Structure-Mutagenicity Relationships of N-Oxidized Derivatives of Aniline, *o*-Toluidine, 2′-Methyl-4-aminobiphenyl, and 3,2′-Dimethyl-4-aminobiphenyl[1]

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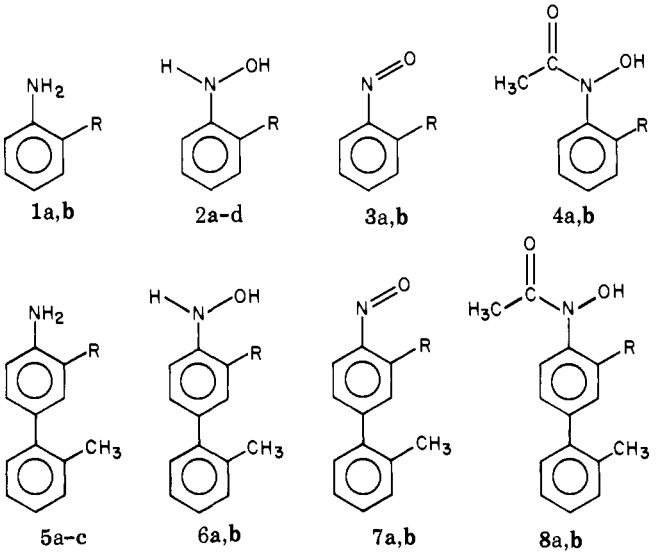
A series of N-oxidized derivatives of aniline (**1a**), 2′-methyl-4-aminobiphenyl (**5a**), and the carcinogens *o*-toluidine (**1b**) and 3,2′-dimethyl-4-aminobiphenyl (**5b**) were prepared and tested for mutagenic activity toward *S. typhimurium*. The compounds tested were the hydroxylamines **2a,b** and **6a,b**; *C*-nitroso compounds **3a,b** and **7a,b**; and the hydroxamic acids **4a,b** and **8a,b** derived from the amines **1a,b** and **5a,b**, as well as the parent amines and *N*-[2-(hydroxymethyl)phenyl]hydroxylamine (**2d**), *N*-(2-methylphenyl)hydroxylamine-*methyl*-d_3 (**2c**), and 3,2′-dimethyl-4-aminobiphenyl-*3-methyl*-d_3 (**5c**). Compounds **2a,b** and **2d–4b** were obtained commercially and purified or were prepared according to previously described procedures, while **2c** was synthesized by nitration of toluene-d_3, separation of the isomers, and reduction. Compounds **5a,b** and **6a–8b** were prepared by standard procedures from 2′-methyl-4-nitrobiphenyl (**9**) and 3,2′-dimethyl-4-nitrobiphenyl (**10**); **5c** was prepared by treatment of **10** with $NaOCD_3$ in CD_3OD to give 3,2′-dimethyl-4-nitrobiphenyl-*3-methyl*-d_3 (**11**), followed by reduction. Aniline (**1a**) and *o*-toluidine (**1b**) were inactive in strains TA 100, TA 1535, and TA 1538, both with or without activation by rat liver homogenate. The hydroxylamine **2b** and *C*-nitroso compound **3b** were mutagenic toward TA 100 and TA 1535 with activation but not toward TA 1538. Hydroxylamine **2d** was less active than **2b** toward TA 100. Hydroxylamine **2a** and *C*-nitroso compound **3a** were not mutagenic toward TA 100, TA 1535, or TA 1538 with activation. When assayed without activation, **2a,b** and **3a,b** were either toxic or nonmutagenic. The hydroxamic acid **4b** was mutagenic toward TA 1535 without activation; **4a** and **4b** did not show activity in TA 100 or TA 1535 with activation. The aminobiphenyls **5a,b** were highly mutagenic toward TA 1538 and TA 100 with activation but not toward TA 1535. Neither **5a** nor **5b** was active in the absence of rat liver homogenate. Hydroxylamines **6a,b**, *C*-nitroso compounds **7a,b**, and hydroxamic acids **8a,b** were mutagenic toward strains TA 1538 and TA 100 with activation; **6b** and **7b** were highly mutagenic toward TA 1538 without activation. The biphenylamine derivatives were more mutagenic than the single ring compounds; the former were frameshift mutagens, while the latter were base-pair mutagens. Among the compounds showing mutagenic activity, the derivatives having a methyl group ortho to the amine functionality were generally more mutagenic, which parallels their carcinogenic activities. Substitution of deuterium for hydrogen in the *o*-methyl groups of **2b** and **5b** resulted in no significant loss of mutagenicity.

Aromatic amines with a methyl group ortho to the amine functionality are often more carcinogenic than the corresponding unsubstituted amines or than the meta- or para-substituted isomers. For example, aniline (**1a**) is not carcinogenic,[3] but *o*-toluidine (2-methylaniline, **1b**) is carcinogenic, inducing predominantly subcutaneous tumors in rats and blood vessel tumors in mice.[4,5] No significant carcinogenic activity was observed for *m*-toluidine or *p*-toluidine in rats, when tested under the same conditions as those used for *o*-toluidine. A second example is found in the aminobiphenyl series. 2′-Methyl-4-aminobiphenyl (**5a**) is not tumorigenic in rats, but 3,2′-dimethyl-4-aminobiphenyl (**5b**) is a potent carcinogen, inducing tumors of the colon in male rats and mammary tumors in females.[6-8] Other *o*-methyl aromatic amines which are more carcinogenic in rats than the unsubstituted analogues include 3-methyl-2-naphthylamine, 4-(acetylamino)-3-methylbiphenyl, and 3,3′-dimethyl-4,4′-diaminodiphenylmethane.[9-11] While the metabolism and mechanism of action of certain aromatic amines have been studied in some detail and are rather well understood, no studies have appeared on the mechanistic aspects of the *o*-methylamino effect. Since N-oxidized metabolites are known to be proximate or ultimate carcinogens and/or mutagens for certain unsubstituted aromatic amines,[12] we have prepared a series of N-oxidized derivatives of aniline (1a), 2′-methyl-4-aminobiphenyl (**5a**), and the corresponding carcinogens *o*-toluidine (**1b**) and 3,2′-dimethyl-4-aminobiphenyl (**5b**). These compounds (**2a,b**, **2d–4b**, and **6a–8b**) and the parent amines have been tested for mutagenicity toward *S. typhimurium* to determine the effects of the *o*-methyl group. The effects on mutagenicity of deuterium substitution in the *o*-methyl groups of **2b** and **5b** have also been examined through assays of **2c** and **5c**.

