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Mutation Research 491 (2001) 183–193

Genetic Toxicology and Environmental Mutagenesis

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Mutagenicity in *Salmonella typhimurium* TA98 and TA100 of nitroso and respective hydroxylamine compounds

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Received 24 October 2000; received in revised form 16 January 2001; accepted 17 January 2001

Abstract

Five aromatic nitroso compounds were prepared and their mutagenicity in *Salmonella typhimurium* strains TA98 and TA100 compared with that of the corresponding hydroxylamines and the previously studied nitroarenes. A remarkable correspondence of the dose-response curves was observed between the nitroso and the respective hydroxylamine compounds. This effect could be observed in TA98 and TA100. It was only marginally dependent on the metabolical activation by rat liver S9-mix. Even the presence of a bulky alkyl substituent either near to the functional group, or far away from it, previously shown to considerably influence the mutagenic properties of nitroarenes, does not remarkably affect the properties of the nitroso and hydroxylamine species. The similarity between the latter two is likely to be due to a fast reduction of the nitrosoarenes to the hydroxylamine species under the test conditions. It seems that enzymes are not responsible for that reduction step, because sterical crowding near the functional group does not influence that behaviour.

The test results of the aromatic hydroxylamines bearing a bulky substituent show that there are at least two ways to influence the mutagenicity of an aromatic nitro compound by such a group. A substituent near the functional group (*ortho*-position) disturbs the enzymatic reduction of the nitro group, because 3-*tert*-butyl-4-hydroxylaminobiphenyl and its corresponding nitroso compound are highly mutagenic, whereas 3-*tert*-butyl-4-nitrobiphenyl was previously shown to be inactive even after addition of S9-mix. In contrast, 4′-*tert*-butyl-4-hydroxylaminobiphenyl with the *tert*-butyl group "far away" from the hydroxylamino functionality clearly shows decreased mutagenic activity suggesting a different influence of a substituent in that position. In addition, the substance shows only little cell toxicity even at higher concentrations. Both effects could be due to a reduced effective dose of the hydroxylamine in the cells compared to the non-alkylated compound, caused by a faster degradation of the hydroxylamine or a hindered interaction between that substance and the cells.

Keywords: *Salmonella*; Nitroaromatics; Alkyl substituent; Metabolites; Hydroxylamines; Nitroso compounds

1. Introduction

A large number of aromatic amines and nitro compounds are still used in industrial processes, such as dye and plastic production, although some of these chemicals are known to be mutagenic and carcinogenic [1]. One possibility to decrease the mutagenicity of a substance is a purposeful structural modification which should be as insignificant as possible in order to keep the desired properties of the compound. We recently reported that the mutagenicity of certain aromatic nitro compounds in *Salmonella typhimurium*

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1383-5718/01/$ – see front matter
PII: S1383-5718(01)00140-1

TA98 and TA100 can be significantly reduced by the introduction of bulky alkyl groups either near the functional group, or far away from it [2,3]. In order to use that kind of structural modification as a general strategy, some further information should be obtained about the effect that is carried out by bulky alkyl groups, which is the purpose of this work.

The mutagenicity of aromatic nitro compounds is thought to be induced by a chemical modification of the deoxyribonucleic acid (DNA), resulting from an electrophilic attack of an activated nitrogen intermediate, as, e.g. *N*-acetoxy-4-aminobiphenyl, at the purine bases of DNA, above all at the C8-position of deoxyguanosine [4,5]. This and other reactive electrophiles are supposed to be generated by two reduction steps from a nitroarene affording first the corresponding nitroso compound, and subsequently the hydroxylamine, which is further activated by *O*-acetylation, *O*-sulfatation or *O*-protonation [6]. Replication failures occurring with the modified DNA are thought to be finally responsible for the tumour induction.

We have already shown that 4′-*tert*-butyl-4-nitrobiphenyl (**7**) (4′-*t*Bu-4-NBp) and the corresponding 4′-*tert*-butyl-4-nitrosobiphenyl (**8**) (4′-*t*Bu-4-NOBp) are almost non-mutagenic even after activation with S9-mix [3]. In contrast, 3-*tert*-butyl-4-nitrosobiphenyl (**5**) (3-*t*Bu-4-NOBp), which is the corresponding nitroso compound of the non-mutagenic 3-*tert*-butyl-4-nitrobiphenyl (**4**) (3-*t*Bu-4-NBp), is highly mutagenic [2]. Hence, different reasons seem to be responsible for mutagenicity, or its suppression, in the two cases. Therefore, we prepared the corresponding hydroxylamines, 4′-*tert*-butyl-4-hydroxylaminobiphenyl (**9**) (4′-*t*Bu-4-NOHBp) and 3-*tert*-butyl-4-hydroxylaminobiphenyl (**6**) (3-*t*Bu-4-NOHBp), as further examples for modification of the directly acting mutagens with a sterical disturbance near the functional group, and far away from it, respectively. We compared their mutagenicity with the activity of their nitroso and their nitro analogues in order to get further insight into the role of the reduction processes. For comparison, we also tested the corresponding non-alkylated hydroxylamines, nitroso and nitro compounds of 4-nitrobiphenyl (**1**–**3**) and some other important mutagenic nitroarenes, i.e. of fluorene (**10**–**12**) and naphthalene (**13** and **14**), which have already been shown to be mutagenic by McCann et al. [7] (Fig. 1).

