adduct detected by ESR from a reaction mixture containing 1 mg/mL microsomal protein, 22.5 mM DPT, 200 mM DMPO, and 1 mM NADPH in 100 mM Chelex-treated phosphate buffer (pH 7.4). (B) Computer simulation of spectrum A. (C) Same as for spectrum A, but without DPT. (D) Same as for spectrum A, but without NADPH. (E) Same as for spectrum A, but without DMPO. (F) Same as for spectrum A, but without microsomes. (G) Complete incubation as for spectrum A with superoxide dismutase SOD (50 μg/mL). (H) Complete incubation as for spectrum A with catalase (50 μg/mL). The instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.250 s; and scan time, 4 min.

dismutase (Figure 1G) or catalase (Figure 1H) did not affect the formation of radical adducts in microsomal incubations.

The amplitude of the spectrum from the phenyl radical adduct generated in microsomal incubations of DPT with DMPO and NADPH (Figures 1A and 2A) was not attenuated by the P450 inhibitor 1-aminobenzotriazole (ABT) (Figure 2B) or by carbon monoxide (Figure 2C), indicating that P450 is not significantly involved in phenyl radical formation.

The formation of a DMPO−phenyl radical adduct was also catalyzed by recombinant human cytochrome P450 reductase (Figure 3C). Addition of anti-rat P450 reductase antibody led to an attenuation of the signal in incubations containing either microsomes (Figure 3B) or reductase (Figure 3D).

The metabolism of DPT under anaerobic conditions was examined in the isolated cecal contents of rats. Low concentrations of DMPO radical adducts were detected in the toluene extract of cecal contents containing DPT and the spin trap (Figure 4A).

The spectrum was the result of at least two radical species. The major species of DMPO radical adduct present in the toluene extract was characterized by the following hyperfine coupling constants: $a^N = 13.9$ G and

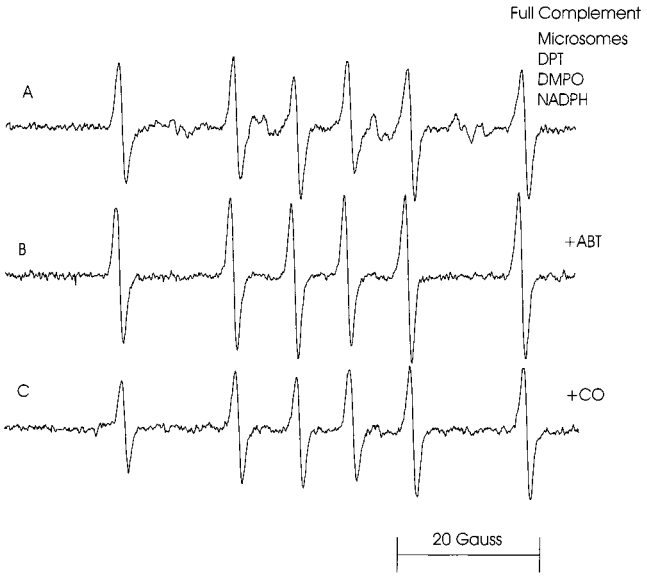


Figure 2. ESR spectra of DMPO−phenyl adducts detected from microsomal incubations of DPT, DMPO, ABT, and CO. (A) DMPO−phenyl radical adduct detected by ESR from a reaction mixture containing 1 mg/mL microsomal protein, 22.5 mM DPT, 200 mM DMPO, and 1 mM NADPH in 100 mM Chelex-treated phosphate buffer (pH 7.4). (B) Same as for spectrum A, but with 1 mM ABT. (C) Same as for spectrum A, but with CO bubbled for 60 s. The instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.250 s; and scan time, 4 min.