Synthesis. The derivatives **2a,b** and **2d–4b** of aniline and *o*-toluidine were obtained commercially or were prepared according to procedures described in the literature. *N*-(2-Methylphenyl)hydroxylamine-*methyl*-d_3 (**2c**) was prepared by nitration of toluene-*methyl*-d_3, separation of the isomers, and reduction of *o*-nitrotoluene-*methyl*-d_3 with Zn and NH_4Cl. The syntheses of **5a**, **6a**, **7a**, and **8a** are outlined in Scheme I. The reactions are typical for the preparation of compounds of these classes. Coupling of *p*-nitrobenzenediazonium tetrafluoroborate with toluene gave a mixture of isomers in which the desired nitro compound **9** predominated and could be separated from the other isomers by chromatography. The yield of **9** was improved by addition of crown ether to the reaction mixture.[13] Reduction of **9** with Zn and NH_4Cl gave hydroxylamine **6a**, which was acetylated to give hydroxamic acid **8a**. Oxidation of **6a** gave the *C*-nitroso compound **7a**, and reduction of **9** gave the parent amine **5a**.



a, R = H; **b**, R = CH_3; **c**, R = CD_3; **d**, R = CH_2OH

The N-oxidized derivatives of 3,2′-dimethyl-4-aminobiphenyl (**5b**) were prepared from **5b** after purification to

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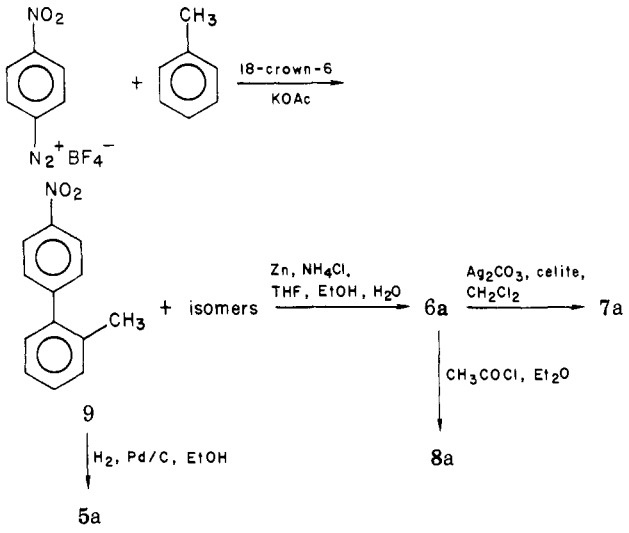
Scheme I



remove traces of other isomers.[14] Controlled oxidation of **5b** with *m*-chloroperbenzoic acid gave **7b**. Further oxidation of **7b** with *m*-chloroperbenzoic acid afforded 3,2′-dimethyl-4-nitrobiphenyl (**10**). Reduction of **10** with Zn and NH_4Cl gave hydroxylamine **6b**, which was acetylated to give hydroxamic acid **8b**. For the preparation of 3,2′-dimethyl-4-aminobiphenyl-*3-methyl-*d_3 (**5c**), **10** was treated with $NaOCD_3$ in CD_3OD to give 3,2′-dimethyl-4-nitrobiphenyl-*3-methyl-*d_3 (**11**). Reduction of **11** with Fe and HCl gave **5c**. Exchange of the protons on the methyl group ortho to a nitro group as described here is a convenient method for the preparation of such deuterated compounds.

Since trace impurities can lead to erroneous interpretation of mutagenicity results, all compounds were carefully checked for purity by GC, TLC, and/or LC. All compounds were at least 99% pure according to these criteria.

Results of Mutagenicity Assays. Aniline (**1a**) and *o*-toluidine (**1b**) were inactive as mutagens toward *S. typhimurium* TA 100, TA 1535, and TA 1538 with or without activation by rat liver homogenate. The hydroxylamine **2b** and the *C*-nitroso derivative **3b** of *o*-toluidine were found to be mutagenic toward both *S. typhimurium* TA 100 and TA 1535 in the presence of rat liver homogenate. The mutagenicity of these N-oxidized derivatives of *o*-toluidine compared to the aniline derivatives **2a** and **3a** is shown in Figures 1 and 2. Under identical conditions, **2b** and **3b** failed to give a positive mutagenic response in TA 1538. The hydroxamic acids **4a,b** showed no significant mutagenic activity in TA 100 and TA 1535 in the presence of hepatic supernatant. When **4a** and **4b** were assayed without activation, only the hydroxamic acid **4b** showed mutagenic activity toward TA 1535 (72 His^+ revertants/plate at a dose of 10 μmol; control = 26). The *C*-nitroso compounds and hydroxylamines in this series were either toxic or nonmutagenic when assayed at doses ranging from 0.05 to 10 μmol in the absence of rat liver homogenate.

The mutagenic activities of the parent biphenylamines **5a,b** toward *S. typhimurium* strains TA 100 and TA 1538 in the presence of hepatic supernatant are shown in Figures 3 and 4. In both strains, 3,2′-dimethyl-4-aminobiphenyl (**5b**) was more mutagenic than 2′-methyl-4-aminobiphenyl (**5a**); neither compound was mutagenic without activation. Neither **5a** nor **5b** was mutagenic toward TA 1535 with or without activation. Figures 5 and 6 summarize the results for hydroxylamines

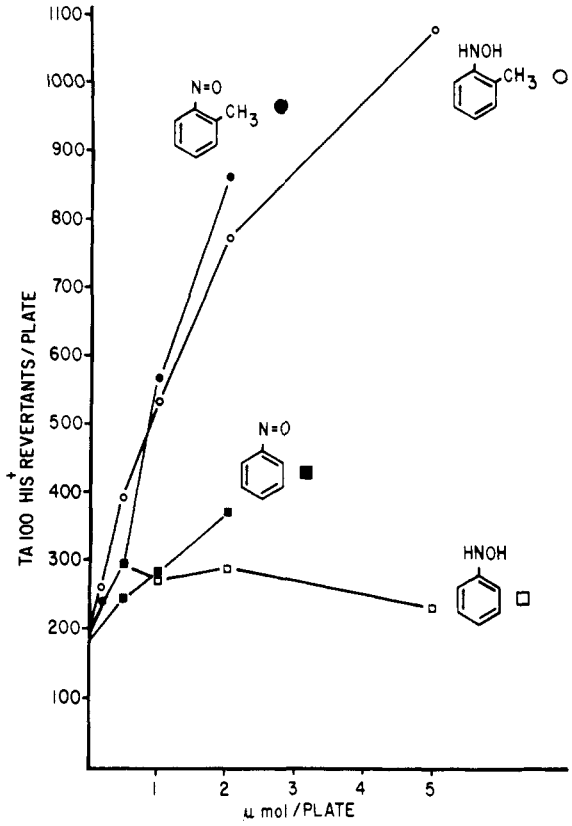


Figure 1. Mutagenicity toward *S. typhimurium* TA 100 of **2a** (□–□), **2b** (○–○), **3a** (■–■), and **3b** (●–●) in the presence of rat liver homogenate. **3a,b** exhibited only toxicity at doses greater than 2 μmol/plate. At the doses shown, the toxicities of **3a** and **3b** were similar, as were **2a** and **2b**.