2. Materials and methods

2.1. Instrumentation

NMR spectra were recorded on a Bruker spectrometer ARX200 and are referenced to tetramethylsilane as an internal standard. Mass spectra were obtained on a Varian MAT CH-7-A (EI, 70 eV). Elementary analyses were recorded by a Heraeus rapid Elementaranalysator. The melting points are uncorrected.

2.2. Chemicals

The aromatic nitro compounds **4** and **7** and the nitroso compounds **2**, **5**, **8** were synthesised according to Klein et al. [2,3], and 2-nitrosofluorene (**11**) was prepared in analogy to that procedure. 4-Nitrobiphenyl (**1**), 2-nitrofluorene (**10**) and 2-nitronaphthalene were purchased from Aldrich chemicals. The hydroxylamines **3**, **6**, **9** and **12** were prepared by reduction of **1**, **4**, **7** and **10**, respectively, with ammonium chloride and zinc dust. The hydroxylamine **14** and the nitroso compound **13** were prepared according to literature procedures [9,10]. All samples that were tested in the Ames assay were repeatedly purified by recrystallisation until they contained no more impurities as judged by TLC and NMR.

2.2.1. 4-Hydroxylaminobiphenyl (4-NHOHBp) (3)

A suspension of 4-nitrobiphenyl (**1**) (10 g, 50.2 mmol) and ammonium chloride (4.6 g, 86.0 mmol) in 200 ml methanol and 20 ml water was preheated to 50°C. Zinc dust (15 g, 229 mmol) was then added in small portions over a period of 2 min. The mixture was vigorously stirred. After 1 min the suspension started boiling, and after further 5 min of stirring the mixture was quickly filtered and poured on ice. The precipitate was extracted with ether, washed with water and brine, dried over magnesium sulphate and evaporated to dryness. The crude product, which contained some amine, was then recrystallised from trichloromethane to yield pure **3** (4.61 g, 48%). Pale yellow plates, ^{1}H-NMR ($CDCl_3$, 200 MHz): $\delta = 8.40$ (s, 2H, NHOH), 7.60–7.28 (m, 7H, H_{arom}), 6.92 (d, 2H, $^{3}J = 8.3$ Hz, H3). ^{13}C-NMR ($CDCl_3$, 50 MHz): $\delta = 151.8$, 140.6, 131.2, 129.0, 127.0, 126.4, 126.0, 113.5. MS (70 eV): *m*/*z* (%): 185 (M^+, 67), 168 (100),

1	4-Nitrobiphenyl (92-92-3)[a]	4-NBp	X=NO_2
2	4-Nitrosobiphenyl (10125-76-5)	4-NOBp	X=NO
3	4-Hydroxylaminobiphenyl (6810-26-0)	4-NHOHBp	X=NHOH
4	3-*tert*-Butyl-4-nitrobiphenyl (70634-28-5)[a]	3-*t*Bu-4-NBp	X=NO_2
5	3-*tert*-Butyl-4-nitrosobiphenyl (-)	3-*t*Bu-4-NOBp	X=NO
6	3-*tert*-Butyl-4-hydroxylaminobiphenyl (-)	3-*t*Bu-4-NHOHBp	X=NHOH
7	4'-*tert*-Butyl-4-nitrobiphenyl (-)[a]	4'-*t*Bu-4-NBp	X=NO_2
8	4'-*tert*-Butyl-4-nitrosobiphenyl (-)	4'-*t*Bu-4-NOBp	X=NO
9	4'-*tert*-Butyl-4-hydroxylaminobiphenyl (-)	4'-*t*Bu-4-NHOHBp	X=NHOH
10	2-Nitrofluorene (607-57-8) [a]	2-NF	X=NO_2
11	2-Nitrosofluorene (2508-20-5)	2-NOF	X=NO
12	2-Hydroxylaminofluorene (53-94-1)	2-NHOHF	X=NHOH
13	2-Nitrosonaphthalene (6610-08-8)	4-NONp	X=NO
14	2-Hydroxylaminonaphthalene (613-47-8)	4-NHOHNp	X=NHOH

Fig. 1. Compounds studied in this work (**2**, **3**, **5**, **6**, **8**, **9**, **11**, **12**, **13**, **14**) together with others used for comparison (**1**, **4**, **7**, **10**). Letter 'a' in superscript indicates mutagenicity data taken from [2] and [3].

152 (49), 141 (53), 115 (36). Calculated: C 77.81, H 5.99, N 7.56; found: C 77.74, H 5.95, N 7.37.

*2.2.2. 2-Hydroxylaminofluorene (2-NHOHF) (***12***)*

This compound was prepared from **10** in analogy to the procedure described for **3**.