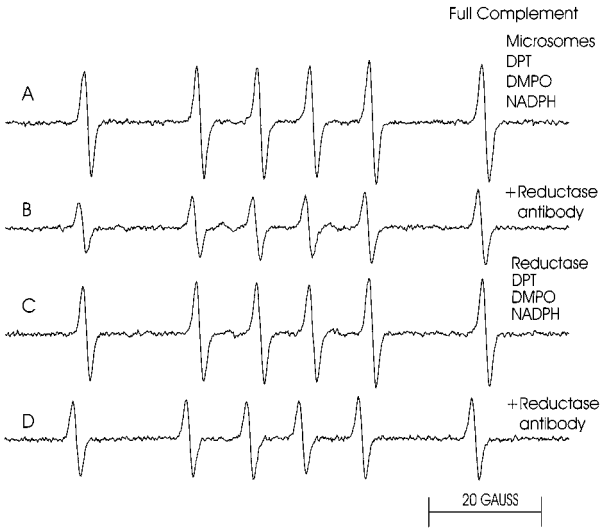


Figure 3. ESR spectra of DMPO adducts detected from microsomal incubations of DPT, DMPO, NADPH, cytochrome P450 antibody, and NADPH-cytochrome P450 reductase. (A) DMPO−phenyl radical adduct detected by ESR from a reaction mixture containing 1 mg/mL microsomal protein, 22.5 mM DPT, 200 mM DMPO, and 1 mM NADPH in 100 mM Chelex-treated potassium phosphate buffer (pH 7.4). (B) Same as for spectrum A, but with rat cytochrome P450 reductase antibody (3 μL/15 μg of microsomal protein). (C) Same as for spectrum A, but with NADPH-cytochrome P450 reductase replacing microsomes. (D) Same as for spectrum C, but with rat cytochrome-P450 reductase antibody. The instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.250 s; and scan time, 4 min.

a_{β}^{H} = 19.4 G; these values are consistent with the spectrum of the DMPO−phenyl radical adducts in benzene (*16*, *17*). These radical adducts were not detected in the absence of cecal content (Figure 4B), DPT (Figure 4C), DMPO (Figure 4D), or toluene (Figure 4E) or in the presence of air (Figure 4F).

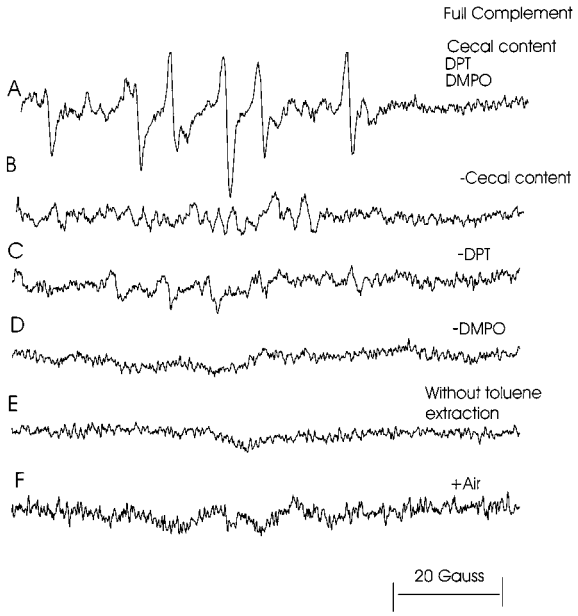


Figure 4. ESR spectra of DMPO radical adducts formed anaerobically by in vitro incubation of cecal contents. (A) ESR spectra obtained from cecal contents (ca. 100 mg/mL) containing 19.2 mM DPT and 200 mM spin trap in 100 mM potassium phosphate buffer (pH 7.4). An incubation mixture (1.06 mL) was added anaerobically to 3 mL of deoxygenated toluene, and the ESR analysis was performed on the toluene extract. (B) Same as for spectrum A, but without cecal content. (C) Same as for spectrum A, but without DPT. (D) Same as for spectrum A, but without DMPO. (E) Same as for spectrum A, but without toluene extraction. (F) Same as for spectrum A, but the experiment was performed aerobically. The instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.250 s; and scan time, 4 min.

