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Reduction of Aromatic and Heterocyclic Aromatic *N*-Hydroxylamines by Human Cytochrome P450 2S1

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Abstract

Many aromatic amines and heterocyclic aromatic amines (HAAs) are known carcinogens for animals and there is also strong evidence for some in human cancer. The activation of these compounds, including some arylamine drugs, involves *N*-hydroxylation, usually by cytochrome P450 enzymes (P450) in Family 1 (1A2, 1A1, and 1B1). We previously demonstrated that the bioactivation product of the anti-cancer agent 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203), an *N*-hydroxylamine, can be reduced by P450 2S1 to its amine precursor under anaerobic conditions and, to a lesser extent, under aerobic conditions (Wang, K., and Guengerich, F. P. (2012) *Chem. Res. Toxicol. 25*, 1740–1751). In the present study, we tested the hypothesis that P450 2S1 is involved in the reductive biotransformation of known carcinogenic aromatic amines and HAAs. The *N*-hydroxylamines of 4-aminobiphenyl (4-ABP), 2-naphthylamine (2-NA), and 2-aminofluorene (2-AF) were synthesized and found to be reduced by P450 2S1 under both anaerobic and aerobic conditions. The formation of amines due to P450 2S1 reduction also occurred under aerobic conditions but was less apparent because the competitive disproportionation reactions (of the *N*-hydroxylamines) also yielded amines. Further, some nitroso and nitro derivatives of the arylamines could also be reduced by P450 2S1. None of the amines tested were oxidized by P450 2S1. These results suggest that P450 2S1 may be involved in the reductive detoxication of several of the activated products of carcinogenic aromatic amines and HAAs.

INTRODUCTION

P450 enzymes catalyze a variety of reactions and are of significant importance in the areas of drug metabolism and toxicology. [1, 2] Based on analyses of marketed drugs, ~75% of drug metabolism reactions involve P450s; five P450s–1A2, 2C9, 2C19, 2D6, and 3A4–account

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ASSOCIATED CONTENT

Supporting Information

HPLC chromatograms of anaerobic incubations of HONH-phenacetin with P450 2S1, HPLC chromatograms of aerobic incubations of 4-HONH-biphenyl with P450 2S1, HPLC chromatograms of anaerobic incubations of 2-HONH-naphthalene with P450 2S1, mass spectra of incubation products of 2-HONH-naphthalene with P450 2S1, HPLC chromatograms of anaerobic incubations of 2-HONH-fluorene with P450 2S1, mass spectra of incubation products of 2-HONH-fluorene with P450 2S1, LC-MS chromatograms of HONH-IQ and IQ, UV spectra of 5F 203, HONH-5F 203, and NO-5F 203, UV spectra of 4-ABP, 4-HONH-biphenyl, 4-NO-biphenyl, 4-NO_2-biphenyl, and azoxybiphenyl, UV spectra of 2-NA, 2-HONH-naphthalene, 2-NO-naphthalene, 2-NO_2-naphthalene, and azoxynaphthalene, UV spectra of 2-AF, 2-HONH-fluorene, 2-NO-fluorene, 2-NO_2-fluorene, and azoxyfluorene, HPLC chromatograms of anaerobic incubations of HONH-5F 203 with P450 1A1 and 2W1, HPLC chromatograms of anaerobic incubations of 4-HONH-biphenyl with P450 1A1, 1A2, 2S1, 2W1, and 3A4, and HPLC chromatograms of anaerobic incubations of 2-HONH-fluorene with P450 1A1, 1A2, 2S1, 2W1, and 3A4. This material is available free of charge via the Internet at http://pubs.acs.org.

for 90% of the P450 reactions. [3, 4] Another recent analysis indicated that 66% of carcinogens are bioactivated by P450s, and three Family 1 P450s show dominant roles, involved in ~50% of the total activations attributed to P450s (1A1, 20%; 1A2, 17%; 1B1, 11%). [5]

Aromatic amines (arylamines, Scheme 1; e.g., 4-aminobiphenyl, 4-ABP; 2-naphthylamine, 2-NA; 2-aminofluorene, 2-AF) are important industrial intermediates for the production of azo dyes and complex chemicals and are also used as antioxidants in rubber manufacturing processes.[6, 7] Rehn first reported the association of exposure to aromatic amines in 1895 with the incidence of bladder cancer among German and Swiss workers in aniline dye factories.[8] Further studies have shown that aromatic amines induce tumors at multiple sites in rodents, dogs, and other experimental laboratory animals as well. [6, 7, 9–11]

Heterocyclic aromatic amines (HAAs, Scheme 1; e.g., IQ, 2-amino-3-methylimidazo[4,5-*f*]-quinoline; MeIQx, 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine) are produced in grilled and charred meats.[12, 13] Several HAAs have also been detected in beer, wine,[14] and cigarette smoke condensate.[15, 16] The Maillard reaction is believed to be involved in the formation of the imidazo-containing HAAs when amino acids and carbohydrates are heated together,[17, 18] and other HAAs may form through the pyrolysis of amino acids and proteins.[19] Extensive studies have shown that HAAs are highly genotoxic in bacterial and mammalian cells and exhibit strong carcinogenic effects in experimental animals.[20–23]

The role of P450s in the bioactivation of aromatic amines and HAAs has been well-documented[24–26] and reviewed.[7, 13, 27] P450-catalyzed *N*-hydroxylation is usually involved as the initial step in the process of chemical carcinogenesis with such compounds (Scheme 2).[26, 28] The *N*-hydroxylamines can be further activated to more reactive esters, by *N*-acetyltransferases (NAT), sulfotransferases (SULT), and other enzymes.[7] Subsequently, the resulting ester breaks down to yield an extremely unstable intermediate, a nitrenium ion, which reacts with DNA predominantly at the C8 position of guanine bases and potentially causes DNA mutations (alternatively, the nitrenium ion can rearrange and the activate molecule will react with a N2 guanine atom).[29] Family 1 P450 enzymes (1A1, 1A2, and 1B1) are responsible for the metabolic activation of many aromatic amines, HAAs, and polycyclic aromatic hydrocarbons.[24, 30–36] The expression of P450 genes in this family is regulated by the aryl hydrocarbon receptor (AhR).[37] P450 1A2 is expressed almost exclusively in liver in humans (10–15% of total P450),[38] but P450 1A1 and 1B1 are generally not present in liver but expressed in extrahepatic tissues at various levels.[39] A number of *in vitro* and *in vivo* studies have shown that P450 1A2 plays a major role in the bioactivation of aromatic amines and HAAs in rodents and humans.[30, 40, 41]

The human genome has 57 P450 genes. P450 2S1 is one of ~13 that are classified as "orphans" with only limited evidence of function.[42] P450 2S1 mRNA has been detected in liver, skin, tumors, and other tissues,[43–46] and the gene is regulated by the AhR.[47, 48] The sites of expression and mode of regulation suggest that P450 2S1 might be involved in the process of chemical carcinogenesis.[45] Recently, attenuation of P450 2S1 by siRNA has been shown to enhance cell proliferation and migration in bronchial epithelial cells.[49] However, a number of carcinogens have been tested and shown not to be activated by P450 2S1.[50] Retinoic acid was reported as a substrate for P450 2S1, [44] but subsequent efforts by others have failed to reproduce these results.[51, 52] The first repeatedly demonstrated reaction for P450 2S1 is the anaerobic reduction of a di-*N*-oxide anti-cancer prodrug, 1,4-bis((2-(dimethylamino-*N*-oxide)ethyl)amino)-5,8-dihydroxyanthracene-9,10-dione (AQ4N), to its amino derivative 1,4-bis((2-(dimethylamino)ethyl)amino)-5,8-dehydroxyanthracene-9,10-dione (AQ4).[51, 52] Previously, we demonstrated that an *N*-hydroxylarylamine, 2-(4-

hydroxylamino-3-methylphenyl)-5-fluorobenzothiazole (HONH-5F 203), can be reduced to its amine precursor 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203, an anticancer agent) by P450 2S1 under anaerobic conditions.[53]

In the present study, several *N*-hydroxylarylamines and nitroso and nitro derivatives of common carcinogenic aromatic amines and HAAs were synthesized and shown to be reduced by P450 2S1. The rates of P450 2S1-catalyzed anaerobic reductions of the *N*-hydroxylarylamines were determined. However, *N*-hydroxyphenacetin (the activated product of the analgesic drug phenacetin[54]) was not reduced by P450 2S1. Our results suggest that P450 2S1 may be involved in the detoxication of the activated products of a number of aromatic amines and HAAs.

EXPERIMENTAL PROCEDURES

Caution

Many of the compounds used in this study are carcinogens and should be handled with care! Gloves, lab coats, and safety goggles should be worn when handling these compounds.

All commercially obtained solvents and other chemicals were used directly without further purification. 4-Nitrophenetole, 1-aminonaphthalene, 2-aminonaphthalene, and 2-nitrofluorene were purchased from Sigma-Aldrich (St. Louis, MO). 2-Nitronaphthalene, 4-nitrobiphenyl, NO_2-IQ, NO_2-MeIQx, and NO_2-PhIP were kindly provided by Dr. Robert. J. Turesky (Wadsworth Center, Albany, NY). NMR data were obtained with a 400 MHz Bruker NMR spectrometer, using the solvents noted, in the Vanderbilt facility. UV spectra were obtained (on-line) with a Waters Acquity UPLC system equipped with a photodiode array detector (Waters, Milford, MA).