Yellow crystals, ^{1}H-NMR ($CDCl_3$, 200 MHz): $\delta = 8.43$ (s, br, 2H, NHOH), 7.75–7.67 (m, 2H, H5, H8), 7.51 (d, 1H, $^3J = 7.4$ Hz, H4), 7.33 (dd, 1H, $^3J = 7.5$ Hz, $^3J = 7.5$ Hz, H6), 7.24 (dd, 1H, $^3J = 7.5$ Hz, $^3J = 7.5$ Hz, H7), 7.10 (s, 1H, H1), 6.89 (d, 1H, $^3J = 7.5$ Hz, H3), 3.85 (s, 2H, CH_2). ^{13}C-NMR (DMSO, 50 MHz): $\delta = 151.9$, 144.1, 142.3, 141.9, 133.1, 126.8, 125.3, 125.0, 120.2, 118.8, 112.1, 109.8, 36.6. Calculated: C 79.16, H 5.62, N 7.10; found: C 78.98, H 5.62, N 7.11.

*2.2.3. 3-tert-Butyl-4-hydroxylaminobiphenyl (3-tBu-4-NOHBp) (***6***)*

To a solution of 3-*tert*-butyl-4-nitrobiphenyl (**4**) (0.3 g, 1.17 mmol) in 10 ml diethyl ether was added ammonium chloride (0.15 g, 2.8 mmol) and then water until all solid had dissolved. The two-phase system was vigorously stirred and charged with zinc dust (0.8 g, 12.2 mmol). The reaction was monitored by TLC (diethyl ether/hexanes 1:2). When

the reaction had finished the reaction mixture was partitioned between 20 ml diethyl ether and 20 ml water. The aqueous phase was repeatedly extracted with ether and the combined organic layer was dried over magnesium sulphate and carefully evaporated to dryness. The crude product, which contained some amine, was then recrystallised from a mixture of ether and hexanes at −50°C to yield **6** (112 mg, 40%).

Colourless crystals, mp 75–77°C (dec.). ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 7.45–7.15 (m, 7H, H_{arom}), 6.60 (d, 1H, ^3J = 8.0 Hz, H3), 3.61 (s, br, 2H, NHOH), 1.36 (s, 9H, $-C(CH_3)_3$). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 144.0, 141.8, 133.8, 131.5, 128.6, 126.5, 126.1, 125.6, 125.6, 118.1, 34.4, 29.6. MS (70 eV): *m/z* (%): 225(M^+ –OH, 86), 210 (100), 195 (14), 182 (28), 178 (18), 169 (21), 126 (25). Calculated: C 79.63, H 7.94, N 5.80; found: C 79.44, H 8.00, N 6.16.

2.2.4. 4′-tert-Butyl-4-hydroxylaminobiphenyl (4′-tBu-4-NOHBp) (**9**)

This compound was prepared from **7** in analogy to the procedure described for **6**.

Colourless crystals, ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 7.60–7.42 (m, 6H, H_{arom}), 7.05 (d, 2H, ^3J = 8.6 Hz, H2), 6.01 (s, br, 2H, NHOH), 1.35 (s, 9H, $-C(CH_3)_3$). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 147.7, 146.7, 135.9, 133.2, 125.5, 124.3, 123.7, 113.0, 32.5, 29.4. MS (70 eV): *m/z* (%): 225 (M^+ –OH, 80), 210 (100), 194 (14), 182 (13), 69 (11). Calculated: C 79.63, H 7.94, N 5.80; found: C 79.44, H 8.04, N 5.55.

2.2.5. 2-Hydroxylaminonaphthalene (2-NHOHNp) (**14**)

This compound was prepared according to [9].

Colourless crystals, mp 135–137°C (dec.). Calculated: C 75.45, H 5.70, N 8.80; found: C 75.12, H 5.54, N 9.12.

2.2.6. 4-Nitrosobiphenyl (4-NOBp) (**2**)

This compound was prepared according to [2].

Green crystals, mp 74°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 7.97 (d, 2H, ^3J = 8.6 Hz, H2), 7.81 (d, 2H, ^3J = 8.6 Hz, H3), 7.64−7.46 (m, 5H, H_{arom}). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 165.0, 148.1, 139.1, 129.1, 128.9, 127.8, 127.5, 121.6. MS (70 eV): *m/z* (%): 183 (M^+, 85), 169 (19), 153 (100), 128 (18), 102 (11), 77 (15), 28 (68).

2.2.7. 2-Nitrosofluorene (2-NOF) (**11**)

This compound was prepared in analogy to the oxidation procedure described in [2] using $Mo[O_2]_2O{\cdot}HMPT{\cdot}H_2O$.

Green crystals, mp 77°C. ^{1}H-NMR ($CDCl_3$, 200 MHz). δ = 8.21 (d, 1H, ^3J = 8.0 Hz, H3), 7.97 (d, 1H, ^3J = 8.0 Hz, H4), 7.96–7.87 (m, 1H), 7.79 (s, 1H, H1), 7.64–7.54 (m, 1H, H_{arom}), 7.48–7.41 (m, 2H, H_{arom}). 4.00 (s, 2H, CH_2). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 166.0, 148.5, 145.8, 143.7, 139.6, 128.9, 127.4, 125.4, 124.4, 121.7, 120.1, 115.4, 37.0. Calculated: C 79.98, H 4.65, N 7.17; found: C 79.94, H 4.79, N 7.00.

2.2.8. 4′-tert-Butyl-4-nitrosobiphenyl (4′-tBu-4-NOBp) (**8**)

This compound was prepared according to [3].

