Preliminary evaluation of mutagenic activity of two amino derivatives of cyclopenta[*c*]phenanthrene*

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ABSTRACT

The mutagenicity of two, newly synthesized amino--derivatives of cyclopenta[*c*]phenanthrene (CP[*c*]Ph) was investigated in the Ames test using TA98 and TA100 histidine dependent *Salmonella typhimurium* strains. Neither of the examined compounds showed mutagenic activity without metabolic activation. The incorporation of an activation system caused high mutagenicity of CP[*c*]Ph derivatives in both *S. typhimurium* strains. The results are compared with the previous data on the mutagenic activity of unsubstituted CP[*c*]Ph and possible mechanisms of activation are discussed.

INTRODUCTION

One of the major problems of society is the growing pollution of the environment. Among the many pollutants, polycyclic aromatic hydrocarbons (PAHs) are implicated in cancer causation more frequently than others. Their mutagenic and, in some cases, carcinogenic activity correlates with the structure of their carbon skeleton and with the substitution pattern. Among many PAH derivatives, aromatic amines are considered to be highly mutagenic and carcinogenic structures and have been widely found in the environment; the contamination by amino and nitro derivatives of PAHs is mostly correlated with anthropogenic sources (Dimashki et al. 2000; Murahashi et al. 2001). Usually, these compounds arise from the incomplete combustion of fossil fuels, production of mineral oil (International Agency for Research on Cancer (IARC) – Summaries & Evaluations 1987a), and from the rubber industry (IARC – Summaries & Evaluations 1987b). Consequently, they are present in wastewaters (Zhao et al. 1998), but unexpectedly, also in hair dyes (IARC – Summaries & Evaluations, 1993; Yu et al. 2002), and cigarette smoke (Forehand et al. 2000; IARC – Summaries & Evaluations 2002).

The high mutagenic and carcinogenic potential of amino- and nitro-PAHs, puts these compounds among the most dangerous to human life and health (Grimmer et al. 2000). Detailed studies of a number of chemical carcinogens, including PAHs, have shown that after these molecules enter the tissue of test animals they are metabolized into yet other chemical species. Thus, evaluation of the mutagenic effects caused by these bioactivated molecules, helps one to understand whether an arrangement is, or is not, potentially carcinogenic (Łuczyński et al. 2005).

The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay, which measures $his^- \rightarrow his^+$ reversion induced by chemicals that cause mutations, frameshift or base changes, in the genome of this bacteria. *S. typhimurium* reverse mutation assay detects mutation in a gene of a histidine--requiring strain to produce a histidine independent strain of this organism (Ames et al. 1975). Briefly, the rat liver homogenate is mixed with the test compound, which often results in the conversion of the test compound to a chemically activated stage. This mixture is applied to a dish of mutant *Salmonella* bacteria which require histidine in their culture medium to grow up. Because histidine is out of the medium, only those bacteria that are mutated to his^+ genotype will be able to grow. The yielded colonies are counted, the number of which indicates the mutagenicity of the tested compound. In the years that followed, the Ames test was employed to screen various substances for their carcinogenic powers, and thus for their threat to human health.

So far, the mutagenic potential of a number of derivatives of cyclopenta[*a*]phenanthrene and chrysene has been examined.

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For example, Catterall et al. (2001) presented data on the mutagenicity of the mentioned compounds functionalised with amino group. Surprisingly, few data are available on cyclopenta[*c*]phenanthrene (CP[*c*]Ph) – only Marrocchi et al. (1996) have reported moderate mutagenic activity. In 2005, we began research on the chemical and biological properties of CP[*c*]Ph and its derivatives (Brzuzan et al. 2006). So far, two pathways of conversion of inactive compounds to their electrophilic metabolites have been proposed with respect to the mutagenic activity of amino-derivatives of PAHs. Whereas, one model is based on the activation of a ring A of a PAH molecule, which causes the formation of bay or fjord region diol-epoxides, the other is based on the activation of the amino function to nitrenium ion (Dipple 1995). In this study we designed and synthesized two compounds based on the CP[*c*]Ph substituted in the way allowing one to critically test the proportions of mutagenic properties of specific compound derived from the two mentioned metabolic activation pathways. We determined the mutagenic activity of two amino derivatives of cyclopenta[*c*]phenanthrene (CP[*c*]Ph): 5–amino–2,3–dihydro–1*H*–cyclopenta[*c*]phenanthrene (ACP[*c*]Ph) and 5–amino–9–methoxy–2,3–dihydro–1*H*–cyclopenta[*c*]phenanthrene (AMCP[*c*]Ph) in the Ames test using two strains of *Salmonella typhimurium*: TA98, which detects mutagens that cause frameshift mutations, and TA100 detecting base-pair substitutions. The experiments were conducted with and without metabolic activation. Neither of the examined compounds showed mutagenic activity without metabolic activation. However, metabolic activation of either compound resulted in a high number of *Salmonella* revertants indicating their high mutation powers.