Chemical Synthesis

***N*-Hydroxyphenacetin—**The title compound was prepared using modification of a previous reported procedure.[54] 4-Nitrophenetole (0.10 g, 0.60 mmol) was reduced to *N*-hydroxyphenetidine in 2 mL of C_2H_5OH/H_2O (3:1, v/v) by Zn dust (0.16 g, 2.4 mmol) in the presence of 0.032 g of NH_4Cl. The reaction mixture was diluted with 4 mL of cold H_2O and extracted with $(C_2H_5)_2O$ (3 × 5 mL). The combined $(C_2H_5)_2O$ extracts were evaporated to dryness *in vacuo* at 23 °C. The residue was dissolved in 2 mL of $(C_2H_5)_2O$ in the presence of a slurry of $NaHCO_3$ (0.08 g in 0.25 mL water) at 0 °C, and 2.5% acetyl chloride (47 mg, 0.60 mmol) in $(C_2H_5)_2O$ (w/v) was added slowly to the mixture. The resulting mixture was stirred for 2 h and extracted with $(C_2H_5)_2O$ (3 × 5 mL). The combined $(C_2H_5)_2O$ layers were washed sequentially with H_2O and brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo, and flash column chromatography on silica gel using $(C_2H_5)_2O$ yielded *N*-hydroxyphenacetin as colorless crystals (0.053 g, yield 45% from 4-nitrophenetole, mp 101–103 °C, lit. mp 103 °C[55]). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37 (d, J = 8.8 Hz, aromatic, 2H), 6.85 (d, J = 8.9 Hz, aromatic, 2H), 4.01 (q, J = 7.0 Hz, 2H, CH_3CH_2-), 2.16 (s, 3H, CH_3CO-), 1.40 (t, J = 7.0 Hz, 3H, CH_3CH_2-); GC-MS (electron impact, m/z 179, 137, 108).

N-Hydroxylamines and Nitroso Compounds—HONH-5F 203, [53] 4-HONH-biphenyl, 2-HONH-naphthalene, 2-HONH-fluorene, and HONH-PhIP were prepared by the reduction of the corresponding nitro derivatives with hydrazine and Pd/C at 0 °C for 30 min to 2 h.[56, 57] HONH-IQ and HONH-MeIQx were prepared by the reduction of nitro analogs with ascorbic acid in $(CH_3)_2SO$ as described by Turesky *et al*.[57] NO-5F 203 and 4-NO-biphenyl were prepared by the oxidation of HONH-5F 203 and 4-HONH-biphenyl with $K_3Fe(CN)_6$ under ambient conditions for 3 h as reported previously.[53]

Enzyme Preparations—Recombinant human P450 2S1 was expressed and purified in *Escherichia coli* membranes as previously described.[50] NADPH-P450 reductase (NPR) was expressed in *E. coli* and purified as described.[58]

Incubation Conditions—A typical incubation[59] mixture contained purified P450 2S1 (0.5 μM), NPR (1 μM), 160 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine, an NADPH-generating system (10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 4 μg mL^{-1} yeast glucose 6-phosphate dehydrogenase), and the appropriate substrate (100 μM) in 0.10 M sodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) buffer(pH 7.4, containing 1 mM EDTA). The incubations were performed at 37 °C for 30 min. Cold CH_2Cl_2 (2-fold volume) was added to terminate each reaction, and the resulting mixtures were separated by centrifugation at 2×10^3 *g* for 3 min. The lower organic layer was transferred and dried under an N_2 stream. The residue was dissolved in CH_3CN for HPLC and LC-MS analysis.

Anaerobic Incubations—Qualitative anaerobic experiments were performed in Thunberg tubes, as previously described.[53] A typical 500-μL incubation mixture contained 0.5 μM P450, 1.0 μM NPR, 160 μM L-α-1,2-dilauoryl-*sn*-glycero-3-phosphocholine, 100 mM sodium HEPES buffer (pH 7.4), 1 mM EDTA, and 100 μM substrate **(**from a freshly prepared 10 mM $(CH_3)_2SO$ stock). When the desired level of anaerobicity was achieved (after 5 cycles of vacuum and Ar), an NADPH-generating system was added to the incubation mixture from the neck of the tube. The reactions were incubated at 37 °C for 10 to 30 min, terminated by the addition of 1.0 mL of cold CH_3CN, and analyzed by HPLC.

For comparisons, anaerobic incubations were carried out with P450 1A1, 1A2, 2S1, 2W1, and 3A4 at the same concentration (0.2 μM) for 10 min.

Steady-state Kinetic Measurements of Anaerobic Reduction of HONH-5F 203 —Anaerobic incubations were carried out under an N_2 atmosphere in a glove box (Labconco, Kansas City, MO). The concentrations of P450 and the NADPH-generating system and buffer conditions remained the same as those employed in the qualitative studies. A stock solution was made of freshly prepared HONH-5F 203 in $(CH_3)_2SO$ and the concentration was determined colorimetrically.[60] A series of standard solutions of different concentrations was prepared from the stock solution by dilution. Reactions with different substrate concentrations (ranging from 0 to 200 μM) were conducted, in duplicate, at 37 °C for 10 min. After the reactions were quenched with CH_2Cl_2 (2-fold volume), 2-phenylbenzothiazole (5 μL, 0.1 mM) was added to each reaction mixture as an internal standard. The mixtures were centrifuged at 2×10^3 *g* for 3 min, and the organic layers were transferred and dried under a stream of N_2. The residues were dissolved in CH_3CN for HPLC analysis.

Determination of Rates of Anaerobic Reduction of 4-NHOH-biphenyl, 2-NHOH-naphthalene, and 2-NHOH-fluorene to the Corresponding Amines by P450 2S1 —Anaerobic incubations were conducted under an N_2 atmosphere in a glove box, as in the steady-state kinetic studies for HONH-5F 203 reduction. The rates of reduction were measured at a single substrate concentration (10 μM). Standard solutions (1 mM, determined by colorimetric assay) of 4-NHOH-biphenyl, 2-NHOH-naphthalene, and 2-NHOH-fluorene in CH_3CN were prepared from freshly prepared samples. Under the conditions used in this study, optimal P450 2S1 concentrations for 4-NHOH-biphenyl, 2-NHOH-naphthalene, and 2-NHOH-fluorene were 500, 25, and 50 nM, respectively (the corresponding concentrations of NPR were 1, 0.5, and 0.5 μM, respectively). The concentrations of the NADPH-generating system and buffer conditions were the same as in a typical incubation, *vide supra*.

A standard solution (5 μL) was added to each enzyme reaction (volume 500 μL) and was incubated at 37 °C. Reactions were terminated by addition of CH_2Cl_2 (1.0 mL) after 0, 1, 2, 3, 4, and 5 min. For each compound, incubations were performed in duplicate at every time point. Incubations devoid of P450 2S1 were performed as controls. 1-Naphthylamine (5 μL, 500 μM) was added to each sample as an internal standard, and the mixtures were centrifuged. The isolated organic layers were transferred and dried under a stream of N_2, and the residues were dissolved in CH_3CN for HPLC analysis.

HPLC and LC-MS Analysis of the Reduction Reactions—The reduced reaction products of hydroxylamine, nitroso, and nitro compounds were analyzed using a Waters Acquity UPLC system equipped with a photodiode array detector (Waters, Milford, MA) and an octadecylsilane (C_{18}) reversed-phase column (5 μm, 2.1 mm × 250 mm, Hypersil GOLD, Thermo Scientific, Odessa, TX). Absorbance was integrated (210–400 nm). The column temperature was maintained at 25 °C. LC conditions for phenacetin, 4-ABP, 2-NA, 2-AF, and 5F 203 derivatives were as described previously.[53] The solvents used were Solvent A (CH_3CN:H_2O, 5:95, 0.5% HCO_2H, v/v/v) and Solvent B (CH_3CN:H_2O, 95:5, 0.5% HCO_2H, v/v). The gradient started from 5% B (v/v) during the first 2 min (0–2 min), increased linearly to 100% B over 5 min (2–7 min), held for 4.5 min (7–11.5 min), and then returned to 5% B (v/v) over 0.5 min (11.5–12 min) before re-equilibration (12– 15 min), at a flow rate of 0.5 mL min^{-1}. LC conditions for the IQ, MeIQx, and PhIP derivatives were as follows: Solvent A contained 20 mM $NH_4CH_3CO_2$ and 5% CH_3CN (v/v), and Solvent B contained $NH_4CH_3CO_2$ (20 mM, pH 6.8) and 95% CH_3CN (v/v). A linear gradient was employed with a flow rate of 0.5 mL min^{-1}, starting from 5% Solvent B and reaching 100% Solvent B at 12 min, and the percentage of Solvent B returned to 5% (v/v) over 3 min (12–15 min).

LC-MS and LC-MS/MS analyses were performed on a Waters Acquity UPLC system coupled to a Thermo-Finnigan LTQ ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The same LC conditions were used for each sample as in the HPLC analysis. Samples were generally analyzed in the atmospheric pressure chemical ionization (APCI) positive ion mode. MS conditions were optimized for each amine derivative as follows: capillary temperature, 275 °C; vaporizer temperature, 450 °C; source voltage, 6.0 kV; sheath gas flow, 50.0; auxiliary gas flow, 5.0; sweep gas flow, 5.0; and in-source fragmentation, 10 V.

Colorimetric Assay for N-Hydroxyarylamines.[60]—From a typical solution containing 5–10 mM *N*-hydroxyarylamine, a 1–2 μL aliquot was added to a tube containing 0.96 mL of the color reagent.[60] The sample was mixed vigorously using a vortex device. After 3 min, 40 μL of stabilizer (H3PO4, 20 mM) was added to the mixture. The sample was mixed thoroughly and A_{535} was measured, with reference to a sample containing all of the reagents except *N*-hydroxyarylamine as control (ε_{535} 39,200 M^{-1} cm^{-1}).