Dark green crystals, mp 109°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 7.95 (d, 2H, ^3J = 8.6 Hz, H2), 7.81 (d, 2H, ^3J = 8.6 Hz, H3), 7.62 (d, 2H, ^3J = 8.6 Hz, H2′), 7.51 (d, 2H, ^3J = 8.7 Hz, H3′), 1.37 (s, 9H, *t*Bu). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 165.0, 152.3, 147.9, 136.1, 127.5, 127.1, 126.1, 121.7, 34.7, 31.2. Calculated: C 80.30, H 7.16, N 5.85; found: C 79.95, H 7.13, N 5.73.

2.2.9. 3-tert-Butyl-4-nitrosobiphenyl (3-tBu-4-NOBp) (**5**)

This compound was prepared according to [2].

Green crystals, mp 87°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 7.88 (s, 1H, H5), 7.60–7.31 (m, 6H, H_{arom}), 6.08 (d, 1H, ^3J = 8.4 Hz, H2), 1.82 (s, 9H, *t*Bu). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 164.6, 152.9, 147.7, 139.9, 129.0, 128.6, 127.4, 126.5, 124.5, 107.4, 37.1, 33.4. Calculated: C 80.30, H 7.16, N 5.85; found: C 80.01, H 7.32, N 5.65.

2.2.10. 2-Nitrosonaphthalene (2-NONp) (**13**)

This compound was prepared according to [10].

Grey crystals (green melt), mp 62°C. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ = 9.49 (s, 1H, H1), 8.27 (d, 1H, ^3J = 8.8 Hz, H3), 7.85–7.64 (m, 4H, H_{arom}), 6.98 (d, 1H, ^3J = 8.8 Hz, H4). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ = 163.7, 137.3, 136.4, 132.9, 131.0, 130.5, 129.0, 128.1, 127.7, 108.2.

2.3. Mutagenicity tests

The compounds were tested as previously described [2] at the Hygiene-Institut of the University of Heidelberg, Germany, following the standard procedure described by Maron and Ames [8]. The tests were carried out three times per sample and the resulting revertant numbers were averaged. The relation between the revertant number of a substrate and the negative control (pure DMSO or THF, respectively) was declared as the induction factor. The decrease of mutagenicity after the peak of induction that can be observed in most cases is due to a growing influence of cell toxicity at higher doses.

3. Results

3.1. Non-alkylated compounds

4NOBp (**2**) and 4NHOHBp (**3**), which have already been found to be directly mutagenic in *Salmonella typhimurium* TA8 [7], are directly acting mutagens in TA98 and TA100, see Table 1.

4-NBp (**1**) was earlier shown to be a weak direct mutagen in TA98 and TA100 [2,3]. Comparing the dose-response curves of **1**, **2** and **3**, we found the curves of 4-NOBp (**2**) to be very similar to those of 4-NHOHBp (**3**), but not to the results of 4-NBp (**1**). Especially in TA100, with and without S9-mix, there is a remarkable equality between the curves of 4-NOBp (**2**) and 4-NHOHBp (**3**), see Fig. 2. The tests in TA98 show comparable results, but cell toxicity is not as critical as in TA100.

In the case of the fluorene compounds **10**–**12** we also found the same behaviour in TA98 and TA100. Again, the dose-response curves of the hydroxylamine **12** and the nitroso compound **11** are very similar, while the nitro compound **10** behaves differently. High cell toxicity is found at higher doses, especially in the absence of S9-mix, see Fig. 3. A comparable similarity was furthermore found between 2-NONp (**13**) and 2-NHOHNp (**14**), see Table 1.

In all cases, the hydroxylamines and the nitroso compounds are mutagenic even at low concentrations, but the mutagenicity is seriously influenced by cytotoxicity already at moderate doses. The addition of metabolically activating S9-mix seems to have a decreasing effect on the cell toxicity at higher concentrations of the compound, especially in the case of the hydroxylamines **3** (see Fig. 2), **12** (see Fig. 3) and **14** (see Table 1).

Although the same results could be qualitatively determined in TA100 for the fluorene compounds **10**–**12** and the naphthalene derivatives **13** and **14**, the strong influence of cell toxicity does not allow any reliable quantitative statement in that tester strain.

3.2. Alkylated compounds

Regarding the mutagenic activity of 4′-*t*Bu-4-NOHBp (**9**), 3-*t*Bu-4-NOHBp (**6**), and their nitroso analogues **5** and **8**, we found considerable differences with regard to the position of the *tert*-butyl groups, see Table 2.

Although 3-*t*Bu-4-NBp (**4**) is not mutagenic at all, in the presence of S9-mix or without, the corresponding hydroxylamine **6** and nitroso compound **5** are both heavily mutagenic. The curves in TA98 are shown in Fig. 4. TA100 leads to comparable results.

It should be noted that the curves with and without S9-mix are almost the same. In both cases, cell toxicity is more important for the hydroxylamine **6**. In contrast to these results, the 4′-alkylated biphenyls **7**, **8** and **9** show a different behaviour, see Fig. 5.