We have also performed experiments in vivo with rats treated with DPT and the spin trap DMPO. The six-line ESR signal of the DMPO−phenyl radical adduct was readily detected in bile 40−60 min after rats were treated with DPT and DMPO (Figure 5A). The computer simulation (Figure 5B) of the radical adduct detected was calculated using the following hyperfine coupling constants: a^{N} = 15.98 G and a_{β}^{H} = 24.61 G. This radical was assigned to the DMPO−phenyl free radical adduct. The formation of this adduct required an administration of both DPT (Figure 5C) and DMPO (Figure 5D).

Discussion

The proposed pathway for formation of the phenyl radical by the one-electron reduction of DPT is shown in Scheme 1. The experiments described herein provide evidence that the triazene DPT is cleaved reductively to ultimately form phenyl radicals in vivo and in vitro, as depicted in Scheme 1. Assignment of the chemical structure of the DMPO−phenyl radical adduct was achieved by the close agreement of the hyperfine coupling constants with the published literature.

In vitro generation of the phenyl radical required the full complement of NADPH, DMPO, DPT, and either microsomes or cytochrome P450 reductase. Addition of either of the P450 inhibitors, ABT or carbon monoxide, caused no attenuation of phenyl radical formation. Taken together, these data demonstrate that cytochrome P450 reductase, rather than the hemoprotein itself, catalyzed radical formation via reduction. In the bile of rats treated with DMPO and DPT, strong spectra of the DMPO−

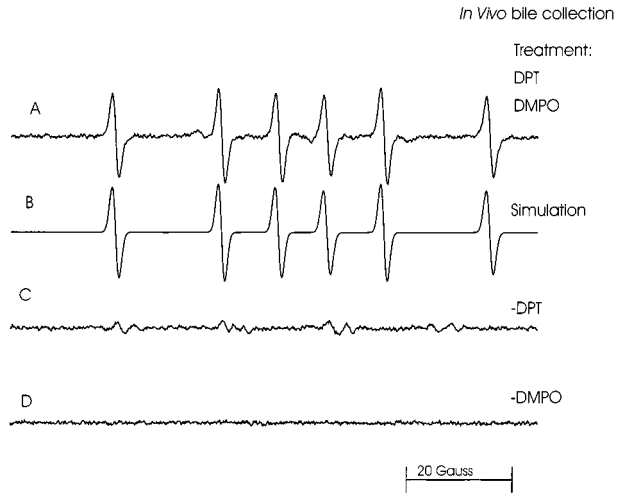
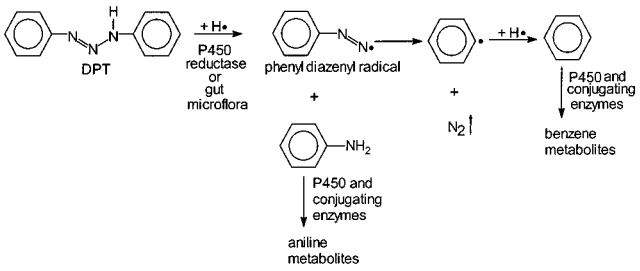


Figure 5. (A) ESR spectra of radical adducts detected in the bile of male Fisher rats 40−60 min post DPT (16 mg/kg ip) and DMPO (1 g/kg ip) administration. Bile was collected into a solution of 30 mM dipyridyl and 30 mM bathocuproine disulfonic acid. (B) Computer simulation of spectrum A. (C) Same as for spectrum A, but rats were not treated with DPT. (D) Same as for spectrum A, but rats were not treated with DMPO. The instrumental conditions were as follows: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 0.250 s; and scan time, 4 min.

Scheme 1. Proposed Pathway for the Metabolism of DPT



phenyl radical adduct were detected as the only radical present. This suggests that phenyl radicals are formed in abundance by reduction of DPT in the hepatocytes and are subsequently excreted into the bile as the radical adduct because the phenyl radical is too reactive to be excreted into bile.