P450 2S1 Binding Assay (with HONH-5F 203)—Binding spectra of P450 with HONH-5F 203 were conducted with purified P450 2S1 (23 °C) using a UV-visible scanning spectrophotometer (Aminco DW-2a, OLIS, Bogart, GA). A baseline was recorded (350–700 nm) using two 1.0-ml glass cuvettes (1-cm path length) containing equal volumes of 100 mM potassium phosphate buffer (pH 7.4, containing 20% glycerol, v/v). Purified P450 2S1 was added to the sample cuvette (final concentration 5 μM). The same volume of the buffer was added to the reference cuvette. Equal amounts of freshly prepared aliquots of HONH-5F 203 (dissolved in $(CH3)_2SO$) were added to both cuvettes. Difference spectra were collected (350 – 700 nm) after an equilibration period of 1 min (total volume of $(CH3)_2SO \leq 2\%$).

RESULTS

Search for Oxidations of Potential Substrates with P450 2S1

No detectable products were observed following the incubation of aniline, 7-ethoxycoumarin, IQ, MeIQx, or PhIP with human P450 2S1, in typical aerobic incubation systems containing NPR and NADPH, as judged by HPLC-UV assays. No products were detected from an incubation mixture of [^{14}C]-lauric acid with P450 2S1 using HPLC (coupled on-line with scintillation counting).

Anaerobic Incubation of N-Hydroxyphenacetin with P450 2S1

N-Hydroxyphenacetin was incubated with P450 2S1 at 37 °C for 20 min under anaerobic or aerobic conditions, with an HPLC peak at t_R 4.7 min corresponding to *N*-hydroxyphenacetin. *N*-Hydroxyphenacetin was stable under all conditions tested in this study (Supporting Information, Figure S1). Neither P450 2S1, NPR, NADPH, nor the combination changed the *N*-hydroxyphenacetin to any products.

Anaerobic Reduction of 4-HONH-Biphenyl, 2-HONH-Naphthalene, and 2-HONH-Fluorene with P450 2S1

N-Arylhydroxylamines are unstable substances and readily undergo disproportionation reactions (Scheme 2) to yield arylamines and nitroso derivatives in neutral or basic media[61, 62] The nitroso products can further condense with *N*-arylhydroxylamines to produce azoxy compounds.[63] In anaerobic incubations of all three *N*-arylhydroxylamines with P450 2S1 (in the presence of NPR and an NADPH-generating system), significantly larger amounts of the corresponding arylamines were formed (than under other conditions when P450 2S1 or an NADPH-generating system was absent, Figures 1, 3, 5), indicating that P450 2S1 reduced the *N*-arylhydroxylamines to arylamines (Scheme 2). Under aerobic incubation conditions (Supporting Information, Figure S2), competitive disproportionations were favored that also yielded arylamines as products. The reduction of *N*-arylhydroxylamines by P450 2S1 was less apparent compared to anaerobic conditions.

HPLC analyses of the incubation of 4-HONH-biphenyl with P450 2S1 showed four major peaks (Figure 1): 4-ABP (λ_{max} 250 nm), 4-HONH-biphenyl (λ_{max} 273 nm), 4-NO-biphenyl (λ_{max} 345 nm), and azoxybiphenyl (λ_{max} 357 nm) were eluted at t_R 4.5, 5.5, 6.8, and 8.2 min, respectively. 4-Nitrobiphenyl (λ_{max} 310 nm) appeared at t_R 6.6 min under the same HPLC conditions (data not shown). 4-ABP (t_R 4.5 min, *m/z* 170, $[MH]^+$, Figure 2A), 4-HONH-biphenyl (t_R 5.5 min, *m/z* 186, $[MH]^+$, Figure 2B), 4-NO-biphenyl (t_R 6.8 min, *m/z* 184 $[MH]^+$, Figure 2C), and azoxybiphenyl (t_R 8.1 min, *m/z* 351 $[MH]^+$, Figure 2D) were detected in LC-MS ($APCI^+$) analyses of the incubation samples. However, 4-NO-biphenyl and azoxybiphenyl were not detected when LC-MS (electrospray ionization, ESI^+) was employed.

The decomposition of 2-HONH-naphthalene was more rapid than that of 4-HONH-biphenyl. HPLC analyses of the incubation mixtures of 2-HONH-naphthalene with P450 2S1 showed four major peaks (Supporting Information, Figure S3), and minor peaks also appeared under some conditions. 2-NA (λ_{max} 275 nm), 2-HONH-naphthalene (λ_{max} 277 nm), 2-NO-naphthalene (λ_{max} 323 nm), and azoxy naphthalene (λ_{max} 346 nm) were eluted at retention times of 3.7, 5.0, 6.4, and 7.9 min. 2-Nitronaphthalene (λ_{max} 307 nm) eluted at t_R 6.3 min under the same HPLC conditions (data not shown). 2-NA (t_R 3.7 min, *m/z* 144 $[MH]^+$, Supporting Information, Figure S4A), 2-HONH-naphthalene (t_R 5.0 min, *m/z* 160 $[MH]^+$, Supporting Information, Figure S4B), 2-NO-naphthalene (t_R 6.4 min, *m/z* 158 $[MH]^+$, Supporting Information, Figure S4C), and azoxy naphthalene (t_R 7.9 min, *m/z* 299 $[MH]^+$,

Supporting Information, Figure S4D) were detected in LC-MS ($APCI^+$) analyses of the incubation samples.

Similarly, HPLC analyses of the anaerobic incubation mixtures of P450 and 2-HONH-fluorene (which disappeared completely after incubations) also showed four major peaks (Supporting Information, Figure S5), suggesting extreme instability under these conditions. 2-AF (λ_{max} 300 nm), 2-HONH-fluorene (λ_{max} 282 nm), 2-NO-fluorene (λ_{max} 372 nm), and azoxyfluorene (λ_{max} 379 nm) were eluted at t_R 4.4, 5.5, 6.8, and 8.3 min, respectively. 2-Nitrofluorene (λ_{max} 335 nm) appeared at t_R 6.75 min under the same HPLC conditions. 2-AF (t_R 4.4 min, *m/z* 182 $[MH]^+$, Supporting Information, Figure S6A), 2-NHOH-fluorene (t_R 5.5 min, *m/z* 198 $[MH]^+$, Supporting Information, Figure S6B), 2-NO-fluorene (t_R 6.8 min, *m/z* 196 $[MH]^+$, Supporting Information, Figure S6C), and azoxyfluorene (t_R 8.3 min, *m/z* 375 $[MH]^+$, Supporting Information, Figure S6D) were observed in LC-MS ($APCI^+$) analyses of the incubation samples.

Anaerobic Reduction of HONH-5F 203, 4-HONH-Biphenyl, and 2-HONH-Fluorene with Other Human P450s

HONH-5F 203 can be reduced by P450 2S1 to 5F 203 under anaerobic conditions and (to a lesser extent) under aerobic conditions, as shown previously.[53] Interestingly, when it was incubated with P450 1A1 or 2W1 at 37 °C for 30 min under anaerobic conditions (Supporting Information, Figure S12), HONH-5F 203 (t_R 6.1 min) was also be reduced to 5F 203 (t_R 6.4 min). To evaluate the relative catalytic efficiency of P450 2S1 for the reduction of aromatic *N*-hydroxylamines, anaerobic incubations of 4-HONH-biphenyl (Supporting Information, Figure S13), and 2-HONH-fluorene (Supporting Information, Figure S14) with P450 1A1, 1A2, 2S1, 2W1, and 3A4 were conducted in parallel. 4-HONH-biphenyl (t_R 5.5 min) disappeared almost completely, and significantly higher amounts of azoxybiphenyl (t_R 8.1 min) were formed in the presence of P450s (except for P450 2S1, Supporting Information, Figures S13A, 13B, 13D, 13E), suggesting higher degrees of disproportionation. In addition to a lower level of disproportionation, more of the reduction product 4-ABP (t_R 4.5 min, Supporting Information, Figure S13C) was generated with P450 2S1. Similar results were observed in the anaerobic reduction of 2-HONH-fluorene with various P450s (Supporting Information, Figure S14), where the largest amount of 2-AF (t_R 4.3 min, Supporting Information, Figure S14C) was produced with P450 2S1.

Reduction of 2-(3-Methyl-4-nitrosophenyl)-5-fluorobenzothiazole (NO-5F 203) and 4-NO-biphenyl by NADPH and P450 2S1

Some aryl nitroso compounds are known to be non-enzymatically reduced by NAD(P)H to *N*-hydroxyarylamines, [64] which could be further reduced by P450 2S1 to arylamines (Scheme 3). Freshly prepared NO-5F 203[53] (containing trace amounts of 5F 203 and HONH-5F 203) was incubated with P450 2S1 at 37 °C for 30 min under anaerobic conditions (Figure 3) and aerobic conditions (data not shown), respectively. The t_R values of 5F 203 (λ_{max} 347 nm), HONH-5F 203 (λ_{max} 338 nm), and NO-5F 203 (λ_{max} 363 nm) were 6.3, 6.0, and 7.4 min, respectively, consistent with our previously reported data.[53] In the absence of P450 2S1, HONH-5F 203 was formed via reduction of NO-5F 203, as the most abundant peak (Figure 3B, in the presence of NPR and an NADPH-generating system), whereas in the absence of an NADPH-generating system HONH-5F 203 was less abundant than 5F 203 (Figure 3C), suggesting that NADPH was capable of non-enzymatically reducing NO-5F 203 to HONH-5F 203. When P450 2S1 was added, NO-5F 203 disappeared and the predominant product was 5F 203 (the arylamine), indicating that P450 2S1 can catalyze the reduction of HONH-5F 203 to 5F 203 (Figure 3D). These results were in accord with our previous observations.[53] Freshly prepared 4-NO-biphenyl (containing trace 4-ABP and azoxybiphenyl) was incubated with P450 2S1 at 37 °C for 30 min under anaerobic

(Figure 4) and aerobic conditions (data not shown), respectively. 4-ABP (λ_{max} 250 nm), 4-NHOH-biphenyl (λ_{max} 273 nm), 4-NO-biphenyl (λ_{max} 345 nm), and azoxybiphenyl (λ_{max} 357 nm) were eluted at t_R 4.5, 5.5, 6.8, and 8.2 min, respectively. The reduction of 4-NO-biphenyl occurred at an appreciable level only when NPR and an NADPH-generating system were present (Figure 4B, 4D), suggesting that the reduction of 4-NO-biphenyl is enzymatic. 4-NO-biphenyl disappeared completely with P450 2S1 in the presence of NPR and an NADPH-generating system (Figure 4D). The ability of NPR to reduce 4-NO-biphenyl was not very obvious due to the high instability of 4-NHOH-biphenyl (rapid disproportionation) and the competitive condensation reaction between 4-NHOH-biphenyl and 4-NO-biphenyl to form azoxybiphenyl. Nevertheless, it was clear that a greater amount of 4-ABP was formed in the presence of P450 2S1 (Figure 4C, 4D), indicating that P450 2S1 can catalyze the reduction of NHOH-biphenyl to 4-ABP.