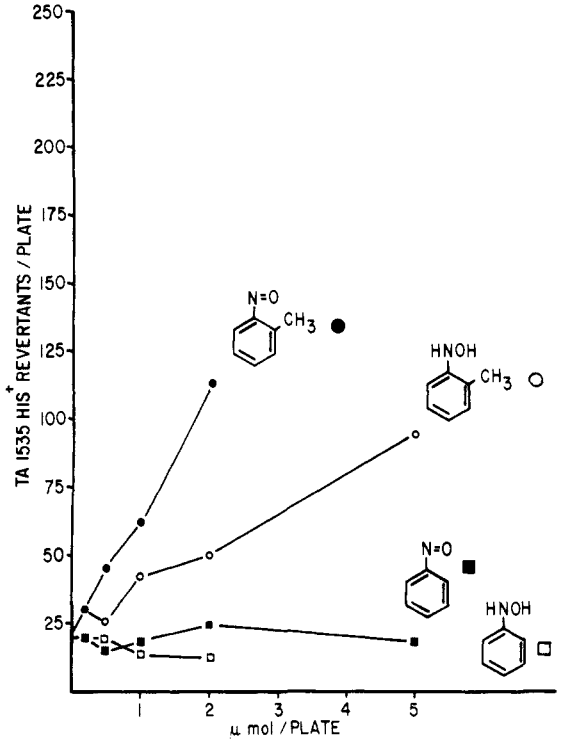


Figure 2. Mutagenicity toward *S. typhimurium* TA 1535 of **2a** (□–□), **2b** (○–○), **3a** (■–■), and **3b** (●–●) in the presence of rat liver homogenate. At doses higher than those shown, only toxicity was observed. At the doses shown, the toxicities of **3a** and **3b** were similar, as were **2a** and **2b**.

6a,b in *S. typhimurium* TA 100 and TA 1538. In each case, **6b** was more mutagenic than **6a**; **6b** was highly

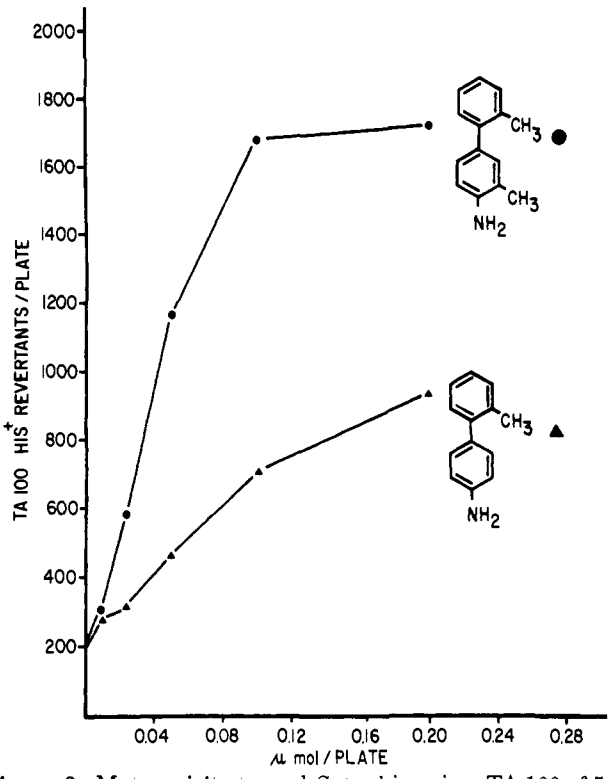


Figure 3. Mutagenicity toward *S. typhimurium* TA 100 of **5a** (▲-▲) and **5b** (●-●) in the presence of rat liver homogenate. No significant toxic effects were observed.

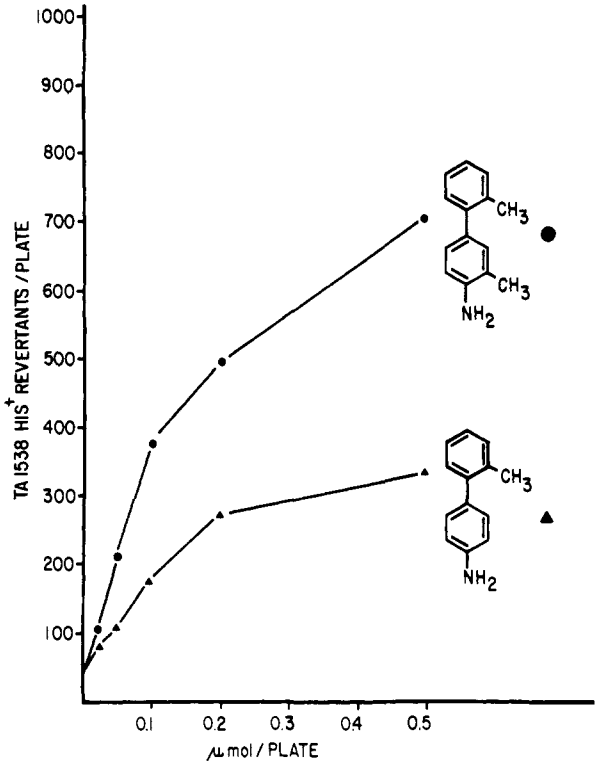


Figure 4. Mutagenicity toward *S. typhimurium* TA 1538 of **5a** (▲-▲) and **5b** (●-●) in the presence of rat liver homogenate.

mutagenic toward TA 1538 both with and without activation. The mutagenicity of **6a** and **6b** toward TA 100 was increased in the presence of hepatic supernatant. Similar results were obtained for *C*-nitroso compounds **7a,b**, as shown in Figures 7 and 8. The *o*-methyl-*C*-nitroso compound **7b** was more mutagenic than **7a** in both strains; **7b** was highly mutagenic toward TA 1538 both with and

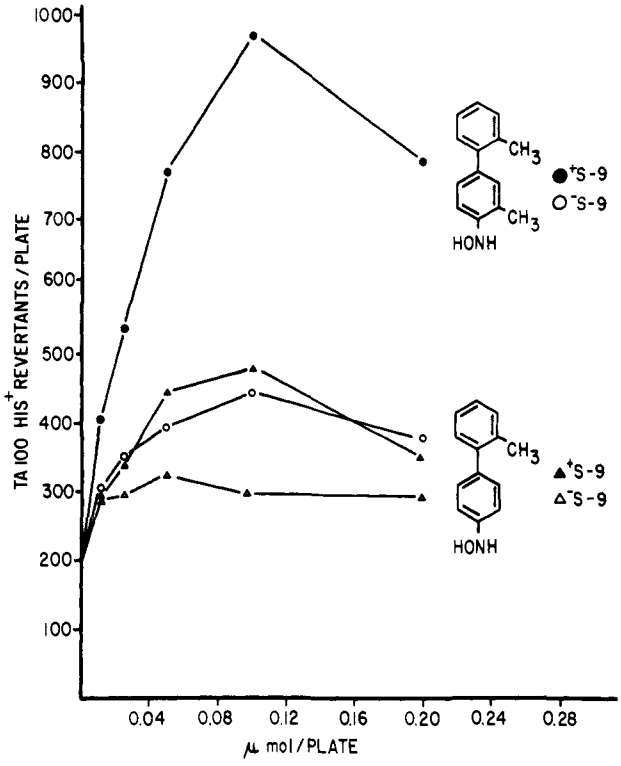


Figure 5. Mutagenicity toward *S. typhimurium* TA 100 of **6a** in the presence (▲-▲) or absence (△-△) of rat liver homogenate and of **6b** in the presence (●-●) or absence (○-○) of rat liver homogenate. No toxicity was observed for **6a,b** in the presence of liver homogenate, but **6a** was toxic in the absence of liver homogenate.