These results are consistent with recent reports from this laboratory of the metabolism of DPT in rats and mice in vivo and in human liver slices to produce benzene, aniline, and their known metabolites (*4*).

The reduction of arene diazonium ions to form phenyl radicals and subsequent C^8-arylguanine adducts in calf thymus DNA has been reported (*18*). Decomposition of arene diazonium adducts of adenine in the same system was also postulated to involve phenyl radicals, which also led to alkylation of the purine in the C^8 position and the generation of apurinic sites (*19*). Both processes were suggested to contribute to the genotoxicity of arylhydrazides, arylhydrazines, and arenediazonium ions (*20*, *21*). Similarly, the triazeno moiety of the anti-HIV drug zidovudine (AZT) is also metabolized by reduction to form the amine metabolite AMT in a reaction that is at least partially catalyzed by cytochrome P450 reductase (*22*−*24*). Additionally, the 1-aryl-3,3-dimethyltriazenes are antitumor compounds whose action lies in their capacity to alkylate DNA (*25*, *26*). It has been postulated that decomposition of their monodemethylated metabolites produces anilines and diazomethane, but alkyl radicals could also be invoked in this process as has been shown in these studies for DPT.

This paper and previous reports (*4*) from this laboratory provide a comprehensive description of the mechanism and products of the metabolism of DPT. It is likely that other linear triazenes are metabolized in a similar fashion to produce an amine and a diazenyl radical, which decomposes to form reactive alkyl radicals. In addition to the benzene and aniline metabolites of DPT, the formation of phenyl radicals and probable phenyl DNA adducts may indicate a toxic potential for this chemical.

Acknowledgment. We thank Ms. Mary J. Mason for editorial assistance.

References

(1) Palmer, S., and Mathews, R. A. (1986) The role of non-nutritive dietary constituents in carcinogenesis. *Surg. Clin. North Am.* **66**, 891−915.

(2) Bailey, J. E., Jr. (1985) Determination of 1,3-diphenyltriazene and azobenzene in D&C Red No. 33 by solvent extraction and reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **321**, 185−197.

(3) NLM (2000) *HSDB (Hazardous Substance Database)*, Bethesda, MD (record 5410).

(4) Mathews, J. M., and De Costa, K. S. (1999) Absorption, metabolism, and disposition of 1,3-diphenyl-1-triazene in rats and mice after oral, i.v., and dermal administration. *Drug Metab. Dispos.* **27**, 1499−1504.

(5) Galli, C. (1988) Radical reactions of arenediazonium ions: an easy entry into the chemistry of the aryl radical. *Chem. Rev.* **88**, 765−792.

(6) Reszka, K. J., and Chignell, C. F. (1995) One-electron reduction of arenediazonium compounds by physiological electron donors generates aryl radicals. An EPR and spin trapping investigation. *Chem.-Biol. Interact.* **96**, 223−234.

(7) Fujita, S., and Peisach, J. (1977) Electron transfer between liver microsomal cytochrome b5 and cytochrome P-450 in the azo reductase reaction. *Biochem. Biophys. Res. Commun.* **78**, 328−335.

(8) Scheline, R. R. (1973) Metabolism of foreign compounds by gastrointestinal microorganisms. *Pharmacol. Rev.* **25**, 451−523.

(9) Reddy, B. G., Pohl, L. R., and Krishna, G. (1976) The requirement of the gut flora in nitrobenzene-induced methemoglobinemia in rats. *Biochem. Pharmacol.* **25**, 1119−1122.

(10) Facchini, V., and Griffiths, L. A. (1981) The involvement of the gastro-intestinal microflora in nitro-compound-induced methaemoglobinaemia in rats and its relationship to nitrogroup reduction. *Biochem. Pharmacol.* **30**, 931−935.

(11) Rickert, D. E., Long, R. M., Krakowka, S., and Dent, J. G. (1981) Metabolism and excretion of 2,4-[^{14}C]dinitrotoluene in conventional and axenic Fischer-344 rats. *Toxicol. Appl. Pharmacol.* **59**, 574−579.