Reduction of NO_2-IQ and 2-Nitronaphthalene by NADPH and P450 2S1

Some heterocyclic and aryl nitro compounds could be reduced by P450 2S1, as exemplified by NO_2-IQ and 2-nitronaphthalene (Scheme 3). NO_2-IQ was incubated without (Figure 5A) or with P450 2S1 (Figure 5B) in the presence of NPR and an NADPH-generating system at 37 °C for 20 min under anaerobic conditions. In the absence of P450 2S1, NO_2-IQ was partially reduced to yield HONH-IQ ($[M+H]^+$, *m/z* 215, Supporting Information, Figure S7A) as the only product; however in the presence of P450 2S1, NO_2-IQ disappeared completely and IQ ($[M+H]^+$, *m/z* 199, Supporting Information, Figure S7B) was produced, in addition to HONH-IQ, indicating that P450 2S1 can catalyze the reduction of HONH-IQ to IQ (the amine). Similar to the results observed for NO_2-IQ, in the absence of P450 2S1, 2-nitronaphthalene was also partially reduced to yield 2-NHOH-naphthalene as the major product (Figure 5C). However in the presence of P450 2S1, 2-NA was formed almost exclusively and no 2-NHOH-naphthalene was present at a discernable level (Figure 5D), suggesting that P450 2S1 can catalyze the reduction of 2-NHOH-naphthalene as well.

HONH-5F 203 Binding to P450 2S1

Titration of HONH-5F 203 with P450 2S1 resulted in a concentration-dependent decrease in absorbance at 417 nm and an increase at 458 nm (Figure 6), indicative of interactions between HONH-5F 203 and the heme iron.

Steady-state Kinetic Data for Anaerobic Reduction of HONH-5F 203 and Rates of Reduction of 4-NHOH-Biphenyl, 2-NHOH-Naphthalene, and 2-NHOH-Fluorene by P450 2S1

Steady-state kinetic analysis of reduction of HONH-5F 203 with P450 2S1 yielded a rather linear rate/substrate relationship, showing little saturation with HONH-5F 203 over a 0–200 μM concentration range (Figure 7). k_{cat} and K_m values could not be determined by this method, but the slope provides an estimate of the catalytic efficiency (k_{cat}/K_m) of P450 2S1 ($5.5 \pm 0.4 \times 10^4$ M^{-1} min^{-1}, or ~ 10^3 M^{-1} s^{-1}). The rates of reduction of 4-NHOH-biphenyl (0.18 ± 0.01 min^{-1}), 2-NHOH-naphthalene (2.4 ± 0.8 min^{-1}), and 2-NHOH-fluorene (0.34 ± 0.12 min^{-1}) by P450 2S1 were measured at a single substrate concentration of 10 μM, considering only the amine formation contributed by P450 2S1 catalysis (subtracting the amounts of amines in control incubations in which P450 2S1 was absent).

DISCUSSION

In this study, P450 2S1 did not exhibit oxidation activity with several common substrates for P450s or a number of aromatic and heterocyclic amines. Although some catalytic activity was reported in the absence of NPR when oxygen surrogates were added, [65, 66] substrates for P450 2S1-catalyzed oxidation under standard conditions have not been identified so far.

The reduction of *N*-hydroxylated compounds in rat and hog liver extracts was noted several decades ago.[67–70] For the enzymatic reduction a microsomal (three-component enzyme system containing cytochrome b_5, NADH-cytochrome b_5 reductase, and an unidentified third component)[67, 68] and a mitochondrial[69, 70] system have been proposed. In a study of the reduction of amidoxime prodrugs, stearoyl-CoA desaturase was proposed to be involved in the microsomal reductions.[71] The third component for the reduction of *N*-hydroxylamines in hog liver microsomes was isolated and characterized as a P450 Subfamily 2D enzyme.[72] However, a human homolog has not been found so far. A novel molybdenum-containing enzyme from the mitochondrial fraction, mitochondrial amidoxime reducing component (mARC), was also suggested.[73] Subsequent incubations with cytochrome b_5, NADH cytochrome b_5 reductase, and mARC exhibited amidoxime reductase activity *in vitro*.[74, 75] The reduction of *N*-hydroxylamines in the absence of the third component was also reported, (i.e., only cytochrome b_5 and NADH cytochrome b_5 reductase).[76, 77] One caveat of these latter studies is that the 4-HONH-biphenyl used was not freshly prepared, and even in the presence of ascorbic acid the disproportionation of 4-HONH-biphenyl (which also yields 4-ABP) was still significant under the experimental conditions.

Although most P450 reactions are oxidations, [5] P450s can also catalyze a wide range of reductions, [1] as summarized by Wisloki et al.[78] and more recently by Testa.[79] Generally in the P450 catalytic cycle, the ferrous P450 species binds O_2 tightly and oxidation occurs rapidly (after the addition of the second electron).[1] However, in tissues where only a low concentration of O_2 is present, e.g. in hypoxic cancer cells, [80] reduction becomes more favorable. Ferrous P450, $E_{m,7} \sim -300$ mV, [81, 82] should be a reasonably good reducing agent if the substrate is in an appropriately bound form and has the potential to accept electrons. Alternatively, the binding of some substrates may block (or partially block) the incorporation of O_2, which should allow the reduction to occur even under aerobic conditions.[1] It was shown earlier that AQ4N (a di-*N*-oxide) can be reduced by both P450 2W1 and 2S1 under anaerobic conditions.[51] Subsequently, we found that 5F 203 can undergo P450 1A1- and 2W1-mediated bioactivation in the presence of O_2, and HONH-5F 203 (one of the reactive intermediates) can be reduced by P450 2S1 under anaerobic conditions and, to a lesser extent, under aerobic conditions.[53] In the present report, we show that P450 1A1 and 2W1 can also catalyze the reduction of HONH-5F 203 anaerobically, suggesting that the biotransformation pathways might be influenced by the O_2 tension.

The analgesic phenacetin is *N*-hydroxylated in rodents and humans.[54, 55] Another metabolic pathway for phenacetin is *O*-deethylation, catalyzed by P450 1A2 to yield acetaminophen.[83] Although acetaminophen is hepatotoxic at high doses, phenacetin induces a high incidence of tumors in the case of chronic use of large doses to animals[84] and was withdrawn from the market due to the concern of possible carcinogenicity.[85] The biotransformation pathways that lead to the formation of DNA adducts for the *N*-hydroxyphenacetin are similar to that for *N*-acetyl-2-AF (AAF) and many aromatic amines. [26, 55] We did not observe any reduction of *N*-hydroxyphenacetin by P450 2S1, under any conditions used, suggesting that P450 2S1 probably is not involved in the biotransformation of *N*-hydroxyphenacetin. However, it should be pointed out that *N*-hydroxyphenacetin is a hydroxamic acid, not a hydroxylamine, and we did not pursue the issue of whether P450 2S1 (or other P450s) reduces other hydroxamic acids.

The results in the present study demonstrate that human P450 2S1 is capable of catalyzing the reduction of several *N*-hydroxy aromatic amines and HAAs, especially under anaerobic conditions. P450 2S1 appeared to be the most efficient enzyme among several P450s examined, at least for the anaerobic reductions of 4-HONH-biphenyl and 2-HONH-fluorene. It is worth noting that 4-HONH-biphenyl, 2-HONH-naphthalene, and 2-HONH-fluorene are very unstable molecules, even in the absence of O_2, and readily undergo disproportionation

reactions to yield amine, nitroso, and azoxy derivatives. Thus, the formation of aromatic amines from *N*-hydroxylamines does not necessarily mean that reduction is occurring, i.e., the difference between the amounts of amine formed with and without P450 2S1 reflects the enzymatic activity and therefore the rates of anaerobic reductions of 4-HONH-biphenyl, 2-HONH-naphthalene, and 2-HONH-fluorene were measured in this way. The presence of NADPH slowed the disproportionation to various extents (Figures 1B, 3B, 5B) when compared to the incubation controls (Figures 1A, 3A, 5A), presumably due to non-enzymatic reduction of nitroso species. On the contrary, P450 2S1 accelerated the process of disproportionation (Figures 1C, 3C, 5C), and some non-reductive pathways for the reaction of oxidized amines have been presented earlier.[86]

The nitroso derivatives of 5F 203 and 4-ABP were reduced by NADPH with NPR to *N*-hydroxylamines (Figures 7B, 8B) under anaerobic conditions. In the presence of P450 2S1, as well as NPR, NO-5F 203 was reduced to 5F 203 (amine) almost completely (Figure 3D). P450 2S1-catalyzed anaerobic reduction of 4-HONH-biphenyl occurred but was not as efficient as with HONH-5F 203 (Figure 4D). A greater amount of azoxybiphenyl (Figure 4D) compared to the incubation control (Figure 4A) is due to the fact that no disproportionation can occur because no 4-HONH-biphenyl is formed in the control reaction. Nitroso derivatives of aromatic amines and HAAs can react with hemoglobin¯β Cys-93 in the blood stream to form arylamine-hemoglobin sulfinamide adducts in experimental animals.[87, 88] 4-ABP has been reported to have a high fraction of dose (> 5%) bound to hemoglobin as sulfinamide adducts.[89] Reduction of nitroso compounds may decrease the formation of hemoglobin adducts. The nitroso derivative of IQ has been shown to be reduced by NPR to form the *N*-hydroxylamine and a trace amount of IQ.[64] Similarly, the nitro derivatives of IQ and 2-NA are also reduced by NPR to *N*-hydroxylamines as major products (Figure 5A, 5C) under anaerobic conditions. If P450 2S1 is also present, *N*-hydroxylamines are reduced to yield IQ and 2-NA (amines, Figure 5B, 5D).