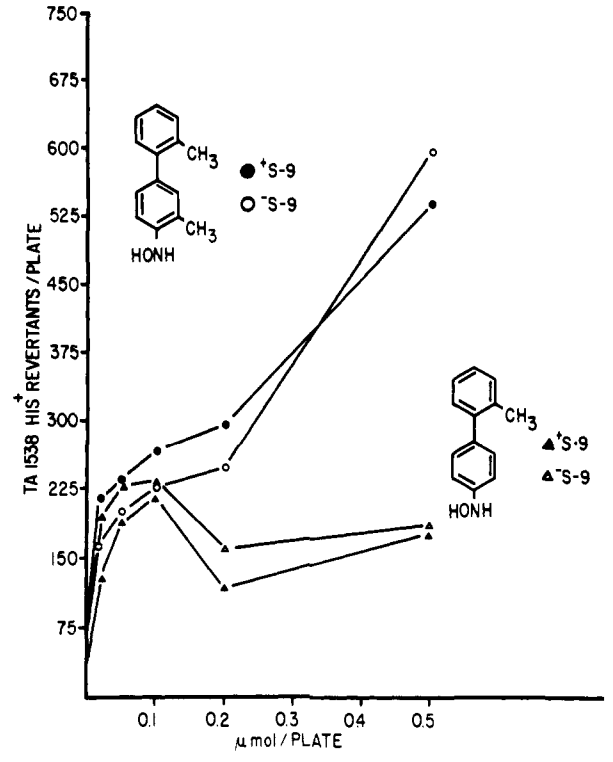


Figure 6. Mutagenicity toward *S. typhimurium* TA 1538 of **6a** in the presence (▲-▲) or absence (△-△) of rat liver homogenate and of **6b** in the presence (●-●) or absence (○-○) of rat liver homogenate.

without activation. The hydroxamic acids **8a,b** showed comparable mutagenic activity toward *S. typhimurium* TA 100 with activation, as shown in Figure 9. They were also active toward TA 1538 (96 His^+ revertants/plate for **8a**

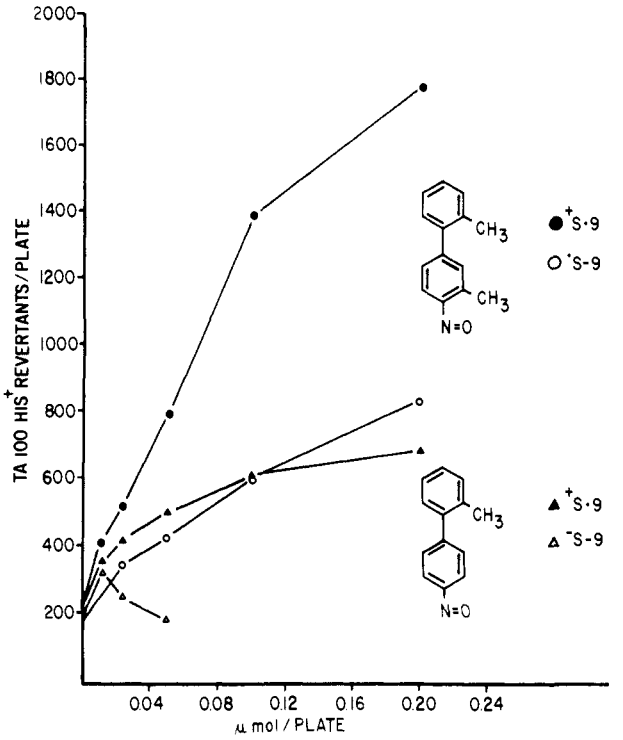


Figure 7. Mutagenicity toward *S. typhimurium* TA 100 of **7a** in the presence (▲–▲) or absence (△–△) of rat liver homogenate and of **7b** in the presence (●–●) or absence (○–○) of rat liver homogenate. No toxicity was observed for **7a,b** in the presence of liver homogenate, but **7a** was toxic in the absence of liver homogenate.

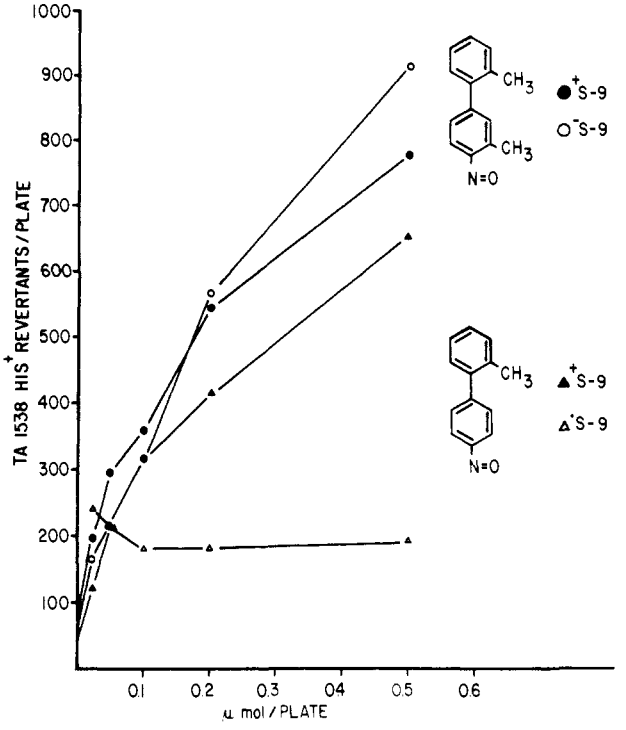


Figure 8. Mutagenicity toward *S. typhimurium* TA 1538 of **7a** in the presence (▲–▲) or absence (△–△) of rat liver homogenate and of **7b** in the presence (●–●) or absence (○–○) of rat liver homogenate.

and 90 His^+ revertants/plate for **8b** at doses of 0.2 μmol). Neither **8a** nor **8b** showed mutagenic activity without activation.

When the deuterated hydroxylamine **2c** was compared to **2b** in strain TA 100 with activation, inconsistent results

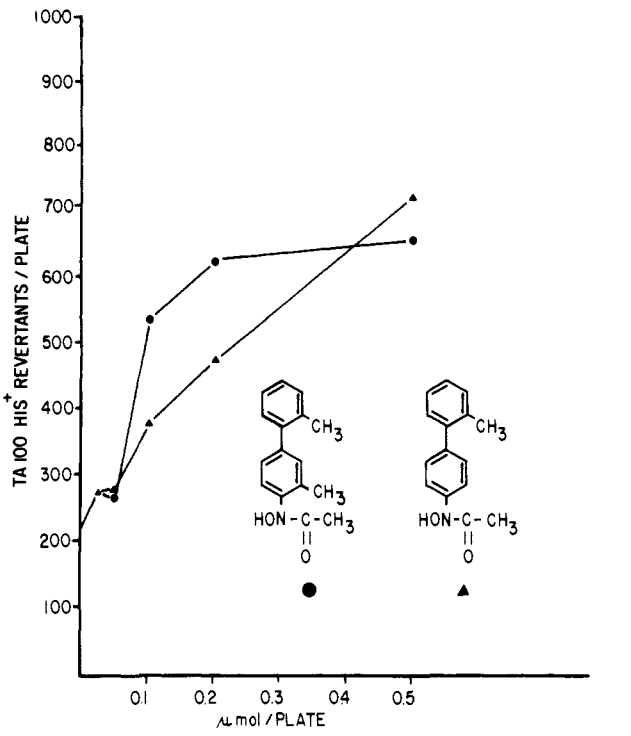


Figure 9. Mutagenicity toward *S. typhimurium* TA 100 of **8a** (▲–▲) and **8b** (●–●) in the presence of rat liver homogenate. No significant toxicity was observed.

