ELSEVIER

Dyes and Pigments 48 (2001) 197–207

DYES and PIGMENTS

www.elsevier.com/locate/dyepig

N-hydroxy-monomethoxy-4-aminoazobenzenes: a computational study

Krishna L. Bhat, Mendel Trachtman, Charles W. Bock *

Department of Chemistry, School of Science and Health, Philadelphia University, Philadelphia, PA 19144, USA

Received 7 September 2000; received in revised form 15 November 2000; accepted 4 December 2000

Abstract

Structures and electronic properties of the *N*-hydroxy metabolites (N–OH–OMe–AAB) of the monomethoxy-4-aminoazobenzene dyes (OMe–AAB) have been calculated using density functional theory with a basis set that includes polarization functions on all the atoms. Positional isomers with the methoxy group *ortho* to the hydroxyamino group (*N*–OH–3(5)-OMe–AAB) are found to be lower in energy than isomers with the methoxy group *meta* to the hydroxyamino group (N–OH–2(6)-OMe–AAB). The geometrical parameters of the phenyl rings for the corresponding isomers of OMe–AAB and N-OH–OMe–AAB are very similar. The length of the C–NHOH bond, however, is significantly longer than the length of the C–NH_2 bond. The energies of the frontier orbitals are more negative for each of the positional isomers of N–OH-OMe-AAB than for the corresponding isomer of OMe–AAB. In particular, the energy of the lowest unoccupied molecular orbital decreases a few kcal/mol for each isomer, suggesting that these *N*-hydroxy metabolites are slightly more electrophilic.

Keywords: Aminobenzene dyes; *N*-hydroxylation; Density functional theory

1. Introduction

Small structural changes can radically alter the carcinogenic behavior of aminoazo dyes [1], e.g. 3-methoxy-4-aminoazobenzene (3-OMe–AAB) is a potent hepatocarcinogen in rats and a mutagen in *Escherichia coli* and *Salmonella typhimurium*, whereas 2-methoxy-4-aminoazobenzene (2-OMe–AAB) is a non-carcinogen and a nonmutagen under similar conditions [2]. It is generally believed that these positional isomers of OMe–AAB require metabolic activation to their *N*-hydroxy derivatives, N–OH–3-OMe–AAB and N–OH–2-OMe–AAB, respectively, prior to reaction with cellular macromolecules [3]. This conclusion is in accord with the observation that 3-OMe–AAB is essentially nonmutagenic on the *Salmonella* mammalian mutagenicity assay (Ames test) unless it is activated with S-9, the 9000 g supernatant fraction of liver homogenate [4,5]. On the other hand, N–OH–3-OMe–AAB is strongly mutagenic without S-9 activation [4,5]. (It has also been observed that injecting rats with *N*–OH–3-OMe–AAB induced sarcoma at the injection site, whereas injecting 3-OMeO–AAB did not [6].) In contrast to the different behavior of 3-OMe–AAB and N–OH–2-OMe–AAB on the Ames test, neither 2-OMe–AAB nor

* Corresponding author. Tel.: +1-215-951-2876; fax: +1-215-951-6812.

E-mail address: chuck@larry.philau.edu (C.W. Bock).

0143-7208/01/$ - see front matter
PII: S0143-7208(00)00104-2

N–OH–2-OMe–AAB is mutagenic on this test even after treatment with S-9.

Hashimoto et al. [7] established that the ubiquitous cytochrome P-450 enzymes are responsible for the mutagenic activation of 3-OMe–AAB and that, in contrast to other carcinogenic aromatic amines, the activation is mediated by phenobarbital-P-450 (not by 3-methyl-cholanthrene-P-450). The precise mechanism by which these cytochrome P-450 enzymes catalyze the stereospecific *N*-hydroxylation of 3-OMe–AAB to N–OH–3-OMe–AAB at physiological temperature, however, is not completely understood [8,9]. Kojima et al. [2] studied the effects of N–OH–2-OMe–AAB and N–OH–3-OMe–AAB on DNA synthesis *in vivo* and determined that they both preferentially form guanine adducts under the action of seryl-tRNA synthetase (from yeast). Although neither the structure of these adducts nor the mechanism of their formation was determined, a ^{32}P-post labeling assay [10] revealed that N–OH–3-OMe–AAB gave 20-fold more DNA adducts than did N–OH–2-OMe–AAB. Furthermore, in DNA from rats treated with 3-OMe–AAB, five different adducts were observed, whereas only one adduct was apparent in the DNA of rats treated with 2-OMe–AAB [11]. These results suggest that the observed difference in the hepatocarcinogenic activity of 2-OMe–AAB and 3-OMe–AAB is related to the inhibitory effects of their *N*-hydroxy derivatives on DNA replication.

The only other monomethoxy AAB dye that has been studied in detail is 4′-OMe–AAB, which is carcinogenic, but to a lesser degree than 3-OMe–AAB. Interestingly, 4′-OMe-AAB is slightly mutagenic in TA98 even without S-9 activation [6]. It becomes more mutagenic in the presence of S-9, but with only about 7% of the revertants/nmol of dye found with 3-OMe–AAB under similar conditions; N–OH-4′-OMe–AAB is mutagenic without S-9 treatment, but with only about 24% of the revertants/nmol of dye as that observed for N–OH–3-OMe–AAB [9]. Although no data on the carcinogenic/mutagenic behavior of 2′- or 3′-OMe–AAB or their *N*-hydroxy derivatives are currently available, both 2′- and 3′-OMe–4-dimethylaminoazobenzene are significant liver carcinogens in the rat [12]; unfortunately, detailed results of mutagenicity tests on these compounds have not been reported.

A recent computational paper [13] showed that there are electronic and structural features of the OMe–AAB positional isomers that correlate with their carcinogenic behavior: (1) the Kohn–Sham highest occupied molecular orbital (HOMO) of 3-OMe–AAB involved the amino nitrogen lone pair of electrons and the next highest occupied orbital (HOMO{-1}) was localized in the vicinity of the azo bond lone pairs; for 2-OMe–AAB, the order of the corresponding orbitals was reversed; (2) the Kohn–Sham orbital energies of the HOMO and the lowest unoccupied molecular orbital (LUMO) of 3-OMe–AAB are *lower* in energy than those of 2-OMe–AAB; (3) the dipole moment of 3-OMe–AAB is smaller than that of 2-OMe–AAB; (4) the C_ϕ–OMe bond length is longer in 3-OMe–AAB than in 2-OMe–AAB, whereas the C_4–NH_2 bond length is shorter.

