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Brief Communication

**Mutagenic potential of nitrenium ions of nitrobenzanthrones: correlation
between theory and experiment**

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Running title: Calculations on nitrobenzanthrone derivatives

ABSTRACT

The mutagenic activity of nine substituted nitrobenzanthrone (NBA) derivatives was recently established in the Ames assay and ranged from near inactivity to extremely high mutagenic activity (Takamura-Enya et al [2006]: *Mutagenesis* 21: 399-404). Using thermo-chemical and molecular modelling techniques, the activation pathway of these NBA derivatives, namely 1-nitro-, 2-nitro-, 3-nitro-, 9-nitro-, 11-nitro-, 1,9-dinitro-, 3,9-dinitro-, 3,11-dinitro- and 3,9,11-trinitrobenzanthrone, and the formation of the corresponding aryl-nitrenium ions, were investigated using density functional theory (DFT) calculations. The calculated properties of the NBA derivatives were systematically compared with their bacterial mutagenic potency. Accommodation of the ligand substrates into the binding pocket of the bacterial nitroreductases was not sterically inhibited for the NBAs. Moreover, electron affinities, water elimination energies, esterification and solvolysis energies did not reveal any possible links with the observed mutagenic potency of the NBAs. However, a strong negative linear correlation was found when the relative energies of the nitrenium ions of the mono- and di-substituted NBAs were plotted against the logarithm of the mutagenic potency of the NBAs found in the different *Salmonella typhimurium* strains. Therefore, our data clearly indicate that the stability of the nitrenium ions is one critical determinant of the mutagenic potency of NBAs in *Salmonella* tester strains.

INTRODUCTION

Nitro-polycyclic aromatic hydrocarbons (nitro-PAHs) are widely distributed environmental pollutants found in extracts from diesel and gasoline engines, and on the surface of ambient air particulate matter (IPCS, 2003). They have become of enormous concern because of their mutagenicity and carcinogenicity (IARC, 1989; IPCS, 2003). Numerous epidemiological studies have shown increased mortality and morbidity from respiratory and cardiovascular diseases due to ambient air pollution (Vineis and Husgafvel-Pursiainen, 2005; Boffetta, 2006). Although environmental levels of nitro-PAHs are lower than those of unsubstituted PAHs, certain nitro-PAHs exhibit high direct-acting mutagenic potency in bacterial and mammalian bioassays (Purohit and Basu, 2000). Since the direct mutagenicity of known nitro-PAHs such as nitropyrenes, nitrofluoranthenes and nitrodibenzopyranones accounts for only about 40% of the total bacterial mutagenicity of ambient air, the presence of more polar, complex, as-yet-unknown nitro-PAHs have been suggested (Finlayson-Pitts and Pitts, 1997; Umbuzeiro *et al.*, 2008).

In recent years the aromatic nitroketone 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA) has received much attention due to its presence in diesel exhaust and its extremely high mutagenic potency in the Ames assay (Enya *et al.*, 1997; Arlt, 2005). The uptake of 3-NBA in humans has been demonstrated and it is a suspected human carcinogen (Seidel *et al.*, 2002; Nagy *et al.*, 2005b). 3-NBA is a genotoxic mutagen that forms DNA adducts after metabolic activation through reduction of the nitro group (Figure 1) (Arlt *et al.*, 2001; Arlt *et al.*, 2003a; Arlt *et al.*, 2003b; Arlt *et al.*, 2005; Stiborova *et al.*, 2006). The predominant DNA adducts formed are 2-(2'-deoxyguansin-*N*²-yl)-3-aminobenzanthrone and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone, and these are most probably responsible for the induction of GC to TA transversion mutations induced by 3-NBA (Arlt *et al.*, 2004; Arlt *et al.*, 2006; Bieler *et al.*, 2007).