were obtained due to the toxicity of the compounds at doses where differences in activity might have been apparent (2.0–5.0 μmol/plate). At lower doses (0.2–1.0 μmol/plate), **2b** and **2c** showed comparable mutagenicity. The mutagenic activities of **5b** and the deuterated derivative **5c** toward *S. typhimurium* TA 100 with activation were also comparable. Both compounds were highly mutagenic (see Figure 3 for mutagenicity of **5b**).

Discussion

A comparison of the mutagenic activity of the phenylamines with the analogous biphenylamines clearly indicates that the latter are more powerful mutagens. For example, a dose of 0.1 μmol of hydroxylamine **6b** induced as many mutations in TA 100 with activation as did a dose of 5 μmol of **2b**. No activity was observed for aniline and *o*-toluidine, whereas the biphenylamines **5a,b** were both mutagenic toward *S. typhimurium* TA 100 and TA 1538 with activation. Similar results have been obtained in previous studies on the mutagenicity and carcinogenicity of aromatic amines.[12,15–18]

The lack of mutagenic activity toward TA 1535 observed for the biphenylamines **5a,b** is indicative of compounds which are exclusively frameshift mutagens. Conversely, the lack of mutagenicity toward TA 1538 by the hydroxylamine and *C*-nitroso derivatives of *o*-toluidine is indicative that these compounds are exclusively base-pair mutagens. Since *S. typhimurium* TA 100 is sensitive to base-pair and frameshift mutagens,[19] mutagenicity was detected in both series by this strain.

The fact that the hydroxylamine **2b** and the *C*-nitroso compound **3b** derived from *o*-toluidine acted exclusively as base-pair mutagens is in contrast to observations on the "comutagenic activity" of norharmane and *o*-toluidine.[15] In these studies, *o*-toluidine when assayed with norharmane was found to act exclusively as a frameshift mutagen. In addition, these "comutagenicity" assays failed to produce a response toward *S. typhimurium* TA 100. While the mode of action of comutagenesis has not been eluci-

dated, these data indicate that the N-oxidized derivatives **2b** and **3b** are not responsible for the comutagenic activity observed with *o*-toluidine.

Among the compounds showing mutagenic activity, the *o*-methyl derivatives were generally more mutagenic than the related compounds lacking the *o*-methyl group, which is in line with carcinogenicity results. This was observed in the single ring series for the hydroxylamines (**2b** > **2a**) and *C*-nitroso compounds (**3b** > **3a**) in the presence of rat liver homogenate. The hydroxamic acid **4b** was also more mutagenic than **4a** in TA 1535 without activation. In the biphenylamine series, the same trend was observed for the parent amines (**5b** > **5a**), hydroxylamines (**6b** > **6a**), and *C*-nitroso compounds (**7b** > **7a**). The only instances in which differences in toxicity could have influenced these results were in the unactivated assays of **6a** and **7a**. These results indicate that the presence of an *o*-methyl group may facilitate formation of an activated intermediate which can interact with bacterial DNA. Similar considerations may apply to ultimate carcinogens derived from **1b** and **5b**, although differences in the mechanisms of carcinogenicity and mutagenicity by aromatic amines have been observed.[20]

The nature of these activated forms is not presently known, but the relatively high mutagenicity of the hydroxylamines **2b** and **6b** and *C*-nitroso compounds **3b** and **7b** indicates that N-oxidation is required for activation of **1b** and **5b**. The high mutagenicity of hydroxylamine **6b** and *C*-nitroso compound **7b** in TA 1538 without activation is of particular interest and suggests that **6b** or **7b** may be an ultimate mutagen of **5b**. These results must be interpreted with caution because certain *S. typhimurium* strains may be able to interconvert *C*-nitroso compounds and hydroxylamines.[21]

To account for the enhancing effect of the *o*-methyl group, we considered the possibility that the *o*-methyl group could be metabolically oxidized to *o*-hydroxymethyl and that the oxygen atom of the latter could facilitate formation of a nitrenium ion from the neighboring hydroxylamino group via anchimeric assistance. If this were the case, then *N*-[2-(hydroxymethyl)phenyl]hydroxylamine (**2d**) would be expected to be more mutagenic than the hydroxylamine **2b**. However, **2d**, which induced only 360 His^+ revertants/plate at a dose of 5.0 μmol, was significantly less active than **2b** in *S. typhimurium* TA 100, with activation. Thus, such a mechanism appears unlikely.

To determine whether breakage of a C–H bond of the *o*-methyl group might be involved in mutagenesis by these compounds, the mutagenicity of the deuterated hydroxylamine **2c** was compared to that of **2b** and the mutagenicity of dimethylaminobiphenyl **5c** was compared to **5b**. The toxicity of **2b** and **2c** did not allow unambiguous comparison of their mutagenic activities. However, in the case of **5b** and **5c**, both compounds were equally mutagenic. Thus, it is unlikely that a C–H bond of the *o*-methyl group of **5b** was broken during the rate-determining step leading to formation of the ultimate mutagen. Further studies are currently in progress to characterize the active intermediates derived from **1b** and **5b**.

Experimental Section

Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^1H NMR spectra were recorded on a Hitachi Perkin-Elmer R-24 high-resolution NMR spectrometer. Chemical shifts are reported in parts per million relative to Me_4Si as an internal standard. IR spectra were recorded on a Perkin-Elmer Model 267 infrared spectrometer. Mass spectrometry and combined GC–MS were performed with a Hewlett-Packard Model 5982A instrument. For GC analyses, we used a Hewlett-Packard Model 5710A gas chromatograph equipped with a flame-ionization detector and a 6 ft × 0.12 in. column packed with 10% UCW-98 on WHP-7620. The oven temperature was programmed from 100 to 240 °C at 4 °C/min; helium was used as carrier gas at a flow rate of 50 mL/min. Preparative GC was done with a Hewlett-Packard Model 7620A gas chromatograph equipped with a thermal-conductivity detector and a 10 ft × 0.25 in. column packed with 10% Carbowax on chromosorb Q; helium was used as the carrier gas at a flow rate of 50 mL/min. For LC, we used a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000 A solvent delivery system, a Model 660 solvent programmer, a Model U6K septumless injector, a Model 440 UV–visible detector, and a 6 mm × 30 cm microbondapak/C_{18} column with elution by 60% CH_3OH in H_2O at a flow rate of 2.5 mL/min. TLC was carried out with 0.25-mm silica gel 60 F_{254} (E. Merck) glass plates. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and were within ±0.3% of the calculated values.