MATERIAL AND METHODS

Chemistry

5–amino–2,3–dihydro–1*H*–cyclopenta[*c*]phenanthrene (ACP[*c*]Ph) and 5–amino–9–methoxy–2,3–dihydro–1*H*–cyclopenta[*c*]phenanthrene (AMCP[*c*]Ph) (Figure 1) were synthesized from respective naphthalenecyclopentanones (Góra et al. 2005) in the three-step reaction described in our previous study (Brzuzan et al. 2006; Góra et al., unpublished results). These comprised of the condensation of adequate ketones with malononitrile and cyclisation of obtained naphthylcyclopentylidenemalononitriles in strong inorganic acids. The desired amines were obtained by the removal of the nitrile group from synthesized vicinal aminonitriles in the high pressure alkaline cleavage. Both structures were confirmed using spectroscopic methods.

5–amino–2,3–dihydro–1*H*–cyclopenta[*c*]phenanthrene (mp. 133–134°C)

^{1}H NMR ($CDCl_3$) δ [ppm]: 2.22 (quint, 2H, C**H$_2$**, *J*=7.4 Hz); 3.06 (t, 2H, C**H$_2$**, *J*=7.5 Hz); 3.69 (t, 2H, C**H$_2$**, *J*=7.4 Hz), 4.07 (s, 2H, N**H$_2$**), 6.94 (s, 1H, Ar-**H**), 7.55-7.61 (m, 2**H**, Ar-**H**), 7.65 (d, 1H, Ar-**H**, *J*=7.5 Hz), 7.78 (d, 1H, Ar-**H**, *J*=7.5 Hz), 7.87 (dd, 1H, Ar-**H**, *J*=7.85 Hz; *J*=1.5 Hz), 8.80 (d, 1H, Ar-**H**, *J*=8.5 Hz).

^{13}C NMR (CDCl3) δ [ppm]: 25.71, 33.45, 37.22, 109.67, 120.32, 120.56, 124.94, 125.56, 125.86, 127.04, 128.21, 129.18, 130.43, 131.84, 133.14, 141.51, 144.55.

anal. found: C 87.73, H 6.58, N 5.97%; calc. for $C_{17}H_{15}N$: C 87.52, H 6.48, N 6.00%

5–amino–9–methoxy–2,3–dihydro–1H–cyclopenta[*c*]phenanthrene (mp. 122–123°C)

^{1}H NMR (CDCl3) δ [ppm]: 2.26 (quint, 2H, C**H$_2$**, *J*=7.5 Hz), 3.20 (t, 2H, C**H$_2$**, *J*=7.0 Hz), 3,63 (t, 2H, C**H$_2$**, *J*=7.2 Hz), 3.97 (s, 3H, OC**H$_3$**), 4.87 (br. s, 2H, N**H$_2$**), 7.23-7.26 (m, 2H, Ar**H**), 7.64 (d, 1H, Ar**H**, *J*=9.2 Hz), 7.68 (d, 1H, Ar**H**, *J*=9.2 Hz), 8.70 (d, 1H, ArH, *J*=9.8 Hz).