2-Nitrobenzanthrone (2-NBA), an isomer of 3-NBA, has been detected in ambient air particulate matter (Phousongphouang and Arey, 2003; Tang *et al.*, 2004). It has been suggested that 2-NBA might be formed more specifically by atmospheric processes while 3-NBA seems to be formed preferentially by combustion processes, such as in a diesel engine. Although 2-NBA has been shown to be genotoxic *in vitro* (Nagy *et al.*, 2005a; Takamura-Enya *et al.*, 2006; Arlt *et al.*, 2007), a lack of genotoxicity *in vivo* (*e.g.* DNA adduct formation) was reported (Arlt *et al.*, 2007). Density functional theory (DFT) calculations have revealed that the nitrenium ion of the 3-isomer is considerably more stable than that of the 2-isomer, providing a possible explanation for the large differences in DNA adduct formation and mutagenic potency between 2- and 3-NBA (Arlt *et al.*, 2007).

In a recent study by Takamura-Enya and coworkers (Takamura-Enya *et al.*, 2006) the mutagenicity of nine nitrobenzanthrone (NBA) derivatives, namely 1-nitro-, 2-nitro-, 3-nitro-, 9-nitro-, 11-nitro-, 1,9-dinitro-, 3,9-dinitro-, 3,11-dinitro- and 3,9,11-trinitrobenzanthrone was tested in the Ames assay and ranged from near inactivity to extremely high mutagenic activity. Moreover, the authors did not find a correlation between the observed mutagenicity of each NBA derivative and a range of calculated physicochemical properties of NBAs including the lowest unoccupied molecular orbital [LUMO], first reduction potential, hydrophobicity and orientation of nitro substituents (Takamura-Enya *et al.*, 2006). Since these theoretical calculations were based on semi-empirical methods (Stewart, 1989a, Stewart, 1989b), in the present study we performed theoretical calculations based on DFT, which has been used previously to describe properties of electrophilic PAHs relevant to their carcinogenesis (Reynisson *et al.*, 2000; Laali *et al.*, 2001; Okazaki and Laali, 2003; Okazaki *et al.*, 2003; Okazaki and Laali, 2004). The predictive power of the DFT method is well documented, in particular regarding redox properties of organic molecules and in the prediction of bond dissociation energies, which are the basis for the calculations of the reaction energies (Reynisson *et al.*, 2004, Ioakimidis *et al.*, 2008). In addition, *in-silico* docking simulations have been shown to provide further insights into the functional and structural aspects of enzyme-substrate binding (Arlt *et al.*, 2005; Stiborova *et al.*, 2005; Hodek *et al.*, 2007). Therefore, interactions of NBAs with the active centres of bacterial nitroreductases NfsA and NfnB were examined in order to investigate the molecular basis of the reductive activation of NBAs by bacterial enzymes.

MATERIALS AND METHODS

Quantum chemical calculation

The energy calculations and geometry optimizations were performed with the GAUSSIAN 03 program suite (Frisch *et al.*, 2003) utilizing unrestricted DFT. The non-local B3LYP functional hybrid method was employed (Becke, 1988; Lee *et al.*, 1988; Becke, 1993). The standard 6-31G(d,p) basis set (Hariharan and Pople, 1973) was used for the geometry optimization and frequency analysis. The zero-point vibrational energies (ZPE) were scaled according to Wong (0.9804) (Wong, 1996). Subsequent single-point electronic energy calculations were performed with the larger 6-311G(2df,p) basis set. The electron affinities (EAs) were calculated as described (Foresman and Frisch, 1996). The bond dissociation energies (BDE) were calculated as reported (Reynisson and Steenken, 2004), meaning that the water elimination, esterification and solvolysis steps described are basically the total energy of the products minus the total energy of the reactants. The effect of the aqueous phase was simulated with the IEF-PCM (Integral Equation Formalism - Polarized Continuum Model) method (Tomasi *et al.*, 2005). Single-point electronic calculations using the 6-311G(2df,p) basis set were performed on the structures optimized in vacuum. The Gibbs free solvation energy was calculated according to the following equation: $\Delta G_{aq} = E^{PCM-B3LYP} - E^{B3LYP}$.