The purpose of the present paper is to describe the results of a computational study of the *N*-hydroxy metabolites of OMe–AAB at the same computational level employed to study OMe–AAB [13]. The lone pairs of electrons on the hydroxy oxygen atom in N–OH–OMe–AAB provide yet another site for delocalization and interactions that can influence the structure and behavior of these molecules. It is important to note that such *N*-hydroxy derivatives are not usually the ultimate carcinogenic forms of aromatic amines under physiological conditions (very few *N*-hydroxy derivatives of aromatic amines bind to electron-rich biotargets in vitro [14,15]), but are proximate forms that need to be further metabolized [16]. This study will determine the extent to which structural and electronic properties of the positional isomers of OMe–AAB are affected by *N*-hydroxylation.

2. Computational methods

Density functional theory calculations were performed at the BP/DN** computational level with SPARTAN version 5.0 [17]. This level uses the non-local Becke-Perdew (BP) 86 functional and employs the DN** basis set which includes polarization functions on all the atoms [18,19]. Complete optimizations for a variety of conformers of each N–OH–OMe–AAB derivative

were carried out; no symmetry constraints were employed to minimize the likelihood of optimizing to a transition state. In few cases, frequency analyses were performed to ensure that the optimized structures were local minima on the potential energy surfaces. Mulliken and electrostatic charges were also calculated. For comparison, a few optimizations were carried out at the B3LYP/6-31+G* computational level [20] using the GAUSSIAN 98 series of programs [21].

3. Results and discussion

Since no experimental structural data is available on *N*-hydroxy azo dyes, we optimized N–OH–AAB to compare with our calculated structures of AB and AAB [13]. The two lowest-energy conformers we found are shown as structures **1** and **2** in Fig. 1; conformer **2** is about 0.8 kcal/mol higher in energy than conformer **1**, see Table 1. Comparing the structural parameters of AB, AAB and N–OH–AAB in Table 2, it is evident that the amino and hydroxyamino groups induce similar distortions in the phenyl ring parameters of AB. On the other hand, the C_4–N bond length in each of the two conformers of N–OH–AAB, 1.405 and 1.404, is much longer than in AAB, 1.388 Å, i.e. the hydroxyl group withdraws electron density from the C_4–N bond and reduces its double bond character. To compensate for this loss of bonding at C_4, there is a small increase in the double bond character of one of the two carbon–carbon ring bonds involving this carbon atom. The C–C bond in the ring that is shortened most is *syn* to the hydroxy group (C_4–C_5 for **1**, C_3–C_4 for **2**). It should also be noted that the N–O bond length in conformers **1** and **2** of N–OH–AAB, 1.441 and 1.438 Å respectively, is much shorter than its length, 1.468 Å, in hydroxylamine, suggesting that the N–O bond in N–OH–AAB has some double bond character. The shift in electron density out of the C_4–N bond results in an increase in the pyramidal structure of the hydroxyamino group in both conformers of N–OH–AAB, as compared to the pyramidal structure of the amino group in AAB; the sum of the three bond angles about the amino nitrogen atom are 332.5° and 346.7°, respectively. This increase in the tendency of *N*-pyramidalization as a result of *N*-substitution by

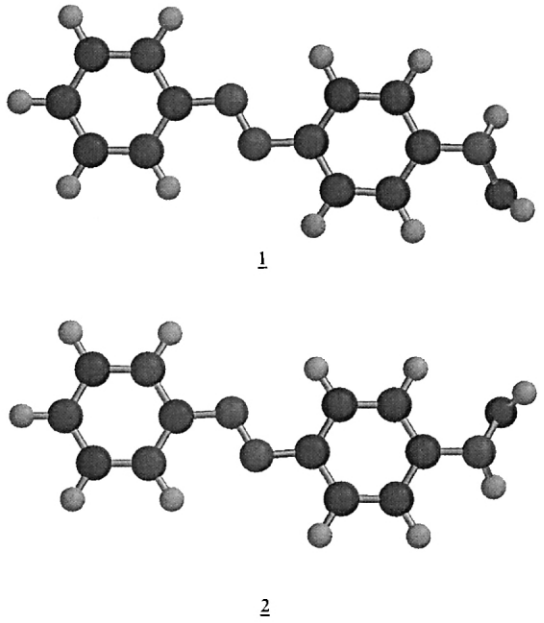

Fig. 1. BP/DN**//BP/DN** optimized structures of conformers **1** and **2** of N–OH–AAB.

Table 1
Total molecular energies (a.u.) and relative energies (kcal/mol) for *N*-hydroxy-*n*-methoxy-4-aminoazobenzenes calculated at the BP/DN**//BP/DN** computational level

n	Total molecular energies (a.u.) (BP/DN**//BP/DN**)	Relative energies (kcal/mol)
2	−818.135537	+6.2
3	−818.145385	0.0
5	−818.143086	+1.4
6	−818.140274	+3.2
2′	−818.140071	+3.3
3′	−818.143644	+1.1
4′	−818.144311	+0.7
5′	−818.143837	+1.0
6′	−818.135106	+6.5
N–OH–AAB (**1**)	−703.569992	0.0
N–OH–AAB (**2**)	−703.568693	+0.8
AAB	−628.365269	–
AB	−572.784293	–
H_2O	−76.471207	–
H_2O_2	−151.636915	–
NH_3	−56.588906	–
NH_2OH	−131.791414	–