In conclusion, our results indicate that human P450 2S1 is capable of catalyzing the reduction of *N*-hydroxylamine, nitroso, and nitro derivatives of at least several carcinogenic aromatic amines and HAAs to yield amines. This may represent a detoxication pathway for aromatic amines and HAAs, although at this time we cannot judge the extent of the *in vivo* contribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2-AAF	2-acetylaminofluorene
4-ABP	4-aminobiphenyl
2-AF	2-aminofluorene
APCI	atmospheric-pressure chemical ionization

AQ4	1,4-bis((2-(dimethylamino)ethyl)-amino)-5,8-dehydroxyanthracene-9,10-dione
AQ4N	1,4-bis((2-(dimethylamino-*N*-oxide)ethyl)amino)-5,8-dihydroxy-anthracene-9,10-dione
ESI	electrospray ionization
5F 203	2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole
HAAs	heterocyclic aromatic amines
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid
HONH-	hydroxylamino
HONH-5F 203	2-(4-hydroxylamino-3-methylphenyl)-5-fluorobenzothiazole
IQ	2-amino-3-methylimidazo[4,5-*f*]-quinoline
LC-MS	liquid chromatography-mass spectrometry
mARC	mitochondrial amidoxime reducing component
MeIQx	2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline
2-NA	2-naphthylamine
NAT	*N*-acetyltransferase
NO-	nitroso
NO-5F 203	2-(3-methyl-4-nitrosophenyl)-5-fluorobenzothiazole
NO_2-	nitro
NPR	NADPH-P450 reductase
PhIP	2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine
SULT	sulfotransferase

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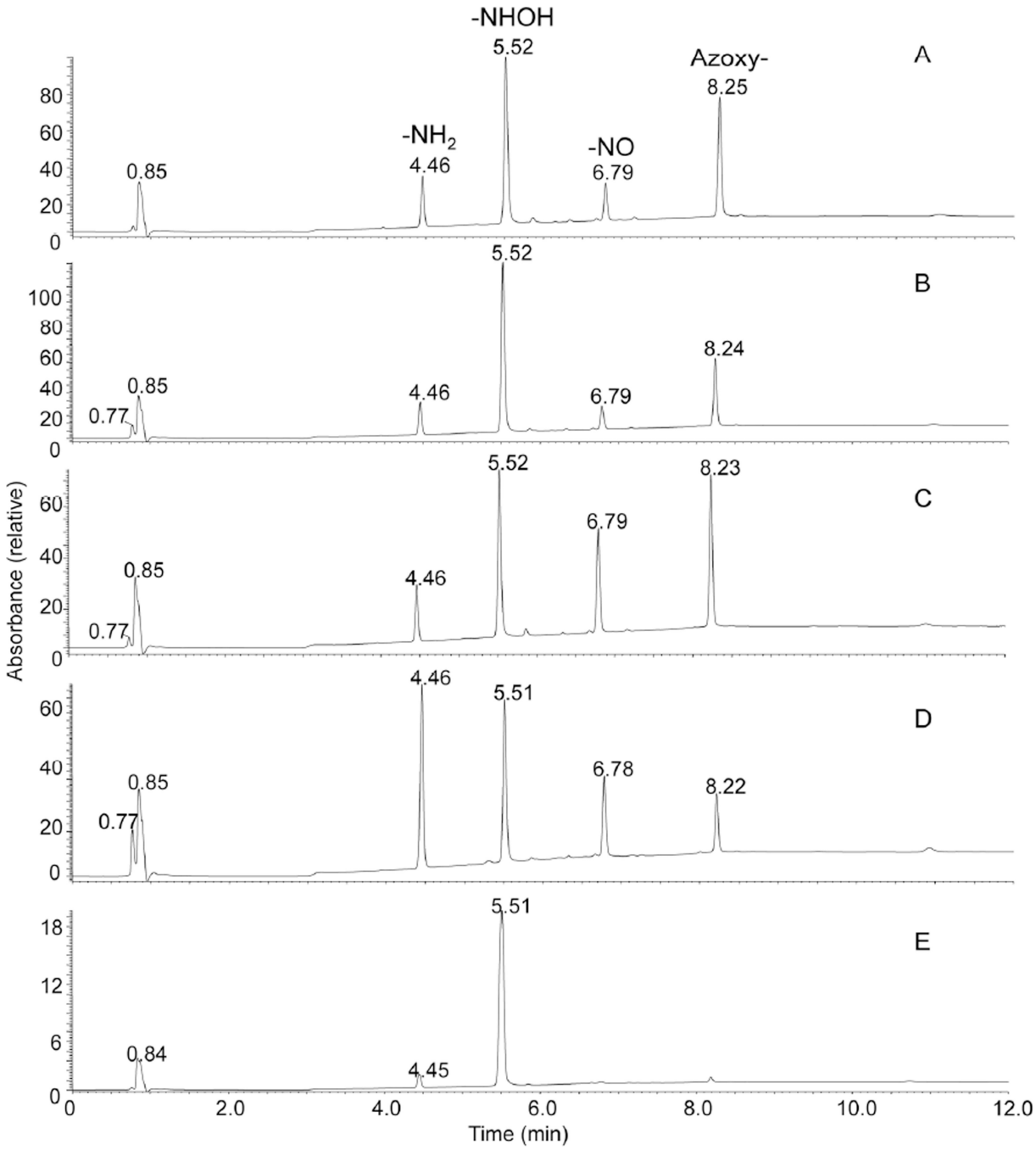


Figure 1.

Anaerobic incubations of 4-HONH-biphenyl with P450 2S1. Freshly prepared 4-HONH-biphenyl (5 μL of a 10 mM solution in $(CH_3)_2SO$) was incubated with P450 2S1 (total volume 0.5 mL) at 37 °C for 15 min under anaerobic conditions. (A) 4-HONH-biphenyl in 100 mM sodium HEPES buffer (pH 7.4) containing 1 mM EDTA, incubated at 37 °C for 15 min. (B) 4-HONH-biphenyl incubated with all P450 system components with the exception of P450 2S1. (C) 4-HONH-biphenyl incubated with all components of the P450 2S1 system, with the exception of the NADPH-generating system. (D) 4-HONH-biphenyl incubated with P450 2S1 in the presence of NPR and an NADPH-generating system. (E) Synthetic standard of 4-HONH-biphenyl. UV absorbance was integrated over the range 200–400 nm.

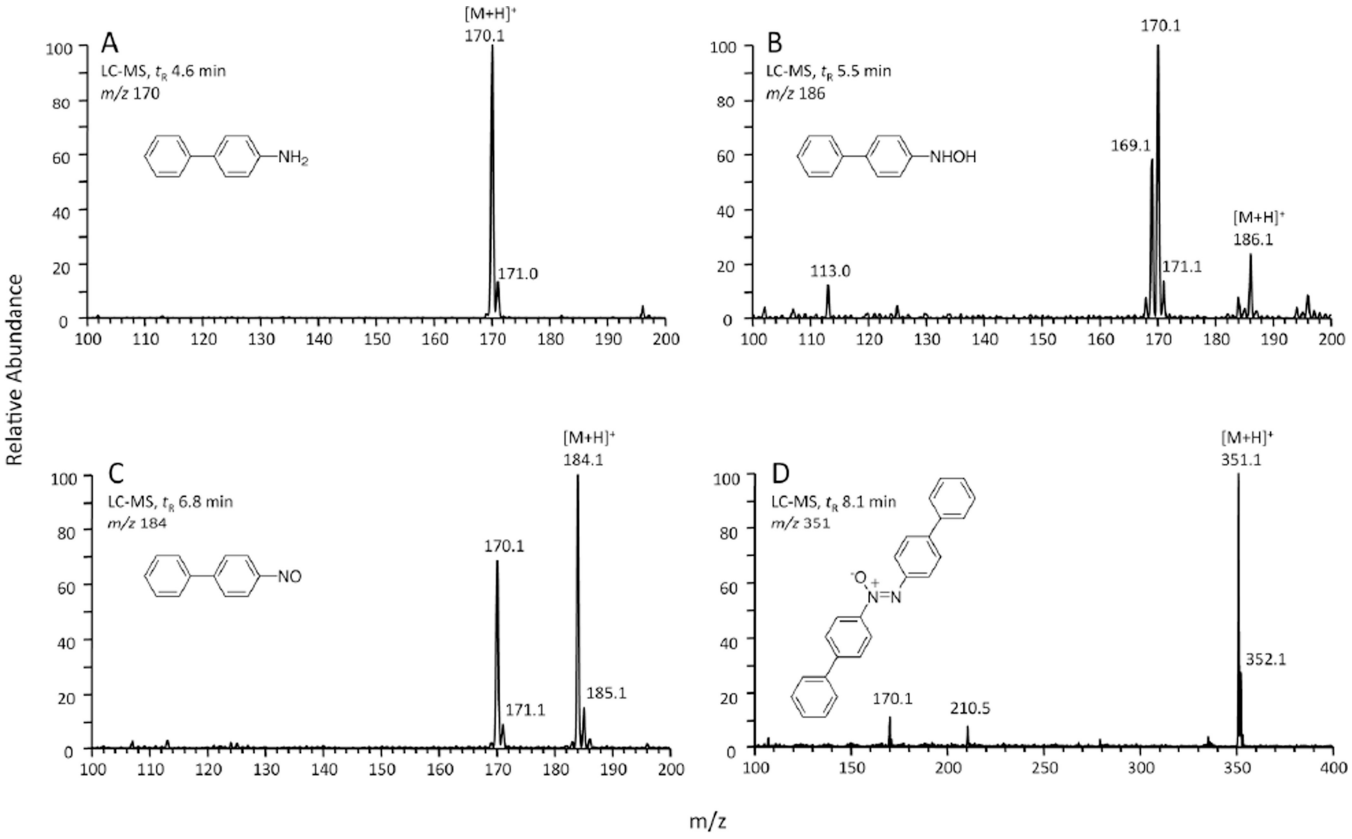


Figure 2.