Aniline (1a), *o*-Toluidine (1b), Nitrosobenzene (3a), and Nitrosotoluene (3b). All compounds were obtained from Aldrich Chemical Co. **1a** and **1b** were purified by distillation and their purities were established by GC. **3a** was recrystallized from cyclohexane–benzene to give pale-yellow crystals: mp 66–67 °C, lit.[22] 64–67 °C; purity was checked by GC and TLC (EtOAc/MeOH, 4:1). **3b** was recrystallized from hexane–benzene: mp 74–75 °C, lit.[23] 72.5 °C; purity was established by GC and TLC (hexane/EtOAc, 6:1).

***N*-Phenylhydroxylamine (2a), *N*-(2-Methylphenyl)-hydroxylamine (2b) and *N*-[2-(Hydroxymethyl)phenyl]-hydroxylamine (2d).** All three compounds were prepared according to previously described methods.[23-25] **2a** was recrystallized from ether–hexane to give colorless crystals: mp 82–83 °C (lit.[24] 82 °C); TLC (hexane/EtOAc, 6:1) and GC analyses showed no impurities; NMR ($CDCl_3$) δ 7.4–6.8 (m, 5 H), 6.67 (br s, 2 H, NHOH); IR (Nujol) 3250, 3150 cm^{-1}; MS *m/e* (rel intensity) 109 (M^+ 79.4), 92 (100).

2b was recrystallized from ether–hexane: mp 43–44 °C (lit.[23] 44 °C); homogeneous by TLC (hexane/EtOAc, 6:1) and LC; NMR ($CDCl_3$) δ 7.3–6.9 (m, 4 H), 6.41 (br s, 2 H, NHOH), 2.10 (s, 3 H, CH_3); MS *m/e* (rel intensity) 123 (M^+ 71), 106 (100).

2d was purified by column chromatography on Silicar CC-7 (Mallinckrodt) with elution by benzene/EtOAc, followed by recrystallization (EtOAc/benzene): mp 103–105 °C (lit.[25] 104 °C); purity >99% by TLC (EtOAc/MeOH, 4:1) and by GC analysis; NMR ($CDCl_3$) δ 8.21 (br s, 1 H, NH), 7.7–7.3 (m, 4 H), 4.72 (s, 2 H, CH_2), 3.25 (br s, 2 H, OH); MS *m/e* (rel intensity) 139 (M^+ 14), 121 (100).

***N*-Acetyl-*N*-phenylhydroxylamine (4a) and *N*-Acetyl-*N*-(2-methylphenyl)hydroxylamine (4b).** Both compounds were prepared according to literature procedures for **4a**.[26,27] **4a** was recrystallized from benzene–hexane: mp 66–67 °C, lit.[28] 67–67.5 °C; homogeneous by TLC (EtOAc/MeOH, 4:1); NMR ($CDCl_3$) δ 9.21 (br s, 1 H, OH), 7.32 (br s, 5 H), 2.05 (s, 3 H, CH_3); MS *m/e* (rel intensity) 151 (M^+ 18), 109 (100). **4b** was recrystallized from benzene/hexane: mp 77–78.5 °C; homogeneous by TLC (EtOAc/MeOH, 4:1); NMR ($CDCl_3$) δ 9.70 (br s, 1 H, OH), 7.30 (br s, 4 H), 2.30 (s, 3 H, CH_3CO), 1.85 (s, 3 H, CH_3); MS *m/e* (rel intensity) 165 (M^+ 15), 123 (100). Anal. ($C_9H_{11}NO_2$) C, H, N.

***N*-(2-Methylphenyl)hydroxylamine-*methyl*-d_3 (2c).** A cold mixture of HNO_3 (2 g, *d* 1.42) and concentrated H_2SO_4 (3 g) was added slowly with stirring to toluene-*methyl*-d_3 (Stohler Isotope Chemicals; 2 g, 0.02 mol). The temperature was gradually raised and maintained at 50 °C for 1 h. Ice–water was added, and the organic layer was separated and washed with H_2O, dilute aqueous $NaHCO_3$ and H_2O. The product was a mixture (by GC analysis) of approximately 50% 2-nitrotoluene-*methyl*-d_3, 27% 4-nitrotoluene-*methyl*-d_3, and 23% 2,4-dinitrotoluene-*methyl*-d_3. The compounds were separated by preparative GC using a temperature program of 190 °C for 15 min to 220 °C at 4 °C/min. In this way, 260 mg (0.0018 mol, 10%) of 2-nitrotoluene-*methyl*-d_3 was obtained: NMR ($CDCl_3$) δ 7.92 (d of d, 1 H, J = 9 and 2 Hz, aromatic H ortho to $-NO_2$), 7.6–7.2 (m, 3 H); MS *m/e* (rel intensity) 140 (M^+ 54), 122 (100), 94 (97). Reduction of 140 mg (0.001 mol) of 2-nitrotoluene-*methyl*-d_3 according to the procedure used for **2b** gave **2c** (70 mg, 50%) as a viscous oil, which was washed several

times with hexane until it was homogeneous by TLC (benzene/EtOAc, 5:1): purity 99% by LC analysis; NMR ($CDCl_3$) δ 7.3–6.8 (m, 4 H), 6.58 (br s, 2 H, NHOH); MS m/e (rel intensity) 126 (M^+ 56), 109 (100).

2′-Methyl-4-nitrobiphenyl (9). Method A.[29] A mixture of *p*-nitrobenzenediazonium tetrafluoroborate (Eastman; 23.7 g, 0.1 mol) and excess dry toluene (50 mL) was stirred vigorously. To this, dry pyridine (7.9 g, 0.1 mol) was added slowly; the reaction flask was then heated between 75 and 80 °C. The diazonium salt gradually dissolved and the pyridinium tetrafluoroborate separated. The color of the reaction mixture changed from yellow to deep red. Stirring was continued for 8 h. The mixture was filtered to remove the pyridinium tetrafluoroborate, which was washed with ether. The combined filtrates were concentrated, and the residue was chromatographed (silica, hexane) to give a mixture (2.5 g, 0.012 mol, 12%) containing three isomers according to GC analysis. Another column chromatography was done (silica, hexane), and fractions were combined after GC analysis. Pure **9** was obtained as pale-yellow crystals, which were recrystallized from benzene–hexane: yield 1 g, 0.0047 mol (4.7%); mp 102–104 °C (lit.[30] 103 °C); NMR ($CDCl_3$) δ 8.15 (d, 1 H, J = 9 Hz, aromatic H ortho to NO_2), 7.4–7.0 (m, 6 H), 2.15 (s, 3 H, CH_3); MS m/e (rel intensity) 213 (M^+ 70), 152 (62), 165 (100).