Table 2
Structural parameters [bond lengths (Å), bond angles (°)] for AB, AAB, N–OH–AAB, and N–OH–*n*-OMe–AAB calculated at the BP/DN**//BP/DN** computational level

n	C_1–C_2	C_2–C_3	C_3–C_4	C_4–C_5	C_5–C_6	C_6–C_1	C_4–N	C_1–N	N=N	O–H	
2	1.438	1.402	1.402	1.409	1.381	1.411	1.405	1.394	1.276	0.974	
3	1.415	1.383	1.427	1.400	1.394	1.402	1.397	1.404	1.275	0.975	
5	1.406	1.388	1.404	1.419	1.387	1.410	1.397	1.403	1.274	0.974	
6	1.409	1.381	1.412	1.402	1.403	1.425	1.405	1.400	1.276	0.974	
2′	1.410	1.388	1.410	1.408	1.389	1.407	1.406	1.408	1.276	0.974	
3′	1.410	1.384	1.408	1.408	1.391	1.406	1.403	1.409	1.273	0.974	
4′	1.410	1.386	1.413	1.403	1.394	1.405	1.407	1.412	1.276	0.974	
5′	1.412	1.384	1.415	1.403	1.392	1.405	1.404	1.408	1.274	0.975	
6′	1.411	1.382	1.413	1.404	1.394	1.404	1.407	1.416	1.276	0.974	
N–OH–AAB (**1**)	1.411	1.386	1.416	1.405	1.390	1.406	1.405	1.406	1.273	0.974	
N–OH–AAB (**2**)	1.409	1.385	1.411	1.409	1.391	1.408	1.404	1.405	1.274	0.974	
AAB[a]	1.412	1.384	1.416	1.411	1.390	1.406	1.388	1.405	1.274	–	
	(1.410)	(1.385)	(1.414)	(1.408)	(1.390)	(1.404)	(1.389)	(1.408)	(1.262)		
AB	1.409	1.390	1.405	1.399	1.396	1.406	–	1.417	1.270	–	

n	$C_{1'}$–N	$C_{1'}$–$C_{2'}$	$C_{2'}$–$C_{3'}$	$C_{3'}$–$C_{4'}$	$C_{4'}$–$C_{5'}$	$C_{5'}$–$C_{6'}$	$C_{6'}$–$C_{1'}$	C_ϕ–O	O–CH_3	Σ N_α[b]	N–O
2	1.418	1.405	1.396	1.400	1.403	1.391	1.410	1.355	1.436	331.5	1.437
3	1.417	1.406	1.396	1.399	1.403	1.393	1.409	1.378	1.435	335.1	1.433
5	1.412	1.404	1.394	1.400	1.405	1.390	1.409	1.377	1.434	332.7	1.439
6	1.412	1.405	1.395	1.399	1.404	1.392	1.409	1.361	1.434	332.8	1.436
2′	1.408	1.425	1.404	1.396	1.400	1.391	1.408	1.363	1.432	333.0	1.438
3′	1.411	1.407	1.396	1.404	1.398	1.395	1.404	1.372	1.432	332.0	1.436
4′	1.406	1.403	1.395	1.402	1.412	1.385	1.412	1.368	1.434	331.1	1.441
5′	1.414	1.402	1.399	1.391	1.410	1.394	1.410	1.374	1.432	332.9	1.437
6′	1.399	1.410	1.389	1.397	1.398	1.404	1.432	1.358	1.433	331.4	1.438
N-OH-AAB (**1**)	1.417	1.405	1.396	1.399	1.404	1.391	1.408	–	–	332.5	1.441
N–OH–AAB (**2**)	1.413	1.405	1.396	1.399	1.403	1.390	1.407			332.3	1.438
AAB[b]	1.416	1.406	1.397	1.399	1.404	1.392	1.409	–	–	346.7	–
	(1.419)	(1.403)	(1.396)	(1.398)	(1.402)	(1.392)	(1.408)			(346.7)	
AB	1.417	1.406	1.396	1.399	1.405	1.390	1.404	–	–	–	–

[a] The values in parentheses are at the B3LYP/6-31 + G*//B3LYP/6-31 + G* level.
[b] Sum of three bond angles at the amine nitrogen atom.

electronegative heteroatom is consistent with earlier observations made by Mislow et al. [22] on substituted hydroxylamines. The particular orientation of the atoms in the –N–O–H linkage in N–OH–AAB (as well as in NH_2OH) reduces the interaction between the lone pair of electrons on the nitrogen atom and the in-plane lone pair on the oxygen atom.

The calculated gas-phase reaction energy for the conversion of AAB to N–OH–AAB using hydrogen peroxide as the oxidizing agent,

$$\mathrm{AAB} + \mathrm{H_2O_2} \rightarrow \mathrm{N{-}OH{-}AAB} + \mathrm{H_2O} \qquad (1)$$

is −24.5 and −23.7 kcal/mol for conformers **1** and **2** respectively; the calculated energy change for the reaction $NH_3 + H_2O_2 \rightarrow NH_2OH + H_2O$ is similar, −23.1 kcal/mol.

In Fig. 2 we show several Kohn–Sham orbitals for conformer **1** of N–OH–AAB; the structures of these orbitals are analogous to those of AAB. The HOMO in N–OH–AAB is primarily a lone pair orbital localized at the azo linkage, 4 kcal/mol above the next highest occupied orbital (HOMO{-1}); the corresponding energy separation in AAB is 3 kcal/mol. The HOMO{-1} in N–OH–AAB involves the hydroxyamino nitrogen atom lone pair, but for the AB backbone, it is essentially a *pi*-bonding