(12) Levin, A. A., and Dent, J. G. (1982) Comparison of the metabolism of nitrobenzene by hepatic microsomes and cecal microflora from Fischer-344 rats *in vitro* and the relative importance of each *in vivo*. *Drug Metab. Dispos.* **10**, 450−454.

(13) Duling, D. R. (1994) Simulation of multiple isotropic spin-trap EPR spectra. *J. Magn. Reson., Ser. B* **104**, 105−110.

(14) Hill, H. A. O., and Thornalley, P. J. (1983) The effect of spin traps on phenylhydrazine-induced haemolysis. *Biochim. Biophys. Acta* **762**, 44−51.

(15) Maples, K. R., Jordan, S. J., and Mason, R. P. (1988) *In vivo* rat hemoglobin thiyl free radical formation following administration of phenylhydrazine and hydrazine-based drugs. *Drug Metab. Dispos.* **16**, 799−803.

(16) Barker, P., Bechwith, A. L. J., Cherry, W. R., and Huie, R. (1985) Characterization of spin adducts obtained with hydrophobic nitrone spin traps. *J. Chem. Soc., Perkin Trans. 2*, 1147−1150.

(17) Augusto, O., Kunze, K. L., and Ortiz de Montellano, P. R. (1982) *N*-Phenylprotoporphyrin IX formation in the hemoglobin-phenylhydrazine reaction. Evidence for a protein-stabilized iron-phenyl intermediate. *J. Biol. Chem.* **257**, 6231−6241.

(18) Gannett, P. M., Lawson, T., Miller, M., Thakkar, D. D., Lord, J. W., Yau, W.-M., and Toth, B. (1996) 8-Arylguanine adducts from arenediazonium ions and DNA. *Chem.-Biol. Interact.* **101**, 149−164.
(19) Gannett, P. M., Powell, J. H., Rao, R., Shi, X., Lawson, T., Kolar, C., and Toth, B. (1999) C^8-Arylguanine and C^8-aryladenine formation in calf thymus DNA from arenediazonium ions. *Chem. Res. Toxicol.* **12**, 297−304.
(20) Lawson, T., Gannett, P. M., Yau, W.-M., Dalal, N. S., and Toth, B. (1995) Different patterns of mutagenicity of arenediazonium ions in V79 cells and *Salmonella typhimurium* TA102: evidence for different mechanisms of action. *J. Agric. Food Chem.* **43**, 2627−2635.
(21) Gannett, P. M., Shi, X., Lawson, T., Kolar, C., and Toth, B. (1997) Aryl radical formation during the metabolism of arylhydrazines by microsomes. *Chem. Res. Toxicol.* **10**, 1372−1377.
(22) Rajaonarison, J. F., La Carelle, B., Durand, A., and Catalin, J. (1993) In-vitro metabolism of zidovudine (AZT) in human. *Therapie* **48**, 341−343.
(23) Eagling, V. A., Howe, J. L., Barry, M. J., and Back, D. J. (1994) The metabolism of zidovudine by human liver microsomes *in vitro*: formation of 3′-amino-3′-deoxythymidine. *Biochem. Pharmacol.* **48**, 267−276.
(24) Veal, G. J., and Back, D. J. (1995) Metabolism of zidovudine. *Gen. Pharmacol.* **26**, 1469−1475.
(25) Mizuno, N. S., Decker, R. W., and Zakis, B. (1975) Effects of 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (NSC-407347), an alkylating agent derived from 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (NSC-45388). *Biochem. Pharmacol.* **24**, 615−619.
(26) Carvalho, E., Iley, J., Perry, M. d. J., and Rosa, E. (1998) Triazene drug metabolites: Part 15. Synthesis and plasma hydrolysis of anticancer triazenes containing amino acid carriers. *Pharm. Res.* **15**, 931−935.

TX000098V