^{13}C NMR (CDCl3) δ [ppm]: 25.61, 33.45, 37.10, 55.28, 108.35 108.76, 115.71, 119.62, 120.92, 124.45, 126.18, 128.56, 129.21, 129.56, 134.65, 141.55, 144.58, 157.43.

anal. found: C 82.26, H 6.33, N 5.50%; calc. for $C_{18}H_{17}NO$: C 82.13, H 6.46, N 5.32%

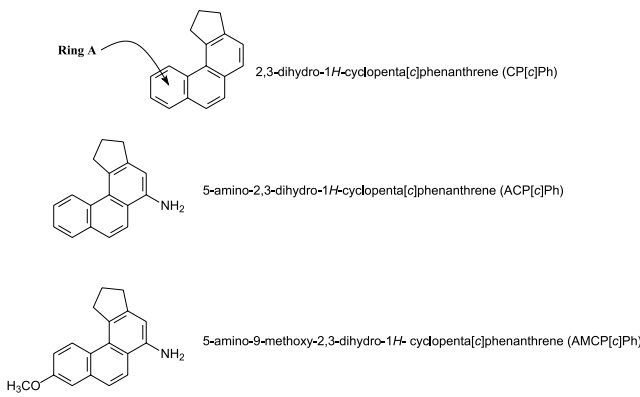

Figure 1. The structure of the examined compounds.

Bacterial mutagenicity assays

The *Salmonella typhimurium* TA98 and TA100 strains and S9 activation system were purchased (Trinova Biochem, Germany).

A plate incorporation test was performed according to the revised method of Maron and Ames (1983). All chemical concentrations studied were examined in triplicate plates, with and without adding S9 mixture. To a test-tube containing 2 ml of molten top agar (0.6% Bacto-agar (Oxoid), 0.5% NaCl), the mixture of 0.04 mM L-histidine and 0.5 mM biotin was added. The test samples (400 μl) consisted of 100 μl of an overnight broth culture of the bacterial tester strain and 0.5 ml of the S9 mix. The S9 solution contained 8 mM $MgCl_2$, 33 mM KCl, 5 mM G-6-P, 4 mM NADP

and 100 mM sodium phosphate (pH = 7.4). The S9 level routinely used was 50 μl/plate corresponding to 3 mg of protein/plate. The mixture was vigorously stirred, poured immediately onto plates of minimal agar and incubated at 37°C. The plates were incubated for 48 h and then histidine-independent revertant colonies were counted with the use of an automatic colony counter. In addition, the number of revertants in positive controls (with diagnostic mutagens) and negative controls ("blank-samples") were determined.

According to the Organisation for Economic Co-operation and Development (OECD 1997) there are several criteria for determining a positive result, such as a concentration--related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate. To select the doses with linear dose vs. response relationship, a method of point rejection was applied (Bernstein et al. 1982). The mutation effect was expressed also as mutagenic rate (MR). MR is a ratio of the number of induced revertants to a number of revertant colonies in the appropriate negative control. The sample was considered evidently mutagenic when its mutagenic rate was MR ≥ 2.

RESULTS

Neither of the examined compounds, ACP[*c*]Ph or AMCP[*c*]Ph, showed mutagenic activity without metabolic activation (-S9). (Both compounds without metabolic activation induced the number revertants similar or little higher than the number of revertans in appropriate controls.)

The three independent preliminary experiments were performed for four concentrations of each compound: 0.10, 1.00, 10.00 and 100.00 μg per plate. Because in the highest dose examined, decreasing number of revertants were observed for both strains, additional experiments were performed for the following doses: 0.05, 0.50, 5.00 and 50.00 μg per plate. Combined data for all four experiments in the form of arithmetic average (± standard error, SE) are shown in Figure 2 (A strain TA98) and (B strain TA100). Number of revertants for 0 dose or (otherwise) the number of revertants in negative controls (DMSO and S9-mix) were for strain TA98: 39.1±15.9 and for TA100: 97.6±66.4. For both strains the number of spontaneous revertants were similar to the number of induced revertants in negative controls (TA98 – 35.0 vs 39.1; Ta100 – 111.5 vs 97.6).

The number of *Salmonella* TA98 revertants yielded by the exposure to ACP[*c*]Ph (+S9) or AMCP[*c*]Ph (+S9) increased gradually for either compound, and showed the following pattern: a slight, but significant increase in the number of revertants induced for the dose 1.00 μg (per plate), a very high increase for the dose 5.00 μg, and a significant decrease for the next doses: 10.00, 50.00 and 100.00 μg.