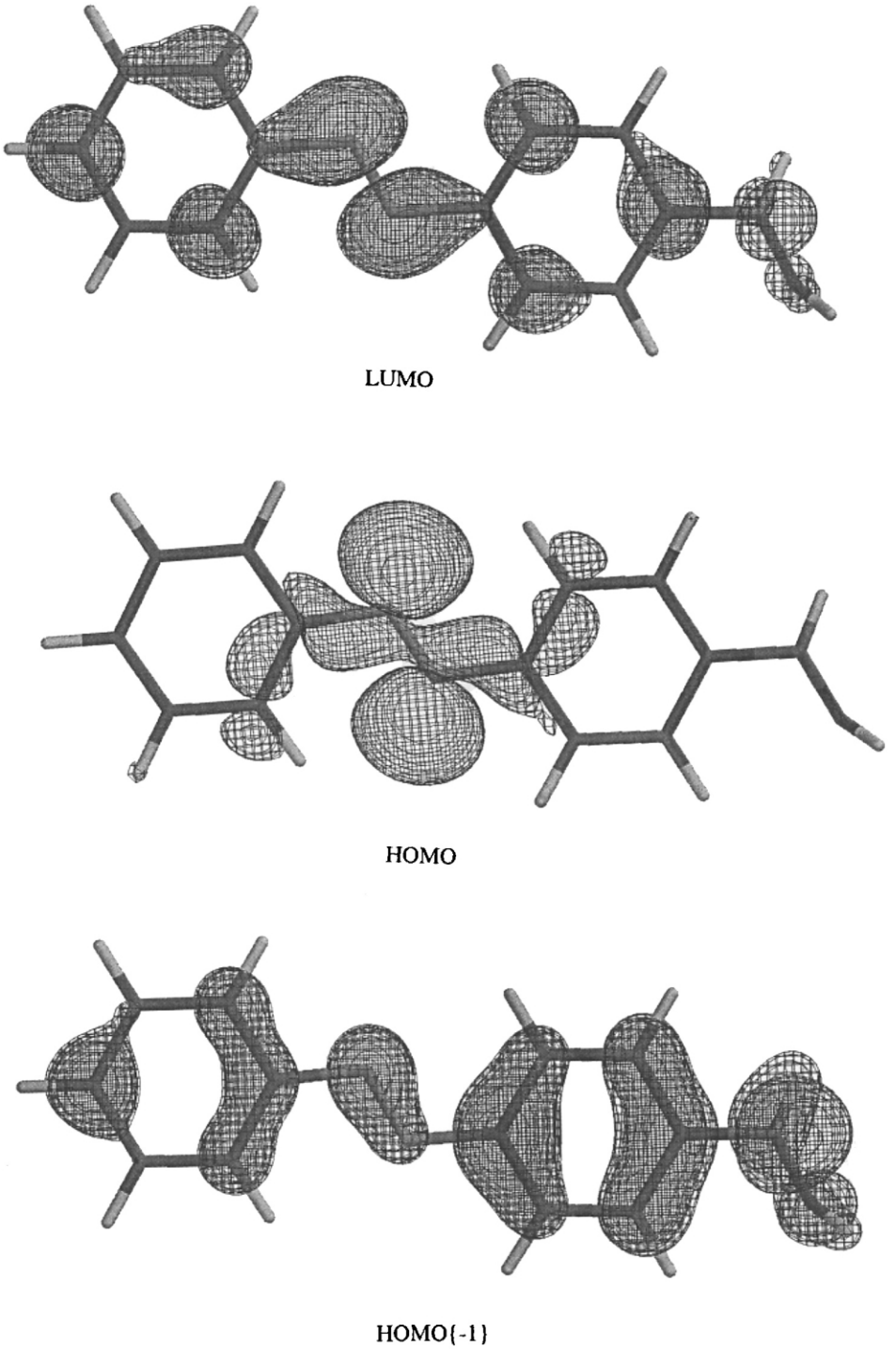

Fig. 2. The Kohn–Sham HOMO{-1}, HOMO and LUMO of conformer **1** of N–OH–AAB calculated at the BP/DN**//BP/DN** computational level.

orbital. The LUMO in N–OH–AAB is *pi*-antibonding for this backbone. Replacing one of the hydrogen atoms in the amino group of AAB by a hydroxy group *lowers* the energy of the Kohn–Sham frontier orbitals, e.g. the LUMO, HOMO and HOMO{-1} of structure **1** of N–OH–AAB are 3.5, 2.9 and 4.5 kcal/mol lower in energy than the corresponding orbitals of AAB. The lower LUMO energy of N–OH–AAB compared to that of AAB suggests that this N-hydroxy metabolite is slightly more electrophilic [23]. Leão and Pavão [24] have recently demonstrated computationally that it is the more electrophilic metabolites of a carcinogenic molecule that are responsible for its biological activity with DNA [25]. Similar conclusions have also been drawn experimentally [26]. There is evidence that N–OH–AAB is slightly mutagenic on *Salmonella typhimurium* TA98 without S-9 activation, whereas AAB is not [3].

For the various positional isomers of N–OH–OMe–AAB, conformers in which the O–Me bond is nearly in the plane of the phenyl ring to which it is bonded are lower in energy than those in which the O–Me bond is oriented nearly perpendicular to the phenyl ring. The specific direction of the methyl group in the various positional isomers of N–OH–OMe–AAB is similar to that for the corresponding OMe–AAB compounds [13]. Since the amino hydrogen atoms are not equivalent in any of the *n*-OMe–AAB isomers, we optimized separately structures in which each amino hydrogen atom was replaced by a hydroxy group. In Tables 1–3, we report energies, structural parameters, and properties only for the lowest-energy forms of N–OH–OMe–AAB that we found at the BP/DN** level.

In most of the experimental literature on OMe–AAB and N–OH–OMe–AAB, no distinction is made between substitution at the 3 and 5 (2 and 6, 3′ and 5′, or 2′ and 6′) positions, see Chart 1A. However, these pairs of compounds are distinct with different electronic and structural properties [13], e.g. adverse lone pair interactions in 2-OMe–AAB make this compound nearly 3 kcal/mol higher in energy than 6-OMe–AAB [13]. Thus, when it is reported in the literature that 2-OMe–AAB is a noncarcinogen, it is not clear if this refers to 2-OMe–AAB or 6-OMe–AAB, or to a