Molecular Modelling

The 3D model of the *Salmonella typhimurium* oxygen-insensitive nitroreductase NfsA (SnrA) was built based on the crystal structure of the highly homologous nitroreductase from *Escherichia coli* NfsA (PDB code: 1F5V, 1.7 Å resolution). The second 3D model representing the *Salmonella typhimurium* oxygen-insensitive nitroreductase NfnB (a “classical” nitroreductase, Cnr) (Watanabe *et al.*, 1998) was based on the crystal structure of nitroreductase from *Enterobacter cloacae* NfnB co-crystallized with an inhibitor benzoate (PDB code: 1KQB, 1.8 Å resolution). For sequence alignments of templates and target proteins see Supplementary Figure S1. Due to the high homology between the template and modeled proteins, all active site residues of modeled enzymes were the same as corresponding residues in the template. Therefore, the original flavin-mononucleotide (FMN) cofactor coordinates could be retained for both models.

The quality of the prepared homology models was evaluated using the program PROCHECK (version 3.4) (Laskowski *et al.*, 1993). The models showed no significant deviations from normality. For the first model (NfsA, SnrA) the Ramachandran plot of the main torsion angles (ϕ , ψ) revealed that 93.8% of non-glycine and proline residues are in the most favoured regions and the remaining 6.2% in allowable regions. The Ramachandran plot of the second model (NfsB, Cnr)

detected 88.8% of non-glycine and proline residues are in the most favoured regions, 10.7 % in allowed regions and 0.5% (peripheral residue Asn179) in disallowed regions. During the model preparation, the polar hydrogen atoms were added and usual protonation states and partial charges were assigned to all residues (His residues were protonated). Mono-substituted NBA (MNBA) ligand geometries and partial charges were obtained via *ab-initio* methodology as implemented in GAUSSIAN 03 program suite (Frisch *et al.*, 2003) using the Hartree-Fock level of theory in conjunction with the 6-31+G(d) basis set.

We employed the hybrid global-local Lamarckian genetic algorithm implemented in the Autodock package (version 4) (Morris *et al.*, 1998) to evaluate ligand-enzyme interaction and preferred binding modes for MNBA derivatives toward the models mentioned above. In order to allow the enzyme to adopt a new (bulkier) ligand (NBA) we utilized the soft-soft docking algorithm recently implemented in the program Autodock. All rotatable bonds of MNBA derivatives and sidechains of residues Ser40, Arg133 and Arg225 of the model *S. typhimurium* nitroreductase NfsA (SnrA) were allowed to rotate freely. In the second model, *S. typhimurium* nitroreductase NfnB (Cnr), the residues Lys14, Ser40, Phe70, Phe124 and Glu165 were treated as flexible.

An extensive search (500 docking runs per compound) was performed with the standard setting including 2,500,000 evaluations and 27,000 generation limits. All 500 resulting binding modes were grouped into clusters upon their Root Mean Square Deviation (RMSD) within 1 Å cut-off. The conformation having the lowest energy in the best binding cluster was chosen as the result.

RESULTS AND DISCUSSION

Figure 1 depicts the metabolic cascade proposed for the NBA derivatives. The first two steps comprise reduction of the nitro group forming the corresponding hydroxylamine. The hydroxylamines are transformed to the nitrenium ions either by direct water elimination or via enzymatic esterification and subsequent solvolysis of the ester.

Electron transfer

The first step in the metabolic activation for the NBA derivatives is reduction, which in *Salmonella* is catalyzed by bacterial nitroreductases (Carroll *et al.*, 2002) such as a SnrA (NfsA) nitroreductase of *Salmonella enterica* serovar Typhimurium TA1535 (Nokhbeh *et al.*, 2002) and a “classical” nitroreductase (Cnr) (Watanabe *et al.*, 1998). These oxygen-insensitive nitroreductases have been reported to share similarities with flavin-dependent nitroreductases of *Escherichia coli*, NfsA and NfsB, respectively (Watanabe *et al.*, 1998; Nokhbeh *et al.*, 2002). Cnr also exhibits a high degree of homology with an oxygen-insensitive nitroreductase of *Enterobacter cloacae* (NfnB) (Haynes *et al.*, 2002). The SnrA and Cnr enzymes of *S. typhimurium* share 87% and 89% sequence identity with the nitroreductases of *E. coli* NfsA (1F5V) and *E. cloacae* NfnB (1KQB), respectively (see Supplementary Figure S1). Both nitroreductases contain FMNH₂ as an electron donor, *i.e.* electron transfer occurs from FMNH₂ to the NBA derivative.