LC-MS spectra of incubation products of 4-HONH-biphenyl with P450 2S1. (A) 4-ABP (t_R 4.6 min). (B) 4-HONH-biphenyl(t_R 5.5 min). (C) 4-NO-biphenyl (t_R 6.8 min). (D) Azoxybiphenyl (8.1 min).

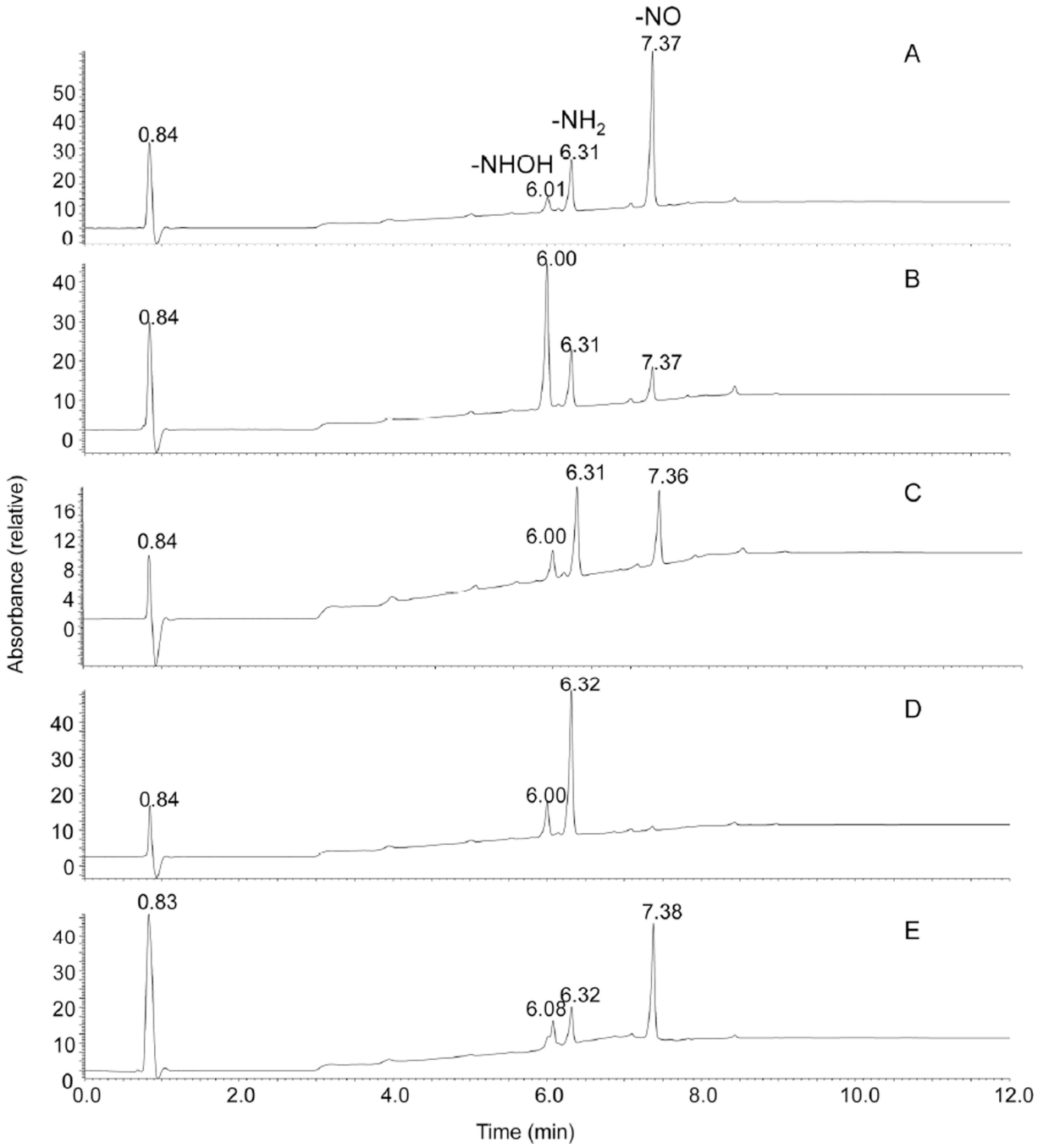


Figure 3.

Anaerobic incubations of NO-5F 203 with P450 2S1. Freshly prepared NO-5F 203 (containing trace amounts of 5F 203 and HONH-5F 203, 5 μL of an approximately 2 mM solution in $(CH_3)_2SO$) was incubated with P450 2S1 (total volume 0.5 mL) at 37 °C for 30 min under anaerobic conditions. (A) NO-5F 203 in 100 mM sodium HEPES buffer (pH 7.4) containing 1 mM EDTA, incubated at 37 °C for 30 min. (B) NO-5F 203 incubated with all P450 system components with the exception of P450 2S1. (C) NO-5F 203 incubated with all components of the P450 2S1 system with the exception of the NADPH-generating system. (D) NO-5F 203 incubated with P450 2S1 in the presence of NPR and an NADPH-generating

system. (E) Synthetic standard of NO-5F 203. UV absorbance was integrated over the range 200–400 nm.

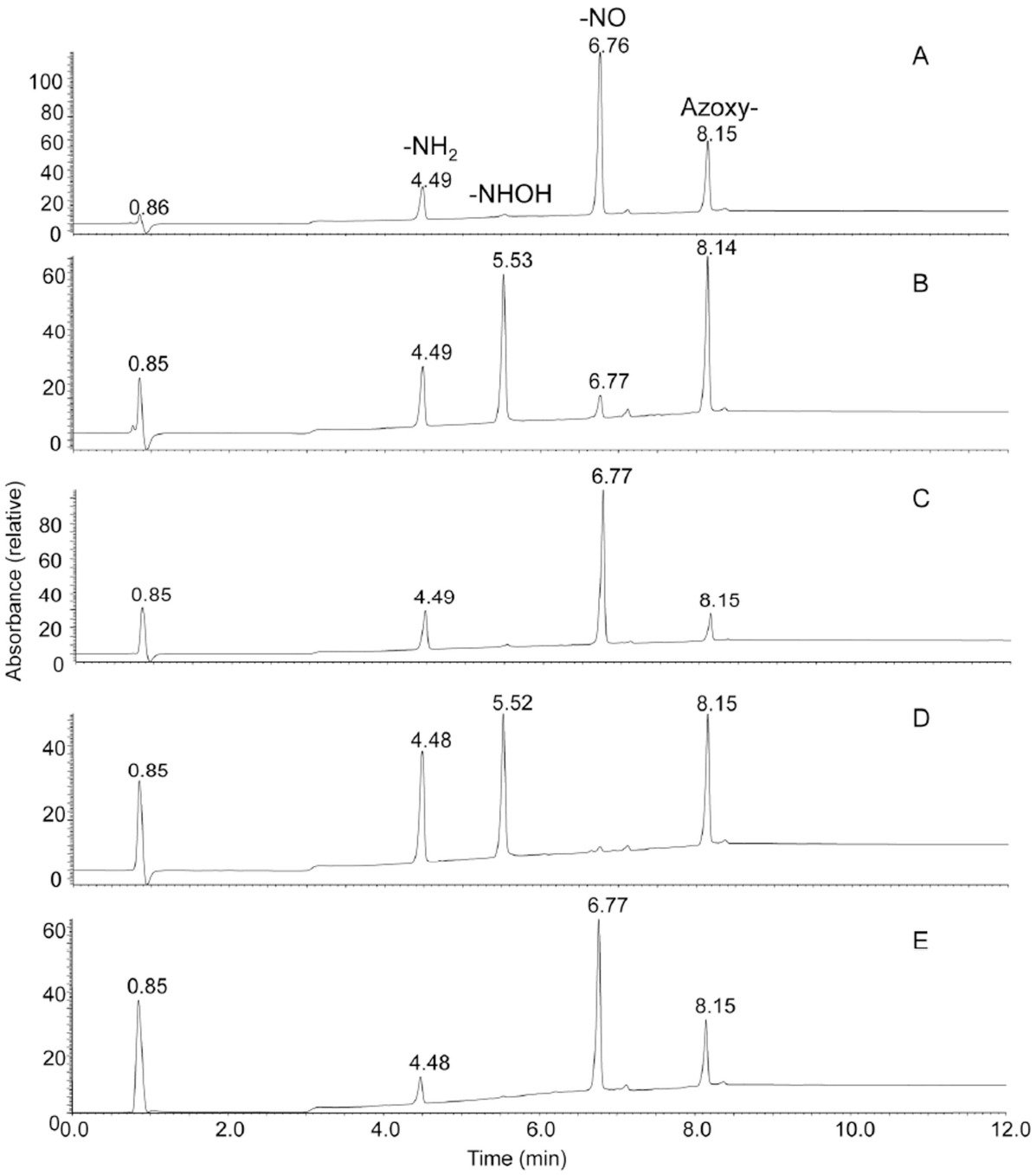


Figure 4.