Interestingly, in the case of the 4′-*tert*-butyl substituted compounds **8** and **9**, the mutagenicity is higher in the absence of S9-mix. Without S9-mix, the dose-response curve of the hydroxylamine **9** shows an almost linear increase, indicating that no cell toxicity influences the measurement up to a dose of 500 μg/50 μl. In contrast to the non-alkylated biphenyls **2** and **3**, the presence of S9-mix reduces the cytotoxic effect. The nitro compounds **4** and **7** do not show any cell toxicity, either.

4. Discussion

4.1. The reduction steps from the nitro group to the hydroxylamine

The dose-response curves of the hydroxylamines **3**, **6**, **9**, **12** and **14** and the corresponding nitroso compounds **2**, **5**, **8**, **11** and **13** are very similar to each other, whereas they are rather different from that of the

Table 1
Mutagenicity of the non-alkylated aromatic hydroxylamines **3**, **12**, **14** and the corresponding nitroso compounds **2**, **11** and **13**

	Compound	Dose (μg/plate)	Revertants			
			TA98		TA100	
			−S9	+S9	−S9	+S9
1[a]	4-NBp	20	96	131	347	488
		100	233	406	795	1285
		500	290	508	885	1294
	DMSO		37	67	207	206
	Positive control		2149[b]	1151[c]	902[d]	795[c]
3	4-NHOHBp	10	288	304	522	716
		20	629	674	502	1086
		50	882	1134	387	1003
		100	849	1176	305	425
		500	0 (tox)	506	0 (tox)	363
	DMSO		32	43	158	146
	Positive control		3136[b]	1628[c]	895[d]	2351[c]
2	4-NOBp	10	249	340	557	769
		20	516	778	311	1112
		50	513	1111	272	1005
		100	347	1104	167	531
		500	0 (tox)	614	0 (tox)	51
	DMSO		32	43	158	146
	Positive control		3136[b]	1628[c]	895[d]	2351[c]
10[a]	4-NF	10	615	809	365	520
		20	1512	1244	834	867
		50	1605	2196	0	1840
	THF		33	38	134	114
	Positive control		1794[b]	1428[c]	836[d]	1774[c]
12	2-NHOHF	10	225	1695	140	613
		20	122	1652	17	462
		50	38	888	0 (tox)	297
		100	0 (tox)	239	0 (tox)	0 (tox)
		500	0 (tox)	0 (tox)	0 (tox)	0 (tox)
	DMSO		25	39	160	164
	Positive control		3474[b]	2171[c]	1266[d]	2285[c]
11	2-NOF	10	329	2116	221	552
		20	355	1363	0 (tox)	562
		50	122	959	0 (tox)	596
		100	0 (tox)	798	0 (tox)	0 (tox)
		500	0 (tox)	0 (tox)	0 (tox)	0 (tox)
	DMSO		25	39	160	164
	Positive control		3474[b]	2171[c]	1266[d]	2285[c]
14	2-NHOHNp	10	257	209	792	503
		20	473	472	1198	899
		50	685	474	1378	791
		100	111	708	1192	0 (tox)
		500	0 (tox)	0 (tox)	0 (tox)	0 (tox)
	DMSO		25	39	160	164
	Positive control		3474[b]	2171[c]	1266[d]	2285[c]
13	2-NONp	10	569	218	746	1269
		20	896	481	1168	1017
		50	194	719	1340	371
		100	0 (tox)	923	893	0 (tox)
		500	0 (tox)	0 (tox)	0 (tox)	0 (tox)
	DMSO		25	39	160	164
	Positive control		3474[b]	2171[c]	1266[d]	2285[c]

[a] Mutagenicity data taken from [3].
[b] 1-Nitropyrene, 2.5 mg.
[c] 2-Aminoanthracene, 2.5 mg.
[d] Sodium azide, 5 mg.

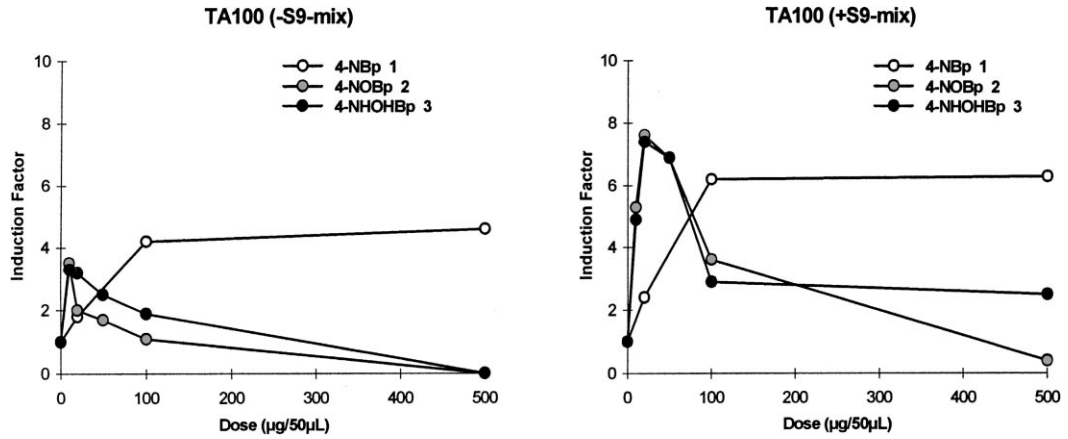

Fig. 2. Comparison of the dose-response curves of 4-nitrobiphenyl (**1**), 4-nitrosobiphenyl (**2**) and 4-hydroxylaminobiphenyl (**3**) in TA100. The tests were carried using the Ames-test [8] and the *Salmonella typhimurium* tester strains TA98 and TA100.