In order to investigate the molecular basis of binding of MNBA derivatives (1-, 2-, 3-, 9- and 11-NBA) to *S. typhimurium* nitroreductases, interaction of these compounds with the active centres of SnrA and Cnr enzymes was examined using docking calculations. Since the crystal structures of both enzymes have not yet been resolved, we constructed homologous models. To construct the most appropriate SnrA and Cnr models, data from the highly homologous *E. coli* NfsA (1F5V) and *E. cloacae* NfnB (1KQB) nitroreductases were employed. Biologically active units of SnrA (NfsA) and Cnr (NfnB) are homodimeric protein complexes. Each complex has 2 active sites, formed by residues of both subunits and FMN cofactor. Therefore, the modelled system used for the docking calculation consists of tightly interacting subunits A and B (in 1F5V.pdb 1KQB.pdb notation). As the docked NBA molecules are larger than the original ligand present in the crystal structure of the template (benzoate), a soft-soft (flexible) docking procedure was employed.

All MNBA derivatives were docked into both SnrA (NfsA) and Cnr (NfnB) nitroreductase models. As exemplified for 2- and 3-NBA the calculated model structures in both nitroreductases are shown in Supplementary Figure S2. All MNBA molecules used for docking fitted into the active sites of both nitroreductases. An extensive search of the lowest energy binding modes found reasonable geometries for all MNBA derivatives showing close contact between ligand (NBA) and

FMNH₂ cofactor. Predicted interaction energies were similar for all MNBA derivatives analyzed and ranged between -5.56 and -7.13 kcal/mol (Table 1). The important result of the docking studies is that all of the MNBA derivatives can form similar tight complexes with the flavin-cofactor in the enzymes and, therefore, their binding affinity seems *not* to be a rate limiting step in the metabolic cascade.

The electron transfer rate (k_{et}) is described by the Marcus theory (Marcus and Sutin, 1985) and the following equation: $k_{et} = \nu_N \kappa \exp(-\Delta G^\ddagger/RT)$ where $\Delta G^\ddagger$ is the activation energy, R is the gas phase constant and T is the temperature. Although no direct information is available on the size of the activation barrier, the calculated EA values give a good indication of its magnitude. As shown in Table 2 the MNBA derivatives have exothermic EA of $\sim$ -40 kcal/mol (around -75 kcal/mol in water), the di-substituted derivatives lie between -55 and -60 kcal/mol (around -88 kcal/mol in water) and the tri-substituted 3,9,11-TNBA has an EA of -66.8 kcal/mol (-94.1 kcal/mol in water). The nitro group is an electron withdrawing group (Hammett constants: $\sigma_I = 0.65$ and $\sigma_R = 0.15$) (Isaacs, 1987) and, as expected, the calculated EA is lowered by its introduction. The oxidation potential of the flavin moiety in the nitroreductase is a constant. For all of the derivatives the calculated EA is exothermic and suggesting that the reduction occurs rapidly, *i.e.* $\Delta G^\ddagger$ is not of significant magnitude. If the ease of reduction was the rate determining factor causing mutational events, 3,9,11-TNBA should be the most potent. However, comparing the data in Table 3 this is not the case. In fact, no correlation is observed between the mutagenic potency observed in *S. typhimurium* TA98 and TA100 and the theoretical EAs (data not shown).