Table 3
Selected properties for AB, AAB, N–OH–AAB and N–OH–*n*-OMe–AAB calculated at the BP/DN**//BP/DN** computational level

n	HOMO{-1} (a.u.)	HOMO (a.u.)	LUMO (a.u.)	Log P[d]	Electrostatic charge on			Dipole moment (D)
					N	O(Me)	O(H)	
2	−0.190880[a]	−0.175956[b]	−0.105674	1.89	−0.53	−0.27	−0.39	4.05
3	−0.190449[c]	−0.189757[c]	−0.113902	1.83	−0.47	−0.25	−0.38	2.80
5	−0.189639[b]	−0.188680[a]	−0.114688	1.92	−0.49	−0.29	−0.38	2.79
6	−0.189450[a]	−0.182742[b]	−0.110815	1.89	−0.52	−0.30	−0.39	3.91
2′	−0.188980[a]	−0.183039[b]	−0.111980	1.80	−0.55	−0.34	−0.39	1.60
3′	−0.194850[a]	−0.190768[b]	−0.115882	1.86	−0.55	−0.35	−0.39	3.19
4′	−0.188308[a]	−0.187042[b]	−0.108944	1.83	−0.55	−0.30	−0.39	1.86
5′	−0.196121[c]	−0.190769[c]	−0.115556	1.83	−0.54	−0.31	−0.39	3.18
6′	−0.188431[a]	−0.177544[b]	−0.107264	1.86	−0.55	−0.34	−0.40	0.91
N–OH–AAB (**1**)	−0.199456[a]	−0.192288[b]	−0.117499	2.03	−0.57	–	0.39	2.36
N–OH–AAB (**2**)	−0.198135[a]	−0.191581	−0.117278		−0.54	–	0.39	2.55
AAB	−0.192206[a]	−0.187648[b]	−0.111910	2.47	−0.72	–	–	3.61
AB	−0.222337[a]	−0.199380[b]	−0.126611	3.30	–	–	–	0.07

[a] Orbital involves amino nitrogen lone pair.
[b] Orbital involves azo bond lone pairs.
[c] Orbital is mixed, see text.
[d] Log P is the logarithm of the octanol–water partition coefficient calculated using the Dixon–Hehre algorithm in Spartan 5.0 [17]. This involves explicit evaluation of $AM1_{oct}$ and $AM1_{aq}$ solvation models. The Ghose–Crippen approach gives log $P = 3.52$ for all the N–OH–*n*-OMe–AAB isomers and 3.64 for N–OH–AAB [28].

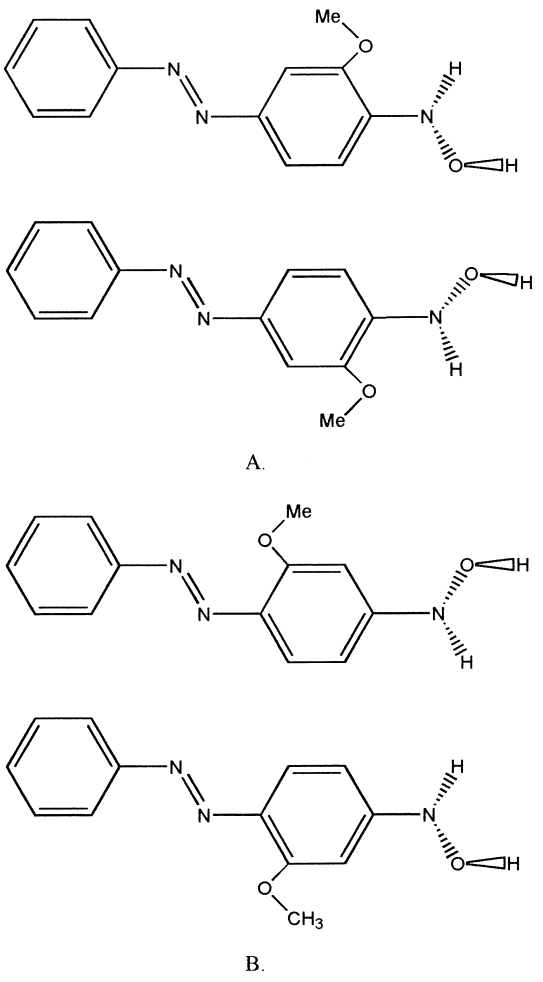

Chart 1.

mixture of these isomers. Experimental structural data on monomethoxy AAB derivatives are clearly needed.

3.1. N–OH–3-OMe–AAB and N–OH–5-OMe–AAB

As can be seen from Table 1, the highly mutagenic compound N–OH–3-OMe–AAB has the lowest total molecular energy among all the positional isomers; the other *ortho* derivative, N–OH–5-OMe–AAB, is only 1.4 kcal/mol higher in energy. This is similar to what we observed for the parent 3- and 5-OMe–AAB azo dyes [13]. For N–OH–3(5)-OMe–AAB the structure of the lowest-energy conformer has the –OH and –OMe groups positioned on opposite sides of the C_4–N bond, see Chart 1A. This arrangement keeps the two negatively charged oxygen atoms further apart.

Many of the structural parameters of N–OH–3(5)-OMe–AAB are very similar to those of 3(5)-OMe–AAB [13]. The C_4–N bond length, however, is some 0.015 Å *longer* in the *N*-hydroxy isomers, and this leads to an increase in the pyramidal structure of the hydroxyamino group compared to that of the amino group; the sum of the three bond angles involving the nitrogen atom is some 14° less in N–OH–3(5)-OMe–AAB than in 3(5)-OMe–AAB. The C_4–N bond length for other positional isomers of N–OH–OMe–AAB are even longer than that found for the 3(5)-isomers, see Table 2. Interestingly, the calculated electrostatic charge on the nitrogen atom in the hydroxyamino group and on the oxygen atom of the methoxy group are less negative for N–OH–3-OMe–AAB than for any of the other positional isomers or for N–OH–AAB, see Table 3. We showed previously that methoxy substitution at the 3(5)-position in AAB reduces the length of the C_4–NH_2 bond slightly [13]. Similarly, the C_4–NHOH bond length in N–OH–3(5)-OMe–AAB, 1.397 Å, is shorter than it is in N–OH–AAB, 1.405 Å (and in C_6H_5–NH_2, 1.408 Å).

The C_ϕ–O bond length in N–OH–3(5)-OMe–AAB, 1.378 (1.377) Å, is relatively long compared to its length in the corresponding 2(6)-isomers, 1.355 (1.361) Å, see Table 2, similar to what we observed for the corresponding isomers of OMe–AAB [13]. The calculated length of the C_ϕ–O bond in 3-OMe–AB, where there is no substitution at the 4-position, is 1.373 Å. Thus, the hydroxyamino group serves to further reduce the double bond character of the C_ϕ–O bond.