Method B.[13] The reaction flask was protected from light and purged with N_2. To a stirred mixtured of *p*-nitrobenzenediazonium tetrafluoroborate (2.4 g, 0.01 mol) and 18-crown-6 (Aldrich Chemical Co.; 0.08 g, 0.0003 mol) in dry toluene (50 mL) was added potassium acetate (0.6 g, 0.0006 mol). The color of the reaction mixture changed from yellow to red within a few minutes. Stirring was continued for 2 h, followed by filtration to remove the solid. The solid residue was washed with benzene (25 mL). The combined filtrates were washed with brine and H_2O, dried (Na_2SO_4), and concentrated. The resulting red oil was chromatographed on silica with elution by hexane. A mixture of three isomers was obtained (0.63 g, 0.003 mol, 30%) in which **9** was about 60%. Another column chromatography (silica, hexane) gave pure **9** (350 mg, 0.0016 mol, 16%).

4-Amino-2′-methylbiphenyl (5a). The nitro compound **9** (213 mg, 1 mmol) was dissolved in 95% ethanol (50 mL) containing HCl (5 mL) and hydrogenated in a Parr shaker with 10% Pd/C (0.05 g) as catalyst. After 45 min, the catalyst was removed by filtration and the solvent evaporated to give a solid, which was washed with benzene. The hydrochloride salt of **9** was recrystallized from CH_3OH/H_2O to give material of mp 290–292 °C dec (lit.[31] 285 °C dec). The salt was treated with 20% aqueous NaOH and the mixture was warmed for 15 min. The free amine **5a** was extracted with benzene, and the benzene layers were dried (Na_2SO_4) and concentrated to give pure **5a** (160 mg, 0.9 mmol, 90%): purity >99% by LC analysis; stored under N_2 at −197 °C; NMR ($CDCl_3$) δ 7.1–6.7 (m, 8 H), 3.30 (br s, 2 H, NH_2), 2.15 (s, 3 H, CH_3).

4-*N*-(2′-Methylbiphenyl)hydroxylamine (6a). The procedure was the same as described below for **6b**. **6a** was obtained from **9** in 38% yield as colorless flakes from benzene–hexane: mp 85–86 °C; purity >99% by LC analysis; NMR ($CDCl_3$) δ 7.10 (br s, 10 H, aromatic H and NHOH), 2.12 (s, 3 H, CH_3); IR (Nujol) 3220 cm^{-1}; MS m/e (rel intensity) 199 (M^+ 33), 182 (100). Anal. ($C_{13}H_{13}NO$) C, H, N.

2′-Methyl-4-nitrosobiphenyl (7a). The procedure was adapted from the literature.[32] To a solution of the hydroxylamine **6a** (190 mg, 1 mmol) in dry CH_2Cl_2 (20 mL) and Celite (500 mg) was added Ag_2CO_3 (Alfa Products; 900 mg, 1.5 mmol) in one portion at 20 °C. The reaction flask was protected from light and shaken throughly for 2–5 min. The precipitate was filtered and washed with CH_2Cl_2 (20 mL). Evaporation of the solvent gave a yellow solid, which was crystallized from benzene–hexane to give the nitroso compound: mp 62–64 °C; pure by LC analysis; NMR ($CDCl_3$) δ 7.7–7.0 (m, 8 H), 2.10 (s, 3 H, CH_3); IR (film) 1485 cm^{-1}; MS m/e (rel intensity) 197 (M^+ 92), 167 (51), 165 (100). Anal. ($C_{13}H_{11}NO$) C, H, N.

***N*-(2′-Methylbiphenyl-4-yl)acetohydroxamic Acid (8a).** The hydroxamic acid **8a** (mp 101–103 °C) was prepared from **6a** in 30% yield as described below for preparation of **8b**: purity >99% according to LC; NMR ($CDCl_3$) δ 9.55 (br s, 1 H, OH), 6.95 (br s, 8 H) 2.05 (br s, 6 H, 2′-CH_3 and CH_3CO); IR (Nujol) 3400–3100, 1650 cm^{-1}; MS m/e (rel intensity) 241 (M^+ 27), 199 (75), 182 (100). Anal. ($C_{15}H_{15}NO_2$) C, H, N.

3,2′-Dimethyl-4-aminobiphenyl (5b). Commercial **5b** hydrochloride (Starks Associates) was treated with boiling 1 N NaOH for 30 min; the aqueous portion saturated with NaCl and free **5b** was obtained. **5b** was homogeneous by TLC (benzene/EtOAc, 9:1; EtOAc/MeOH, 4:1). However, GC analysis showed three peaks; isomers by MS analysis, with the 3,2′ isomer as the major component. Purification of **5b** was achieved as previously described:[14] purity was established >99% by GC and LC; NMR ($CDCl_3$) δ 7.4–7.0 (m, 6 H), 6.25 (d, 1 H, J = 8 Hz, aromatic H ortho to NH_2), 3.45 (br s, 2 H, NH_2), 2.35 (s, 3 H, CH_3), 2.25 (s, 3 H, CH_3 ortho to NH_2).

3,2′-Dimethyl-4-nitrosobiphenyl (7b). A solution of **5b** (197 mg, 1 mmol) in $CHCl_3$ (20 mL) was cooled to 0 °C, and *m*-chloroperbenzoic acid (345 mg, 2 mmol) in $CHCl_3$ (25 mL) was added dropwise under a nitrogen atmosphere.[33] A green color developed as the peracid was added to the amine. After addition of peracid was complete, stirring was continued for 15 min. The reaction mixture was washed with 1 N NaOH, extracted ($CHCl_3$), dried ($CaCl_2$), and concentrated. The residue was purified by column chromatography (silica CC-7; hexane/benzene, 9:1) to give **7b** (120 mg, 0.5 mmol, 50%): crystallized from benzene–hexane to give pale-yellow crystals; mp 90–92 °C; purity confirmed by TLC (benzene/EtOAc; 5:1), GC, and LC; NMR ($CDCl_3$) δ 7.5–7.1 (m, 6 H) 6.35 (d, 1 H, J = 9 Hz, H ortho to N=O), 3.40 (s, 3 H, CH_3 ortho to N=O), 2.32 (s, 3 H, CH_3); IR (film) 1480 cm^{-1}; MS m/e (rel intensity) 211 (M^+ 42), 165 (100). Anal. ($C_{14}H_{13}NO$) C, H, N.

3,2′-Dimethyl-4-nitrobiphenyl (10). To the nitroso compound **7b** (120 mg, 0.6 mmol) in $CHCl_3$ (20 mL) at 20 °C, *m*-chloroperbenzoic acid (173 mg, 1 mmol) in $CHCl_3$ (20 mL) was added dropwise and the reaction mixture was stirred overnight. The mixture was worked up as above. NMR showed the disappearance of the CH_3 group ortho to N=O and the appearance of a CH_3 ortho to NO_2. The crude nitro compound was recrystallized from benzene–hexane to give yellow crystals (70 mg, 0.3 mmol, 50%): mp 54–56 °C (lit.[33] 56 °C); NMR ($CDCl_3$) δ 7.95 (d, 1 H, J = 8 Hz, H ortho to NO_2), 7.20 (br s, 6 H), 2.58 (s, 3 H, CH_3 ortho to NO_2), 2.20 (s, 3 H, CH_3); MS m/e (rel intensity) 227 (M^+ 100), 210 (64), 165 (78). The nitro compound could also be obtained by peracid oxidation of **5b** (5:1 peracid/**5b**; refluxing $CHCl_3$, 8 h) but the yield was lower (<40%).