The electron transfer rate equation contains two pre-exponential factors. The first, ν_N describes the nuclear frequency factor of the reaction and since all of the investigated compounds have the same polycyclic skeleton it can be assumed that they have similar vibrational modes and therefore ν_N does not cause different electron transfer rates for the derivatives. κ is the transmission coefficient of the reaction. It is related to the Hamiltonian (H_{AB}) perturbation coupling of the initial (A) and final (B) states of the reaction system. The value of the H_{AB} depends on the overlap between the electronic-wave functions of the donor and acceptor, which decreases exponentially with distance. According to the modelling calculations all of the NBA derivatives adopted a “sandwich” type conformation with the flavin moiety. This allows for an overlap between their π -systems, *i.e.*, good H_{AB} , facilitating charge transfer.

Another interesting aspect of the charge transfer mechanism is that not all of the NBA derivatives have a planar polycyclic skeleton according to the DFT calculations. This will have the same effect on the Hamiltonian (H_{AB}) perturbation as poor access due to structural features of the

reductase. Four of the investigated NBAs are calculated to form planar systems (2-NBA, 3-NBA, 9-NBA and 3,9-DNBA) whereas the rest (1-NBA, 11-NBA, 1,9-DNBA, 3,11-DNBA and 3,9,11-TNBA) are slightly buckled. There was no correlation between planarity and the mutagenic activities observed in the Ames assay (data not shown) and it can be concluded that the non-planarity of the polycyclic skeleton does *not* seem to affect the mutagenicity of NBA species.

Water elimination

The water elimination is a heterolytic bond dissociation where the negative charge is delocalized onto the hydroxyl moiety, which then acts as a leaving group. In the aqueous phase this step is facilitated by protonation (Kadlubar *et al.*, 1977) where the leaving group is a water molecule. The thermochemistry of this step was calculated with and without solvent simulation and the results are shown in Table 2. Although the inclusion of the water effects in the thermochemical calculations is complicated they can be accommodated. First, the difference in solvation free energy (ΔG_{aq}) for all of the organic species involved was calculated. The ΔG_{aq} for the hydroxylamine derivatives are predicted to be ~ -20 kcal/mol and for the nitrenium ions the ΔG_{aq} lies between -43.3 and -62.3 kcal/mol. Second, the dehydration of the proton, needed to form the water leaving group, must be accounted for and it has been measured as $\Delta G_{\text{aq}} = -263.9$ kcal/mol (Tissandier *et al.*, 1998). Thirdly, a heterolytic bond formation occurs as a water molecule is produced, and is measured as -390.7 kcal/mol (Schulz *et al.*, 1982), and adds to the thermochemical push of the reaction. According to these calculations the heterolytic bond cleavage is endothermic by ~ 200 kcal/mol for all NBA derivatives *in vacuo*. It is not surprising to see such a large number since separation of negative and positive charges must occur, which is energetically very unfavourable. The inclusion of the effects of water drastically reduces the endothermicity of the reaction to 29.0 to 58.0 kcal/mol, which is mainly due to water being the leaving group and therefore no charge separation being necessary for the reaction to occur. When the water elimination data are compared to the mutagenic potency (Table 3) no correlation was observed, for instance, in *S. typhimurium* TA98 and TA100 (data not shown), suggesting that water elimination is *not* the rate determining step for the mutagenicity of NBA species.

Esterification / Solvolysis

The hydroxylamine derivatives can undergo enzymatic esterification, facilitating the production of the nitrenium ion (Carroll *et al.*, 2002). Here the acetyl group of acetyl-CoA in the enzyme is modelled as ethanethioic acid-*S*-methyl ester (Watanabe *et al.*, 1994). A nucleophilic attack occurs on the carboxy group of the acetyl moiety from the oxygen lone pair of the *N*-OH moiety. The products of this reaction are acetylated NBA derivatives as well as methanethiol as the

model compound for HS-CoA. In the calculations, where the solvent effects are taken into account, the ethanethioic acid-*S*-methyl ester and methanethiol are not solvated since they are a part of the protein. The acylated derivatives have predicted solvation energies between -11.5 and -32.9 kcal/mol. The results of the calculation are presented in Table 2.