The reaction energy for the conversion of 3(5)-OMe–AAB to N–OH–3(5)-OMe–AAB,

$$\text{3(5)-OMe–AAB} + H_2O_2 \rightarrow \text{N–OH–3(5)-OMe–AAB} + H_2O \quad (2)$$

is −23.4 (−22.8) kcal/mol, which is similar to that found for the conversion of AAB to N–OH–AAB. Thus, substitution of a methoxy group at the 3(5)-position has relatively little effect on the thermodynamics of *N*-hydroxylation.

Replacing the amino group in 3(5)-OMe–AAB with a hydroxyamino group lowers the energy of the frontier orbitals, see Table 3; the energy of the orbital involving the amino nitrogen atom lone pair of electrons is lowered slightly more than that of the orbital involving the more distant azo lone pairs, e.g. 3.2 kcal/mol vs. 2.7 kcal/mol. Since in 3(5)-OMe–AAB the HOMO involves the amino lone pair of electrons [13], the energy separation between the HOMO and HOMO{-1} is smaller in N–OH–3(5)-OMe–AAB. In N–OH–3-OMe–AAB the HOMO and HOMO{-1} differ in energy by only 0.4 kcal/mol and have become mixed, i.e. both orbitals involve contributions from both the azo and amino lone pairs. The HOMO of N–OH–5-OMe–AAB still involves only the amino lone pair, similar to what we found for 5-OMe–AAB. Although the energy of the LUMO in N–OH–3(5)-OMe–AAB is lower than the energy of the LUMO in 3(5)-OMe–AAB, it is higher in energy than the LUMO in N–OH–AAB. The HOMO–LUMO energy gap in 3(5)-OMe–AAB, N–OH–3(5)-OMe–AAB, AAB and N–OH–AAB are all quite similar, ranging from 46.4 to 47.6 kcal/mol.

3.2. N–OH–2-OMe–AAB and N–OH–6-OMe–AAB

The nonmutagens N–OH–2-OMe–AAB and N–OH–6-OMe–AAB are calculated to be 6.2 and 3.2 kcal/mol, respectively, higher in energy than N–OH–3-OMe–AAB, see Table 1. For these isomers, the –OH and –OMe groups are positioned on the same side of the C_4–N bond, see Chart 1B. The relatively high energy of the 2-isomer is a result of greater adverse interactions involving the lone pairs on the methoxy oxygen atom and one of the azo nitrogen atoms. (This adverse interaction is also evident in 2-OMe–AAB, which is 2.7 kcal/mol higher in energy than 6-OMe–AAB.) These results suggest that the compounds labeled in the experimental literature as 2-OMe–AAB and N-OH–2-OMe–AAB may actually be the 6-isomers, see Chart 1A.

Many of the calculated structural parameters of N–OH–2(6)–OMe–AAB, see Table 2, are similar to their corresponding values in 2(6)-OMe–AAB [13], e.g. the C_1–C_2 bond length for N–OH–2-OMe–AAB, 1.438 Å, is similar to its value in 2-OMe–AAB, 1.436 Å, and is the longest phenyl ring C–C bond we observed in this study. On the other hand, the C_4–N bond length in N–OH–2(6)-OMe–AAB, 1.405 Å, is approximately 0.015 Å longer than its length in 2(6)-OMe–AAB, and 0.008 Å longer than in the *ortho* substituted compounds, N–OH–3(5)-OMe–AAB. The electrostatic charge on the hydroxyamino nitrogen atom in N–OH–2(6)-OMe–AAB is more negative than it is in N–OH–3(5)-OMe–AAB and the corresponding dipole moments are more than 1 debye larger, see Table 3. The C_ϕ–O bond length in N–OH–2(6)-OMe–AAB, 1.355 (1.361) Å, is very similar to its value in 2(6)-OMe–AAB, 1.356 (1.362) Å, and some 0.02 Å shorter than the corresponding bond lengths in N–OH–3(5)-OMe–AAB. Thus, the presence of the hydroxy group does not interfere with delocalization involving the methoxy-oxygen atom out-of-plane lone pair, which gives the C_ϕ–O bond additional double bond character.

The calculated value of the reaction energy for the *N*-hydroxylation of 2(6)-OMe–AAB,

$$\text{2(6)-OMe–AAB} + H_2O_2 \rightarrow \text{N–OH–2(6)-OMe–AAB} + H_2O \quad (3)$$

is −24.1 (−24.2) kcal/mol. These results are similar to those for the corresponding reaction with AAB, and are only marginally more exothermic than those involving 3(5)-OMe–AAB. Thus, the presence of a methoxy group at the 2(6)- or 3(5)-position does not have a significant effect on the energetics of these model oxidation reactions. There is evidence that the dimethoxy compound 2,5-OMe–AAB is resistant to *N*-hydroxylation [6], but the corresponding reaction energy is −24.3 kcal/mol, suggesting that this resistance is not a thermodynamic issue.

The presence of a methoxy group *meta* to the amino or hydroxyamino group alters the two highest occupied Kohn–Sham orbitals differently than when the methoxy group is *ortho* to either of these groups. In the carcinogen 3(5)-OMe–AAB and its *N*-hydroxy metabolite, the HOMO and HOMO{-1} are close in energy, differing by no more than 1.3 kcal/mol, and the orbital involving

the amino or hydroxyamino nitrogen atom lone pair is generally *higher* in energy. In the non-carcinogen 2(6)-OMe–AAB and its *N*-hydroxy metabolite, the HOMO and HOMO{-1} have a greater energy separation, between 3.6 and 9.4 kcal/mol, and the orbital involving the amino lone pairs is consistently *lower* in energy. The azo lone-pair orbitals in 2-OMe–AAB and N–OH–2-OMe–AAB are higher in energy than those for the corresponding 6-isomers. The particular arrangement of atoms near the *trans* azo linkage causes electron overcrowding in this region to be more severe for substitution at the 2-position than at the 6-position. The energy of the LUMO of N–OH–2(6)-OMe–AAB is lower than that of 2(6)-OMe–AAB, but the LUMOs of 2(6)-OMe–AAB and N–OH–2(6)-OMe–AAB are consistently higher in energy than those of the corresponding 3(5)-isomers, suggesting that the 2(6)-isomers of OMe–AAB and N–OH–OMe–AAB are slightly *less* electrophilic than the corresponding 3(5)-isomers.