4-*N*-(3,2′-Dimethylbiphenyl)hydroxylamine (6b). A solution of **10** (500 mg, 2.6 mmol), absolute EtOH (400 mL), H_2O (25 mL), and NH_4Cl (160 mg, 3 mmol) was stirred at 20 °C. THF was added dropwise until a homogeneous solution was obtained. To this mixture, Zn dust (390 mg, 6 mg-atoms) was added during the course of 5 min. As the reduction proceeded, the temperature rose to 35 °C. Stirring was continued for 20 min after all the Zn dust had been added. The mixture was filtered, saturated with NaCl, extracted with benzene, dried (Na_2SO_4), and concentrated to give a pale-yellow solid. The solid was crystallized from benzene–hexane to give colorless flakes (250 mg, 1 mmol, 38%): mp 105–106 °C; purity >99% by LC analysis; NMR ($CDCl_3$) δ 7.28 (s, 7 H), 7.10 (br s, 2 H, NHOH), 2.30 (s, 3 H, CH_3), 2.20 (s, 3 H, CH_3 ortho to NHOH); MS m/e (rel intensity) 213 (M^+ 63), 196 (100). Anal. ($C_{14}H_{15}NO$) C, H, N.

***N*-(3,2′-Dimethylbiphenyl-4-yl)acetohydroxamic Acid (8b).** The hydroxylamine **6b** (100 mg, 0.5 mmol) was dissolved in Et_2O (15 mL), and the solution was cooled to −5 °C and stirred vigorously. To this, acetyl chloride (170 mg, 1.5 mmol) in Et_2O (10 mL) was added dropwise over a period of 5 min. After the addition was complete, stirring was continued for an additional 30 min, during which time the reaction was followed by TLC. The Et_2O was evaporated to give a residue, which was purified by preparative TLC (benzene/EtOAc, 9:1). A pale-yellow solid was obtained (40 mg, 0.16 mmol, 32%): mp 132–133 °C; pure according to LC analysis; compound kept dry at liquid N_2 temperature to avoid decomposition; NMR ($CDCl_3$) δ 9.45 (br s, 1 H, OH), 7.18 (br s, 7 H), 2.20 (s, 3 H, 3-CH_3), 2.15 (br s, 6 H, CH_3CO and 2′-CH_3), MS m/e (rel intensity) 255 (M^+ 26), 213 (83), 196 (100). Anal. ($C_{16}H_{17}NO_2$) C, H, N.

3,2′-Dimethyl-4-aminobiphenyl-*3-methyl-d*$_3$ (5c). Sodium metal (0.23 g, 0.01 g-atom) was added to CD_3OD (Stohler Isotope Chemicals; 2 mL). To this, the nitro compound **10** (0.227 g, 0.001 mol) dissolved in CD_3OD (3 mL) was added after complete

disappearance of the sodium metal. The mixture was refluxed for 30 min until a light brown color developed. The reaction mixture was then added to a minimum amount of ice–water and extracted with benzene (60 mL). The benzene layer was dried (Na_2SO_4) and evaporated to give 3,2′-dimethyl-4-nitrobiphenyl-3-*methyl*-d_3 (**11**) as a yellow solid (0.205 g, 0.0008 mol, 80%): mp 52–54 °C; NMR ($CDCl_3$) δ 7.92 (d, 1 H, J = 9 Hz, aromatic H ortho to NO_2), 7.3–7.0 (m, 6 H), 2.20 (s, 3 H, 2′-CH_3); MS m/e (rel intensity) 230 (M^+ 100), 212 (83), 165 (58). The deuterated nitro compound **11** (90 mg, 0.38 mmol), Fe powder (200 mg, 0.003 g-atom), and 50% aqueous EtOH were added to a three-necked flask, fitted with a reflux condenser, and a mechanical stirrer. THF was then added until the nitro compound dissolved. The mixture was heated under reflux with stirring, and a solution of concentrated HCl (2 mL) in 50% aqueous EtOH (10 mL) was added slowly. The mixture was refluxed for 2 h after the addition of the acid was complete. The reaction mixture was made just alkaline to litmus by the addition of 20% aqueous KOH. The iron was removed by filtration, the filtrate was concentrated, and the residue was extracted several times with benzene. The benzene layers were dried (Na_2SO_4) and concentrated, and concentrated HCl was added dropwise to the oily residue. The amine hydrochloride was washed with Et_2O and benzene. The free amine was obtained by treating the hydrochloride with 20% KOH and extracting with benzene. The benzene layer was dried (Na_2SO_4) and concentrated under reduced pressure to give **5c** as a viscous oil (40 mg, 0.2 mmol, 52%), pure according to GC: NMR ($CDCl_3$) δ 7.2–6.9 (m, 6 H), 6.59 (d, 1 H, J = 9 Hz, aromatic H ortho to NH_2), 3.50 (br s, 2 H, NH_2), 2.20 (s, 3 H, 2′-CH_3); MS (**5c**·HCl) m/e (rel intensity) 200 (M^+ – HCl, 100), 182 (60), 181 (35), 165 (30).

Mutagenicity Assays. Mutagenicity studies were performed using *S. typhimurium* tester strains TA 1535, TA 1538, and TA 100 provided by Dr. Bruce Ames of the University of California, Berkeley. The S-9 fraction routinely employed for microsomal activation was obtained from the livers of male Fischer-344 rats weighing 300–350 g which had been treated 5 days prior to sacrifice with 500 mg/kg Aroclor 1254 (Analabs, Inc.). The S-9 fraction was prepared by centrifugation of the liver homogenate (25% in 0.15 M KCl) at 9000g for 15 min as described by Ames et al.[34] The S-9 fraction was filter sterilized at 4 °C using a 600-mL Millipore pressure filtration apparatus equipped with a 0.45-μm Swinnex filter. Each microsomal preparation was checked for sterility on nutrient agar prior to storage at −80 °C. The S-9 mix contained per milliliter: potassium phosphate buffer (100 μmol), pH 7.4; 8 μmol of $MgCl_2$; 1.65 μmol of KCl; 5 μmol of glucose 6-phosphate; 4 μmol of NADP; and 0.5 mL of S-9 fraction.

The procedure of Ames et al.[35] was employed in performing these assays. In summary, various concentrations of test compound in 50 μL of dimethyl sulfoxide were added to 0.1 mL of an overnight nutrient broth culture of the bacterial tester strain. After addition of 200 μL of S-9 mix and 2 mL of molten top agar at 45 °C, the contents were mixed and poured on minimal glucose agar plates. All compounds were assayed in duplicate at each dose level. Positive controls included *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, picrolonic acid, chrysene, and quinoline. Percent survivors for all compounds assayed was determined by employing dilutions of bacterial broth under identical conditions, with the exception that excess histidine was added to the top agar. Interpretation of the relative mutagenic activities of all compounds was based upon not only the observed mutagenicity but also their relative toxicities. Compounds which showed marked toxicity (less than 50% survival) to the bacterial tester strains are indicated in the figure legends. At least two separate assays were done for these compounds.

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