In the gas phase and when the effects of water are incorporated a modest endothermic reaction (~5 kcal/mol) is observed for the esterification. The solvolysis step is a heterolytic bond cleavage of the N-O bond and lies between 130.9 and 150.9 kcal/mol *in vacuo*. The effects of water reduce the energy needed for the cleavage to ~30 kcal/mol, *i.e.*, considerably lower number than for the water elimination making the ester derivative even more labile than its hydroxylamine counterpart. No correlation of these values was observed with the mutagenicity in *S. typhimurium* TA98, TA100, YG1021 and YG1024 (data not shown) indicating that the solvolysis is *not* the rate limiting step in forming the corresponding reactive nitrenium ions.

Stability of the nitrenium ions

It has been postulated that the stability of the nitrenium ions plays a major role in their biological activity (Kadlubar and Beland, 1985; Borosky, 2007). In order to investigate whether the stability of the ions correlated with the mutagenic potency of the NBAs, the relative energies of the MNBAs and di-substituted NBAs (DNBAs) were plotted against the mutagenic potency measured in *Salmonella* (Table 3). The relative energies are calculated by subtracting the total energy of the ions from the most stable ion. Hence, the most stable ion has a relative energy of zero. This procedure is only possible when the pertinent molecules have the same number of nuclei and electrons and therefore two data sets emerge, one for the MNBAs and one for the DNBAs. As an example, the correlations between the relative energies of the MNBAs and the mutagenic potency in *S. typhimurium* TA98 and YG1024 are shown in Figure 2A and 2B. Similarly, the correlation for the DNBAs is shown in Figure 2C and 2D. A good correlation was observed for the relative energies of the nitrenium ions, both with and without solvent simulation (Table 3). When the 11-NBA is not considered the correlation becomes better. An explanation for the very weak mutagenicity of 11-NBA in the Ames assay awaits further investigation. However, the results shown in Table 3 (see also Figure 2) firmly support the argument that the stability of the nitrenium ions could be one of the major factors in their ability to damage DNA and to subsequently induce mutations.

For the MNBAs a better correlation was observed against *S. typhimurium* TA98 than against *S. typhimurium* TA100 (Table 3). It is noteworthy that *S. typhimurium* TA98 has a *hisD3052* mutation leading to a frame-shift reversion event whereas *S. typhimurium* TA100 has a *hisG46* mutation leading to a base-pair substitution event (Mortelmans and Zeiger, 2000). For the DNBAs

the correlation strongly depended on the calculation mode, namely with or without solvent simulation (Table 3). DNBAAs have two possible activation points, which may both be partially active during the metabolic activation. It is plausible that the complex metabolism of the DNBA is not entirely reflected by the “simplified” thermochemical calculations, resulting in the observed discrepancy. Furthermore, the optimised structures from the *in-vacuo* calculations are used for the water simulations, which might have a considerable effect on the energy minima. Experimental data on a larger number of NBA derivatives would help to substantiate the linear correlations observed and enhance our ability to build theoretical models to understand the genotoxicity of the NBAs.