3.3. N–OH–3′-OMe–AAB and N–OH–5′-OMe–AAB

The positional isomers N–OH–3′(5′)-OMe–AAB are only 1.1 (1.0) kcal/mol higher in energy than N–OH–3-OMe–AAB, see Table 1. In these isomers the hydroxyl group is on the same side of the C_4–N bond as the methoxy group. Methoxy substitution at the 3′- or 5′-position does not lead to any significant interactions among the various lone pairs on the oxygen or nitrogen atoms and does not have much of an effect on the structural parameters of either of the phenyl rings beyond those already present in N–OH–AAB.

N–OH–3′(5′)-OMe–AAB has the second largest dipole moment we observed for the positional isomers of N–OH–OMe–AAB, see Table 3. For 3′(5′)-OMe–AAB and N–OH–3′-OMe–AAB, the HOMO involves the azo lone pairs and the HOMO{-1} involves the hydroxyamino nitrogen atom lone pair. Interestingly, for N–OH–5′-OMe–AAB the HOMO and HOMO{-1} are slightly mixed, i.e. having contributions from both the hydroxyamino and azo lone pairs. Compared to the other positional isomers of N–OH–OMe–AAB the HOMO, HOMO{-1} and LUMO energies of 3′(5′)-OMe–AAB are closer to those of N–OH–AAB. N–OH–3′(5′)-OMe–AAB have the lowest LUMO energies among the N-hydroxy derivatives.

3.4. N–OH–2′-OMe–AAB and N–OH–6′-OMe–AAB

The positional isomers N–OH–2′-OMe–AAB and N–OH–6′-OMe–AAB are calculated to be 3.3 and 6.5 kcal/mol higher in energy than N–OH–3-OMe–AAB. The 6′-isomer is the highest-energy form of N–OH–OMe–AAB we found, 0.3 kcal/mol above N–OH–2-OMe–AAB, see Table 1. The relatively high energy of N–OH–6′-OMe–AAB is again the result of adverse lone pair interactions in this isomer, similar to what we observed N–OH–2-OMe–AAB. The lowest energy conformers of N–OH–2′(6′)-OMe–AAB are such that the −OH and −OMe groups are on the same side of the C_4–N bond.

Many of the structural parameters of N–OH–2′(6′)-OMe–AAB are similar to those of 2′(6′)-OMe–AAB, e.g. the $C_{1'}$–N bond length in N–OH–6′-OMe–AAB is the shortest we observed in this study, 1.399 Å, and its value in 6′-OMe–AAB is 1.401 Å. The C_ϕ–O bond length for 6′-OMe–AAB and N–OH-6′-OMe–AAB, 1.359 [16] and 1.358 Å, respectively, are quite short; the only shorter C_ϕ–O bonds we observed are for the corresponding 2-isomers, 1.356 and 1.355 Å. N–OH–6-OMe–AAB has the lowest dipole moment, 0.9 D, of any of the positional isomers of OMe–AAB or N–OH–OMe–AAB.

The Kohn-Sham HOMO of 2′(6′)-OMe–AAB and N–OH–2′(6′)-OMe–AAB all involve the azo lone pairs. The energy of the HOMO of N–OH–6′-OMe–AAB is extremely high, analogous to that found in N-OH–2-OMe–AAB; these *N*-hydroxy compounds have similar adverse lone pair interactions and have the highest-energy LUMOs we observed for any of the positional isomers of N–OH–OMe–AAB.

3.5. N–OH–4′-OMe–AAB

The mutagenic isomer N–OH–4′-OMe–AAB is only 0.7 kcal/mol higher in energy than N–OH–3-OMe–AAB, making it the second lowest-energy isomer we found, see Table 1. As can be seen in

Table 2, the C_4–N bond length in this isomer, 1.407 Å, is the longest we observed for these N-hydroxy derivatives. As we reported for 4′-OMe–AAB, the Kohn–Sham HOMO and HOMO{-1} are close in energy and involve contributions from both the azo and amino lone pairs [13]. The HOMO in N–OH–4′-OMe–AAB, however, involves only the azo group and the HOMO{-1} involves only the hydroxyamino group. The uncoupling of these orbitals is a result of the hydroxy group, which lowers the energy of the orbital involving the lone pair on the amine nitrogen to a greater extent than the energy of the orbital involved with the azo lone pairs.

4. Concluding remarks

An *N*-hydroxy metabolite of the potent hepatocarcinogen 3-OMe–AAB is found to be lower in energy than any of the other positional isomers of N–OH–OMe–AAB at the BP/DN**//BP/DN** computational level. This conformer of N–OH–3-OMe–AAB has the −OH and −OMe groups arranged on opposite sides of the C_4–N bond which keeps the negatively charged oxygen atoms relatively far apart; the 2(6)-, 2′(6′)-, and 3′(5′)-isomers all have the hydroxy group on the same side of the C_4–N bond as the methoxy group, see Chart 1. The highest-energy positional isomers of the OMe-AAB dyes and their N-hydroxy metabolites involve the methoxy group at the 2- and 6′-positions where the adverse lone pair interactions between the azo linkage and the methoxy group are greatest. These results suggest that the compound referred to as 2-OMe–AAB (N–OH–2-OMe–AAB) in the experimental literature actually corresponds to 6-OMe–AAB (N–OH–6-OMe–AAB) in our numbering system.

The structural parameters of the corresponding positional isomers of N–OH–OMe–AAB and OMe–AAB clearly show that the amino and hydroxyamino groups alter the phenyl ring parameters of AB in a similar way and by a similar amount. As expected, the main structural effect of the hydroxy group is to lengthen the C_4–N bond; this leads to a more pronounced pyramidal structure of the hydroxyamino group [22]. This increase in pyramidalization occurs even though the N–O bond length is shorter for all the positional isomers of N–OH–OMe–AAB than it is in hydroxylamine.