respective nitro compounds **1**, **4**, **7** and **10**. This effect was found in both tester strains, TA98 and TA100, and was shown to be independent of the addition of rat liver S9-mix, see Tables 1 and 2, and Figs. 1–5. Bulky alkyl groups far away from, or near to, the functional group NO_2, which we previously showed to carry out a remarkable influence on the mutagenic properties of aromatic nitro compounds [2,3], do not disturb that effect. The early decrease of mutagenicity induced by cell toxicity in the dose-response curves of the hydroxylamines **3** and **12** and the nitroso compounds **2** and **11**, compared to their corresponding nitro compounds **1** and **10**, shows that the reduction of the nitro group to the nitroso functionality is rather slow. Thus, the metabolically activated reduction procedure of nitro compounds obviously consists of a "difficult" reduction from the nitro to the nitroso species, followed by a more "easier" reduction from the nitroso to the hydroxylamine function. Interestingly, a similar observation has earlier been made in the 1-nitropyrene/1-nitrosopyrene/1-hydroxylaminopyrene system by Heflich et al. [11]. They found that 1-nitrosopyrene is at least 20-fold more mutagenic than 1-nitropyrene in *Salmonella typhimurium* TA1538, with the same

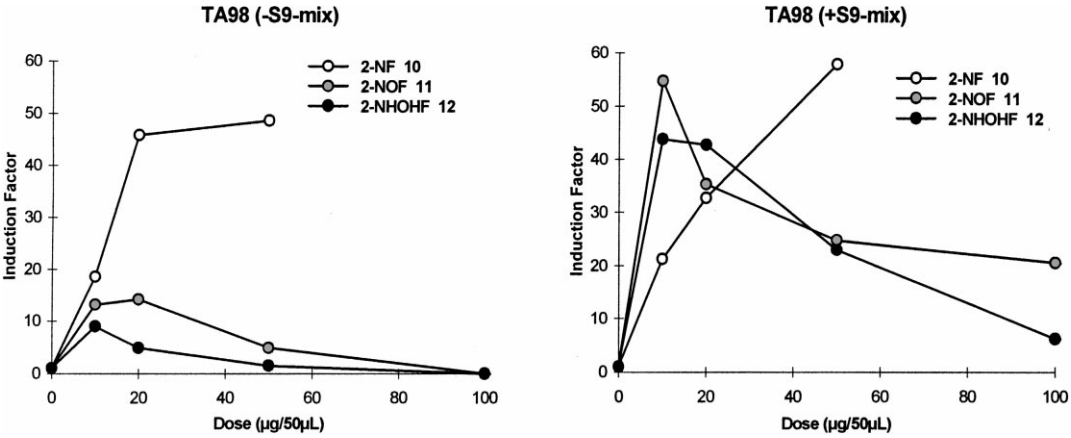

Fig. 3. Comparison of the dose-response curves of 2-nitrofluorene (**10**), 2-nitrosofluorene (**11**) and 2-hydroxlaminofluorene (**12**). The tests were carried using the Ames-test [8] and the *Salmonella typhimurium* tester strains TA98 and TA100.

Table 2
Mutagenicity according to the Ames assay [8] in *Salmonella typhimurium* TA98 and TA100 of the alkylated aromatic hydroxylamines **6** and **9** and the nitroso compounds **5** and **8**[a]

	Compound	Dose (μg/plate)	Revertants			
			TA98		TA100	
			−S9	+S9	−S9	+S9
4[b]	3-*t*Bu-4-NBp	20	31	44	117	131
		100	33	43	124	129
		500	39	43	139	188
	DMSO		30	45	116	117
	Positive control		830[c]	998[d]	864[e]	1033[d]
6	3-*t*Bu-4-NHOHBp	10	132	107	824	517
		20	375	358	1399	1315
		50	640	772	1729	1806
		100	780	1097	1825	1783
		500	442	883	1032	1290
	DMSO		25	39	160	164
	Positive control		3464[c]	2171[d]	1266[e]	2285[d]
5	3-*t*Bu-4-NOBp	10	134	95	679	489
		20	285	283	1202	1022
		50	439	541	1586	1745
		100	785	1162	2360	2099
		500	1146	1829	2050	1261
	DMSO		25	39	160	164
	Positive control		3474[c]	2171[d]	1266[e]	2285[d]
7[b]	4′-*t*Bu-4-NBp	20	45	62	206	209
		100	53	63	203	209
		500	50	68	210	227
	DMSO		37	67	207	206
	Positive control		2149[c]	1151[d]	902[e]	795[d]
9	4′-*t*Bu-4-NHOHBp	10	34	57	167	277
		20	35	65	191	360
		50	55	73	218	352
		100	92	108	258	368
		500	438	205	746	524
	DMSO		25	39	160	164
	Positive control		3474[c]	2171[d]	1266[e]	2285[d]
8	4′-*t*Bu-4-NOBp	10	42	50	220	314
		20	73	62	320	380
		50	86	56	407	446
		100	154	67	1061	546
		500	281	160	1707	978
	DMSO		25	39	160	164
	Positive control		3474[c]	2171[d]	1266[e]	2285[d]

[a] For comparison, the nitro compounds **4** and **7** are also added [2,3].
[b] Mutagenicity data taken from [2] and [3].
[c] 1-Nitropyrene, 2.5 mg.
[d] 2-Aminoanthracene, 2.5 mg.
[e] Sodium azide, 5 mg.