Intuitively, one might associate instability with more DNA damage. However, the most stable nitrenium ion of a MNBA, 3-NBA, induces the highest DNA damage according to the experimental data (Takamura-Enya *et al.*, 2006). This can be explained in terms of nitrenium ion life-time in the cell. A very reactive chemical species has a short life-time and will be more prone to react with biomolecular structures near to where it is formed in the cytoplasm like cytoplasmic proteins. In prokaryotic cells a more stable electrophilic species may be able to diffuse through the cell’s cytoplasm and reach an electron-rich macromolecule, in this case DNA. The selectivity of various nitrenium ions has been extensively studied by Novak and colleagues (Novak *et al.*, 2002a). In these studies the azide anion (N_3^-) has been used in “azide clock” experiments, which provide k_{az}/k_s , a ratio of the second-order rate constant for trapping of the nitrenium ion by N_3^- and pseudo-first-order constant for trapping the solvent (in most cases, water) (Novak *et al.*, 1993). If the k_{az} for these ions is diffusion limited ($\sim 5 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) the life-time of the nitrenium ions can be deduced and it is reported to lie between 10 μs to 1 ms, depending on the structure of the ions (Novak *et al.*, 2002a). Furthermore, the life-time of nitrenium ions has also been measured using laser-flash photolysis and was shown to range between 40 ns to 16 μs (Bose *et al.*, 1999). This huge difference in nitrenium ions’ life-time, dependent on structure, supports the idea presented in our study that more stable ions have a longer life-time and can diffuse some distance within the cells. Also, the k_{az}/k_s ratio, sometimes denoted as log S, can be interpreted as the stability of the nitrenium ion investigated. The logarithm of the k_{az}/k_s ratio has been plotted against the mutagenic potency of different aromatic and heterocyclic amines in *S. typhimurium* TA98 and TA100 and a rough linear correlation was observed (Novak *et al.*, 2002b; Nguyen and Novak, 2007). Collectively, these findings are in line with our results. However, factors other than the stability of the nitrenium ions, *e.g.* the properties of mammalian enzymes, may also play a role in determining the extent of DNA damage (Arlt *et al.*, 2007).

SUMMARY AND CONCLUSIONS

DFT is a widely applicable and robust method to calculate thermochemical parameters. Using the DFT method we found that among the thermochemical parameters calculated a good correlation was observed between the theoretical energetics of the nitrenium ions derived from NBAs and the experimental mutagenic activity of these NBAs measured in the Ames assay. Overall, the relative energies of the nitrenium ions of the MNBAs showed a stronger correlation than those of the DNBAAs. The association between a compound's mutagenic potency and the stability of its corresponding nitrenium ion has also been observed by others. Among a large group of aromatic and heterocyclic aromatic amines, derivatives with the highest electron density on the exocyclic nitrogen, *i.e.* the most stable species, were the most mutagenic ones (Borosky, 2007). Thus, the main conclusion of this work is that the relative stability of the nitrenium ions may be the main factor governing their ability to damage DNA in *Salmonella* tester strains. However, a recent study also suggests that, in mammalian cells, a combination of both physicochemical and enzymatic properties related to 2- and 3-NBA activation may account for the large differences in their genotoxicities (Arlt *et al.*, 2007). Therefore, determining the capability of human enzymes to metabolize NBAs will be important in human risk assessment.

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Table 1. The calculated energies of MNBA binding to nitroreductase models

Derivatives	SrnA (NfsA)	Cnr (NfnB)
	Intermolecular Energy [kcal/mol]	Intermolecular Energy [kcal/mol]
1-NBA	-5.56	-5.97
2-NBA	-5.93	-6.10
3-NBA	-5.89	-5.59
9-NBA	-5.68	-6.23
11-NBA	-5.72	-7.13

Table 2. The calculated electron affinities (reduction), water elimination, esterification and solvolysis energies, and the relative energies of the nitrenium ions

Derivatives	Reduction [kcal/mol] ¹		Water Elimination [kcal/mol]		Esterification [kcal/mol]		Solvolysis [kcal/mol]		Nitrenium ions [kcal/mol]	
1-NBA	-82.7	-44.3	40.2	201.2	-2.3	-6.9	25.4	133.2	7.1	5.4
2-NBA	-71.6	-42.8	41.8	212.2	-3.1	-7.2	33.2	144.5	8.4	12.2
3-NBA	-77.4	-47.6	29.0	196.6	-3.8	-8.9	22.5	130.9	0.0	0.0
9-NBA	-77.3	-42.2	41.3	203.4	-3.1	-7.0	27.3	135.6	2.9	3.0
11-NBA	-75.6	-37.9	34.3	197.6	-7.4	-10.6	24.6	133.4	7.5	6.5
1,9-DNBA	-88.5	-56.1	44.0	209.1	-3.6	-4.0	33.2	138.2	0.0 ³	6.7
			49.7	212.7	-5.0	-3.8	35.2	141.6		
3,9-DNBA	-88.4	-59.5	49.7	206.3	-5.3	-1.5	44.3	140.1	0.1	0.0
			44.2	214.1	-11.8	-8.7	32.4	140.7		
3,11-DNBA	-88.0	-55.0	41.3	203.5	-9.6	-7.6	39.2	130.3	2.9	7.0
			45.6	210.9	-14.9	-1.7	38.2	143.7		
3,9,11-TNBA	-94.1	-66.8	48.9	217.8	-3.5	-7.9	34.6	148.4	NA ⁴	NA
			58.0	223.4	— ²	-1.3	—	149.8		
			51.9	217.8	0.2	-5.5	35.3	150.9		