Comparing the electronic features of the corresponding conformers of OMe–AAB and N–OH–OMe–AAB shows that the hydroxy group lowers the energies of the frontier orbitals. For example, in going from 3-OMe–AAB to N–OH–3-OMe–AAB, the energy of the LUMO decreases by 3.1 kcal/mol. The energy of the orbital involved with the lone pair on the amine nitrogen atom is typically lowered by about 1 kcal/mol more than the energy of the orbital involved with the lone pairs at the azo linkage. The calculated electrostatic charge on the nitrogen atom of the hydroxyamino group in N–OH–OMe–AAB is some 25% less than the electrostatic charge on the nitrogen atom of the amino group in the corresponding isomer of OMe–AAB. Methoxy substitution at the 3-position of N–OH–AAB, which leads to the most carcinogenic monomethoxy form, has the *least* negative charge on both the hydroxyamino nitrogen and methoxy oxygen atoms for any of the N–OH–OMe–AAB positional isomers. The dipole moment of each positional isomer of N–OH–OMe–AAB is smaller than that of the corresponding isomer of OMe–AAB, with reductions in the range from 16 to 55%. The largest dipole moments we observed in this study are found for the noncarcinogens N–OH–2-OMe–AAB and N–OH–6-OMe–AAB.

The lower LUMO energies in the *N*-hydroxy derivatives of OMe–AAB suggest that each of these positional isomers is somewhat more electrophilic than the corresponding isomer of OMe–AAB. Since the OMe–AAB dyes that have been tested to date do not appear to be mutagenic per se on the Ames test, whereas some of their *N*-hydroxy metabolites are mutagenic, this increase in the electrophilic character of the *N*-hydroxides is in accord with expectations based on decades of experimental work by the Millers [26] and the recent computational results by Leão and Pavão [24]. However, in going from any one of the positional isomers of OMe–AAB to its corresponding *N*-hydroxy compound, the energy of the LUMO only decreases by 4–5%. Such a small decrease is consistent with the idea that these *N*-hydroxy derivatives are only the first step in the metabolic

activation of these monomethoxy AAB dyes [7]. There is some evidence that N–OH–OMe–AAB compounds undergo further activation *in vivo* via esterification [27]. We are currently investigating the structures and electronic features of *N*-acetyl and *N*-acetoxy derivatives of OMe–AAB dyes.

Acknowledgements

The authors would like to thank Revathy Iyer from the University of Pennsylvania and Brandon Francis from Philadelphia University for technical assistance. We would also like to acknowledge the National Textile Center (Grant Nos. COO-PO1 and I98-PO1) for financial support of this work.

References

[1] Miller JA, Miller EC. Cancer Res 1961;21:1068.
[2] Kojima M, Degawa M, Hashimoto Y, Tada M. Biochem Biophys Res Commun 1991;179:817.
[3] Hashimoto Y, Degawa M, Watanabe HK, Tada M. Gann 1981;72:937.
[4] Degawa M, Miyairi S, Hashimoto Y. Gann 1978;69:367.
[5] Degawa M, Shoji Y, Masuko K, Hashimoto Y. Cancer Lett 1979;8:71.
[6] Hashimoto Y, Watanabe HK, Degawa M. Gann 1981;72:921.
[7] Degawa M, Kojima M, Hashimoto Y. Mutat Res 1985;152:125.
[8] Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG. Science 2000;287:1615.
[9] Guengerich FP. J Biol Chem 1991;266:10019.
[10] Kojima M, Degawa M, Hashimoto Y, Tada M. Can Lett 1991;58:199.
[11] Kojima M, Degawa M, Hashimoto Y, Tada M. Env Health Perspectives 1994;102(Suppl. 6):191.
[12] Miller JA, Miller EC, Finger GC. Cancer Res 1956;16:387.
[13] Bhat KL, Freeman HS, Velga J, Sztandera L, Trachtman M, Bock CW. Dyes and Pigments 2000;46:109.
[14] Marroquin F, Coyote N. Chem Biol Interact 1970;2:151.
[15] Irving CC, Veazey RA, Hill JT. Biochem Biophys Acta 1969;179:189.
[16] Searle CE, editor. Chemical carcinogens. 2nd ed. Revised and expanded in ACS Monograph 1984;1:179.
[17] Spartan v. 5.0, Wavefunction Inc., 18401 Von Karmen Avenue, Suite 370, Irvine, CA 92612.
[18] Perdew JP. Phys Rev 1986;B33:8822.
[19] Perdew JP. Phys Rev 1987;B34:7046.
[20] Gill PMW, Johnson BG, Pople JA, Frisch M. J Chem Phys Lett 1992;l97:499.
[21] Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Zakrzewski VG, Montgomery JA, Stratmann RE, Burant JC, Dapprich S, Millam JM, Daniels AD, Kudin KN, Strain MC, Farkas O, Tomasi J, Barone V, Cossi M, Cammi R, Mennucci B, Pomelli C, Adamo C, Clifford S, Ochterski J, Petersson GA, Ayala PQ, Cui Q, Morokuma K, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Cioslowski J, Ortiz JV, Stefanov BB, Lui G, Liashenko A, Piskorz P, Komaromi I, Gomperts R, Martin RL, Fox DJ, Kieth T, Al-Laham MA, Peng CY, Nanayakkara A, Gonzalez C, Challacombe M, Gill PMW, Johnson BG, Chen W, Wong MW, Andres JL, Head-Gordon M, Replogle ES, Pople JA. Gaussian Inc. Pittsburgh, PA, 1998.
[22] Rauk A, Allen LC, Mislow K. Angew Chem, Int Ed Engl 1970;9:400.
[23] Flemming I. Frontier orbitals and organic chemical reactions. NY: Wiley, 1976.
[24] Leão MBC, Pavão AC. Int J Quant Chem 1997;62:323.
[25] Miller EC. Cancer Res 1978;38:1479.
[26] Miller JA, Miller EC. Ultimate chemical carcinogens as reactive mutagenic electrophiles. In: Hiatt HH, Watson JD, Winsten JA, editors. Origins of human cancer. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1977. p. 605.
[27] Lin JK, Schmall B, Sharpe ID, Miuva I, Miller JA, Miller EC. Cancer Res 1975;35:832.
[28] Ghose AK, Pritchett A, Crippen GM. J Comput Chem 1988;9:80.