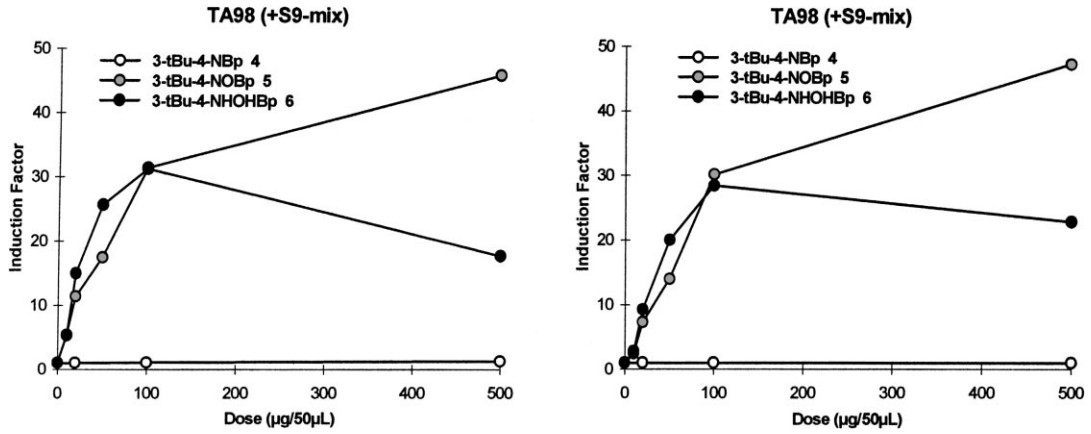

Fig. 4. Comparison of the dose-response curves in TA98 of 3-*t*butyl-4-hydroxylaminobiphenyl (**6**), with its nitroso (**5**) and nitro (**4**) analogues. The tests were carried using the Ames-test [8] and the *Salmonella typhimurium* tester strains TA98 and TA100.

deoxyguanosine-adduct being formed as from the corresponding hydroxylamine. These authors proposed the nitroreductase, which is responsible for the initial reduction of 1-nitropyrene, to be the rate-limiting factor. Howard et al. found that 1-nitropyrene is rapidly reduced to 1-aminopyrene by the strains TA98 and TA100 of *Salmonella typhimurium*, confirming that these tester strains are able to reduce aromatic nitro and nitroso compounds without metabolically activating S9-mix [12]. The ability to reduce these functional groups is dependent on the size of the aromatic system.

We have reported before that a bulky alkyl group close to the functional group of 4-NBp (**10**) disturbs the enzymatic reduction from the nitro to the nitroso compound, making the substrate non-mutagenic [2]. This is either due to a sterical inhibition of the nitroreductase by the bulky group, or it might be caused by an "out of plane" orientation of the nitro group with respect to the ring planarity [2]. However, the reduction of the nitroso compound **5** to the directly mutagenic hydroxylamine **6** is not at all influenced by the bulky group, and S9-mix does not change the dose-response curves, see Fig. 3. This means that the second

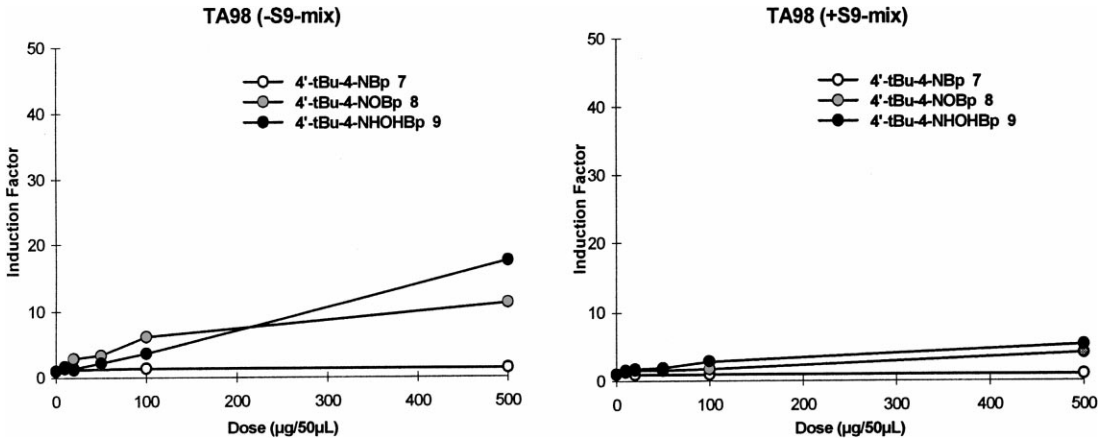

Fig. 5. Comparison of the dose-response curves in TA98 of 4′-*t*butyl-4-hydroxylaminobiphenyl (**9**) with its nitroso (**8**) and nitro (**7**) analogues. The tests were carried using the Ames-test [8] and the *Salmonella typhimurium* tester strains TA98 and TA100.

reduction step from the nitroso to the hydroxylamine functionality might be rather induced by some smaller non-enzymatic substance than by the interaction with a "larger" enzyme. This seems also to hold in the case of other nitro, nitroso and hydroxylamine compounds, see Table 1 and Fig. 3. Mutagenicity tests carried out by Wirth et al. support that hypothesis. These authors did not find a significant difference in the mutagenicity of 2-NOF (**11**) in either nitroreductase proficient TA98 nitroreductase deficient TA98FR [13].