¹ Physicochemical parameters have been calculated as described in Material and Methods. The first values given in each column are with solvent simulation whereas the second values given are without solvent simulation, *i.e. in vacuo*.

² Water simulation did not converge.

³ For DNBA's only the most stable ions is given.

⁴ Not applicable.

Table 3. Correlation of the relative energies of the nitrenium ions with the natural logarithm of the mutagenic potency of the MNBA and DNBA observed in different *Salmonella typhimurium* strains

Derivatives	Mutagenic potency (revertants/nmol, -S9 mix) ¹					
	TA98	TA100	TA98NR	TA98/1,8-DNP ₆	YG1021	YG1024
1-NBA	5560	4590	5230	4780	8480	19830
2-NBA	160	390	8	160	1820	3060
3-NBA	208400	29790	150200	52330	128900	6292000
9-NBA	84870	3270	27490	21530	26100	490400
11-NBA	6	6	3	2	10	46
1,9-DNBA	41500	280	3150	3280	17640	268900
3,9-DNBA	46560	4340	57550	51140	25070	223700
3,11-DNBA	3330	360	2520	4770	3110	12200
3,9,11-TNBA	29000	174	2400	660	8270	230800
$r(\text{MNBA})^2$	-0.84 ³	-0.69	-0.85	-0.76	-0.72	-0.88
(n=5)	(-0.72)	(-0.56)	(-0.83)	(-0.63)	(-0.54)	(-0.70)
$r(\text{DNBA})$	-1.0	-0.39	-0.55	-0.35	-0.99	-1.0
(n=3)	(-0.56)	(-0.99)	(-1.0)	(-0.98)	(-0.64)	(-0.48)

¹ The mutagenic potency data have been reported recently (Takamura-Enya *et al.*, 2006). TA98: standard strain (~TA1538 plus plasmid pKM101, which contains a gene encoding a translesion-synthesis DNA polymerase); TA98NR: TA98-derived clone with nitroreductase deficiency; TA98/1,8-DNP₆: TA98-derived clone resistant to 1,8-dinitropyrene due to lack of acetyltransferase; YG1021: TA98 with plasmid-mediated strong overexpression of nitroreductase; YG1024: TA98 with plasmid-mediated strong overexpression of acetyltransferase. All TA98 strains contain the same frameshift mutation at *hisD3052*. TA100: standard strain (~TA1535 plus plasmid pKM101), which contains a base-substitution mutation at *hisG46*.

² R is the Pearson's correlation coefficient and n number of data points.

³ Values given are with solvent simulation; values given in parentheses are without solvent simulation, *i.e. in vacuo*.

Figure 1: The proposed metabolic activation and DNA adduct formation of the NBAs. See text for details. Inset: structure of benzanthrone indicating positions of substitution with 1, 2, or 3 nitro groups.

Figure 2: The correlation of the relative energies of the nitrenium ions in water and the natural logarithm of the mutagenic potency in *Salmonella typhimurium* strains TA98 (A and C) and YG1024 (B and D). In the *upper panels* (A and B) correlations are shown for MNBAs, whereas in the *lower panels* (C and D) correlations for both DNBA are presented. R, Pearson's correlation coefficient; N, number of data points.