Anaerobic incubations of 4-NO-biphenyl with P450 2S1. Freshly prepared 4-NO-biphenyl (containing trace 4-ABP and azoxybiphenyl, 5 μL of an approximately 2 mM solution in $(CH_3)_2SO$) was incubated with P450 2S1 (total volume 0.5 mL) at 37 °C for 30 min under anaerobic conditions. (A) 4-NO-biphenyl in 100 mM sodium HEPES buffer (pH 7.4) containing 1 mM EDTA, incubated at 37 °C for 30 min. (B) 4-NO-biphenyl incubated with all P450 system components with the exception of P450 2S1. (C) 4-NO-biphenyl incubated with all components of the P450 2S1 system with the exception of the NADPH-generating system. (D) 4-NO-biphenyl incubated with P450 2S1 in the presence of NPR and an

NADPH-generating system. (E) Synthetic standard of 4-NO-biphenyl. UV absorbance was integrated over the range 200–400 nm.

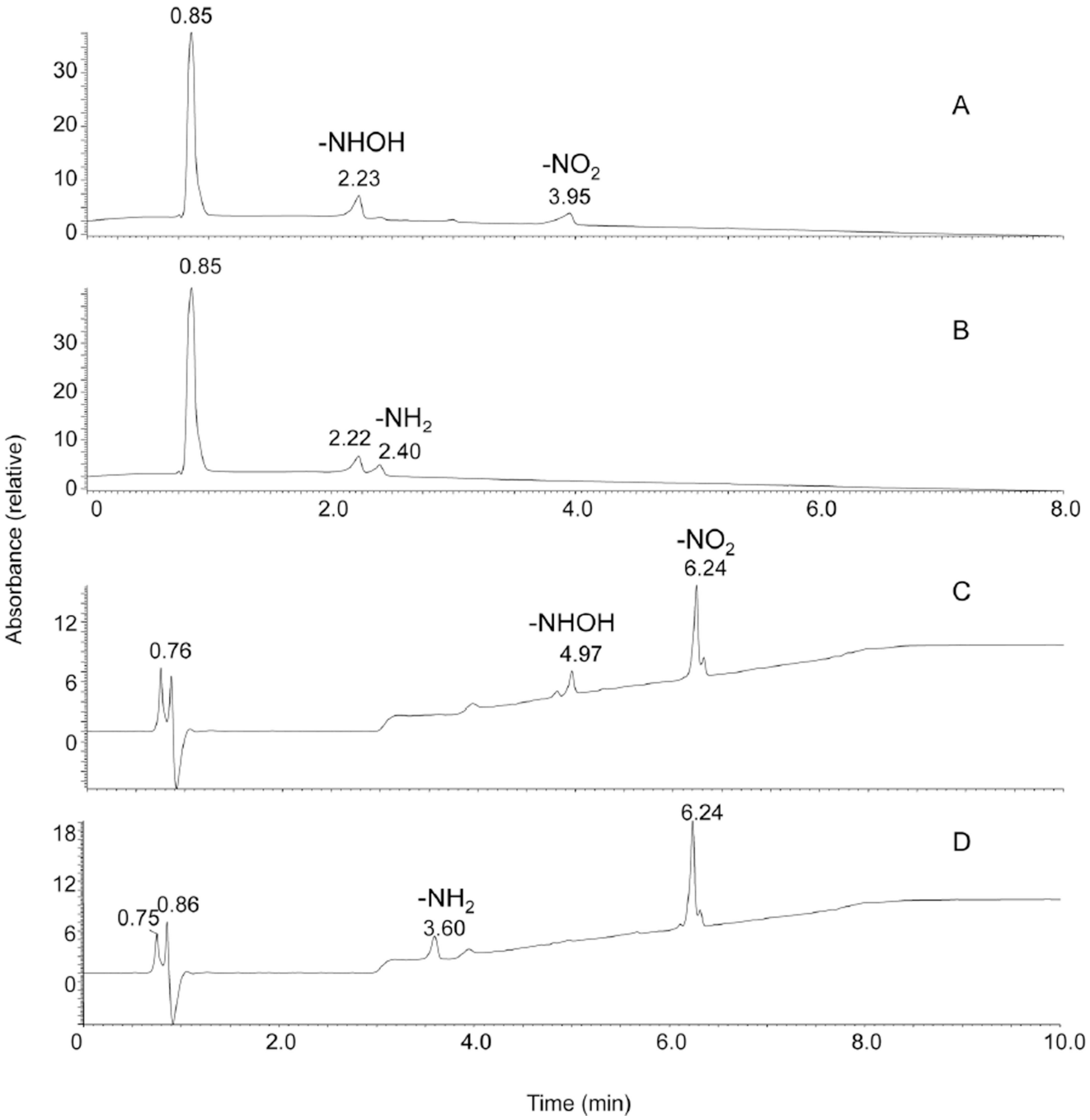


Figure 5.

Anaerobic incubations of NO_2-IQ and 2-NO_2-naphthalene with P450 2S1. NO_2-IQ (5 μL of a 10 mM solution in $(CH_3)_2SO$) was incubated with P450 2S1 (total volume 0.5 mL) at 37 °C for 20 min under anaerobic conditions. (A) NO_2-IQ incubated with all P450 system components with the exception of P450 2S1. (B) NO_2-IQ incubated with P450 2S1 in the presence of NPR and an NADPH-generating system. (C) 2-NO_2-naphthalene incubated with all P450 system components with the exception of P450 2S1. (D) 2-NO_2-naphthalene incubated with P450 2S1 in the presence of NPR and an NADPH-generating system. UV absorbance was integrated over the range 200–400 nm.

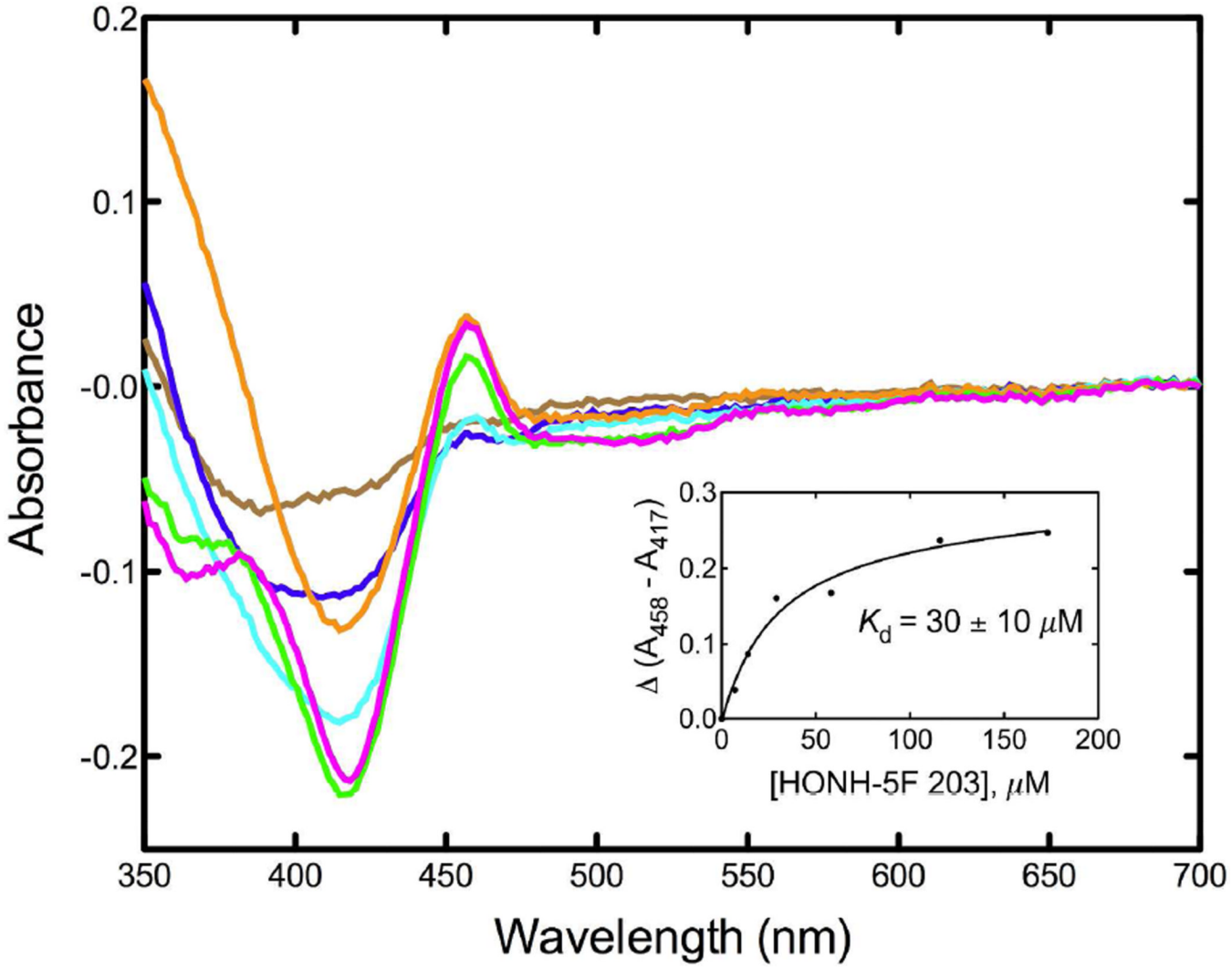


Figure 6.
Binding spectra for titration of P450 2S1 with HONH-5F 203. The calculated K_d was 30 ± 10 μM.