*4.2. The influence of a bulky alkyl group in 4′-position of nitrobiphenyl (**1**)*

4′-*tert*-Butyl-4-nitrosobiphenyl (**8**) and 4′-*tert*-butyl-4-hydroxylaminobiphenyl (**9**) are much less mutagenic in TA98 and TA100 than their non-alkylated parent compounds **2** and **3**, see Fig. 5, although the sterical influence of the *tert*-butyl group is located far away from the functional group. Even in the absence of S9, **9** shows some mutagenic activity at higher concentrations. The loss of cell toxicity in the case of **9** might indicate a decreased effective dose of the substrate **9** in the cells. The interaction between the substrates and the cells could in some way be disturbed by the bulky substituent, probably during the penetration of the compound through the cell wall. As a consequence, the hydroxylamine **9** is degradated before larger amounts are able to reach the cell. Larger amounts of the hydroxylamine **9** within the cell would be likely to carry out at least some cytotoxic effect at higher concentrations. Addition of S9-mix decreased the mutagenicity because the hydroxylamine seems to be more quickly degraded to non-mutagenic by-products. What are the reasons for the 4′-alkylated nitro compound **7** to be non-mutagenic at all, see Fig. 5? First, the enzymatic reduction step from 4′-*t*Bu-4-NBp (**7**) to 4′-*t*Bu-4-NOBp (**8**) seems to be affected by the alkyl substituent, because **7** does not show any mutagenicity at all, even not on addition of S9-mix, whereas the hydroxylamine **9** and the nitroso analogue **8** both show at least some mutagenicity. Secondly, most of the hydroxylamine **9** seems to be degraded before it can pass the cell wall and form adducts with DNA. Besides that, there is the possibility that the attack of the hydroxylamine **9** on the DNA is sterically disturbed, or that DNA modified with **9** is harmless, because of some conformational difference to DNA which has formed an adduct with the non-alkylated biphenyl **3**. Thus, an exact explanation for the suppression of the mutagenicity of 4′-*t*Bu-4-NBp (**7**) cannot be given as yet. The analysis of reactions between the hydroxylamines **3** and **9**, respectively, and DNA, or *Salmonella* bacteria, might give some additional information. Appropriate in vitro experiments are under investigation.

4.3. Substitution of hydroxylamines by nitroso compounds in the Ames assay

Aromatic hydroxylamines are interesting substrates in the Ames assay, because they give important information about the mutagenic properties of intermediates arising from aromatic amines and nitro compounds after metabolic activation in vivo. Nevertheless, in most cases their preparation and their purification is not easy because aromatic hydroxylamines easily undergo disproportionation yielding the corresponding amine and the azoxy compound [14]. Nitrosoarenes can more easily be prepared from the amines by oxidation using a solution of $Mo[O_2]_2O{\cdot}HMPT{\cdot}H_2O$ in 30% H_2O_2 [15]. They are stable up to room temperature and can be purified using silica gel chromatography. Since their dose-response curves are very similar to that of the hydroxylamines without any exception, see compounds **2** and **3**, **5** and **6**, **8** and **9**, **11** and **12** in Figs. 2–5, the investigation of the nitroso compounds in the Ames assay might be worthwhile in order to get information about the corresponding hydroxylamine, if the latter is difficult or impossible to obtain.

In conclusion, we showed that the aromatic hydroxylamines **3**, **6**, **9**, **12**, **14** and their corresponding nitroso compounds **2**, **5**, **8**, **11**, **13** yield almost identical results in the Ames assay using TA98 and TA100 without any exception. Even bulky alkyl substitution (*tert*-butyl group) does not change that results, although it was previously shown that alkyl substitution has a remarkable influence on the mutagenicity of an aromatic nitro compound [2,3]. Thus, when a hydroxylamine is difficult to prepare, the corresponding nitroso compound might give a hint about the mutagenicity of the hydroxylamine. Furthermore, it was found that bulky alkyl groups like the *tert*-butyl group can interfere with the mutagenicity of 4-NBp (**1**) in at least two different ways, depending on the location

of that group in relation to the functional group. A bulky group close to the functionality (ortho-position) appears to disturb enzymatic reduction. When the alkyl substituent is located far away from the functional group, e.g. in the 4′-position of 4-nitrobiphenyl (**7**), the influence is not yet understood. However, our results suggest that a decrease in the effective concentration of the test compound, or a conformational difference of the adduct as compared to the non-alkylated adduct, might be responsible.

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