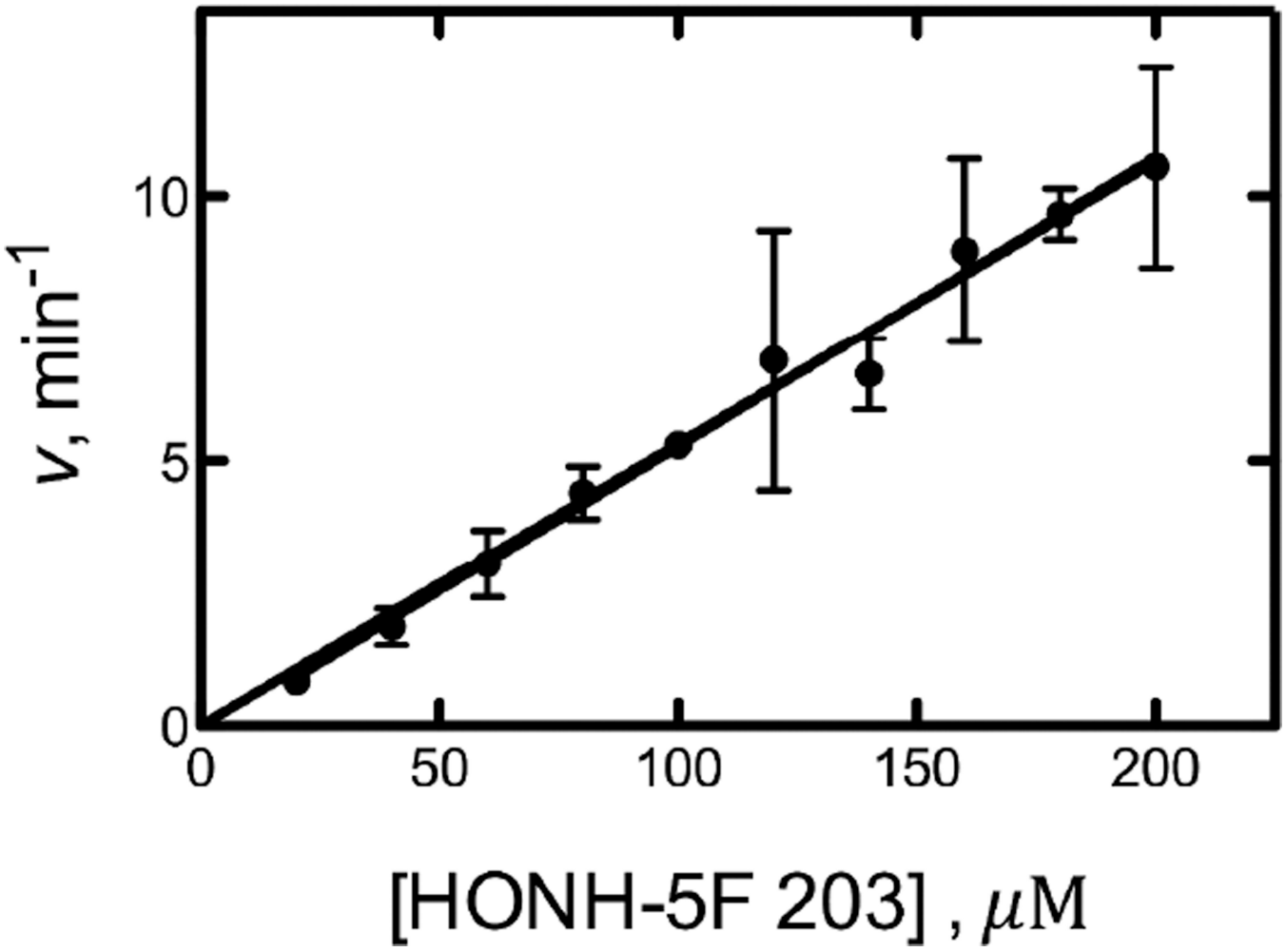
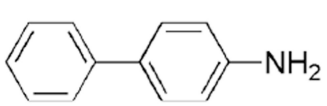
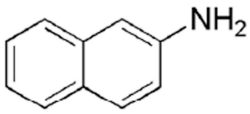


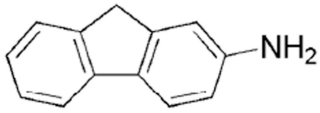
Figure 7.
Catalysis of anaerobic reduction of HONH-5F 203 by P450 2S1 as a function of substrate concentration.



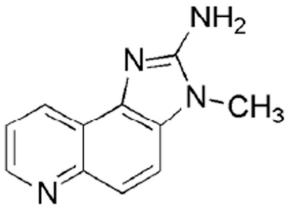
4-aminobiphenyl (4-ABP)



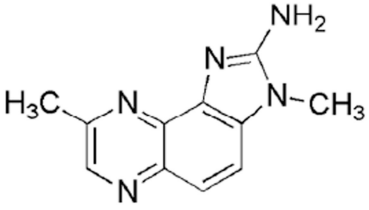
2-naphthylamine (2-NA)



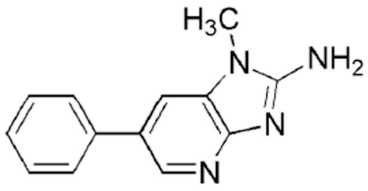
2-aminofluorene (2-AF)



2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ)

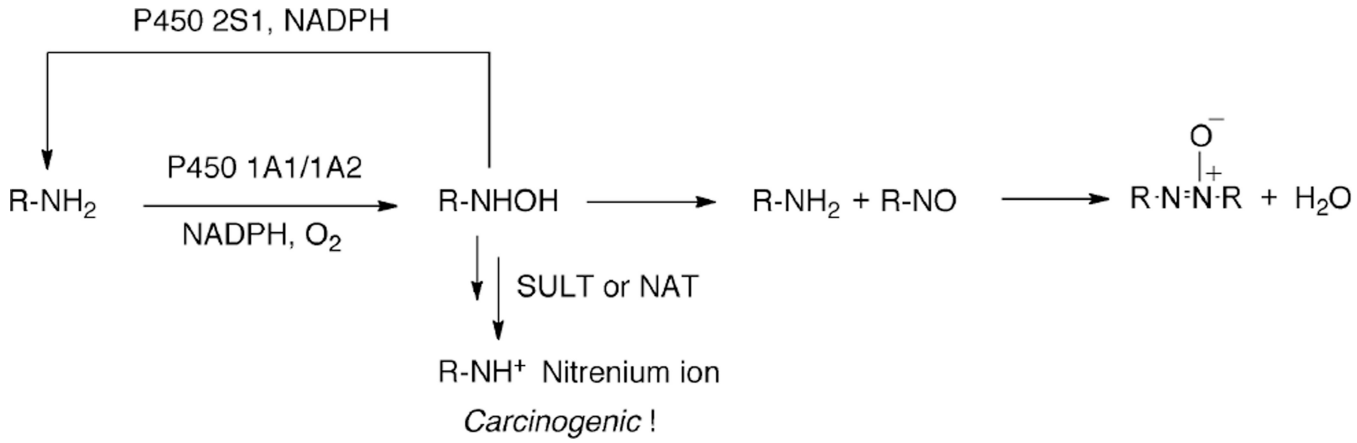


2-amino-3,8-di-methylimidazo-[4,5-*f*]quinoxaline (MeIQx)

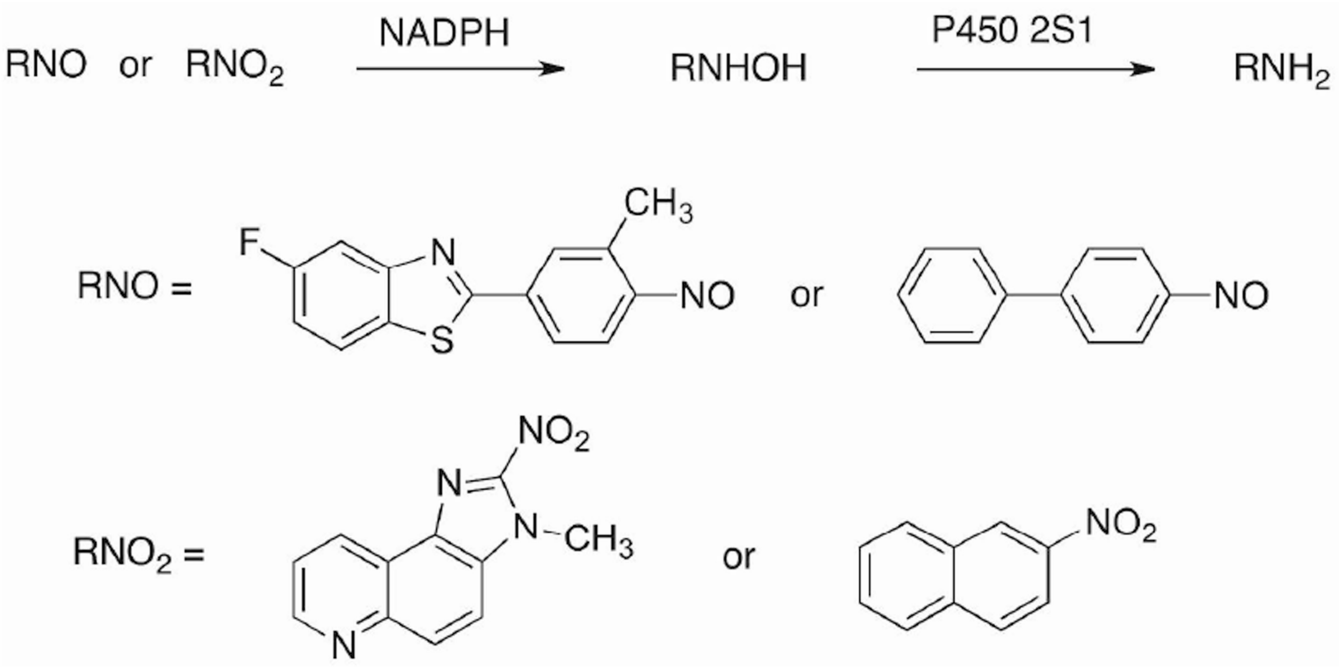


2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine (PhIP)

Scheme 1.
Structures of carcinogenic aromatic and heterocyclic aromatic amines



Scheme 2.
Biotransformation pathways of aromatic and heterocyclic aromatic amines



Scheme 3.
Reduction of aromatic nitroso and nitro compounds by NADPH and P450 2S1