In case of the strain TA100 both compounds induced also the highest number of revertans for the dose of 5.00 μg (per plate). For the highest doses of ACP[*c*]Ph, a slow decrease

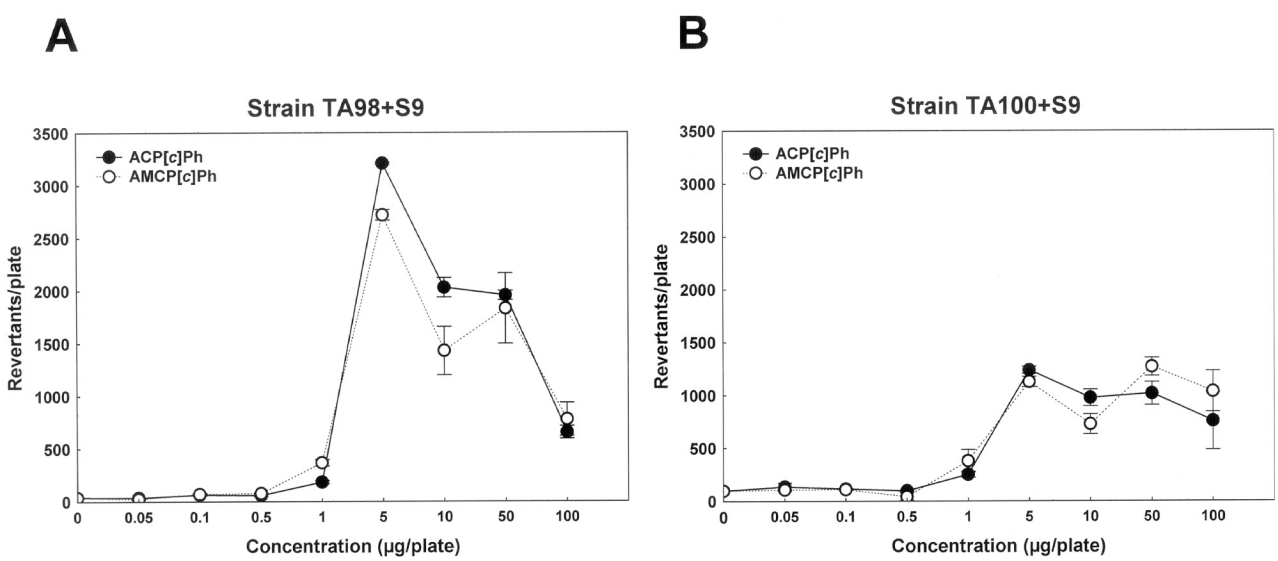

Figure 2. Mutagenicity of ACP[*c*]Ph and AMCP[*c*]Ph in the presence of an activation system in the *Salmonella typhimurium* strains TA98 (graph A) and TA100 (graph B). Results are presented as mean ± S. E. of triplicate plates.

Table 1. Number of induced revertants (IR) per μg and mutagenic rate (MR) for ACP[*c*]Ph and AMCP[*c*]Ph in the presence of an activation system S9 mix Trinova Biochem.

Strain	ACP[*c*]Ph		AMCP[*c*]Ph	
	IR/μg	MR/μg	IR/μg	MR/μg
TA98+S9	578.3	13.8	470.6	11.0
TA100+S9	309.4	2.3	278.0	1.8

in the number of revertants was observed. For the doses 10.00, 50.00 and 100.00 μg of AMCP[*c*]Ph per plate the plateau effect (fluctuation with tendency to stabilisation) was observed.

The number of spontaneous revertants (SR) were different and variable, therefore the value of mutagenic effect of the discussed PAH derivatives was also expressed as relative mutagenic rate (MR) of 1 μg. Since both experiments revealed significant dose-response relationships for ACP[*c*]Ph or AMCP[*c*]Ph and the *Salmonella* strains used, we estimated the number of revertants induced per 1.00 μg of either compounds to compare their mutagenic effect. The number of induced revertants per μg as well as the mutagenic rate for examined compounds are shown in Table 1.

DISCUSSION

The PAH molecules designed for this study had the amino function in the same position as the CP[*c*]Ph skeleton, but the possible activation of *pseudo*-fjord region in one of them was blocked by substitution with inactive methoxy group. The metabolism of amino-PAHs may include the oxidation of the amino function by cytochrome P450 enzymes to form hydroxylamine which can be transformed into *N*-esters in the enzyme-catalysed reaction. *N*-hydroxyl, *N*-acetoxy ester as well as *N*-sulfonate ester can subsequently form the nitrenium ion, a potent electrophile. It is possible that both, the epoxidation of ring A and oxidation of amino function may occur. Both metabolites are probably able to form a covalent bond with the bases within DNA. This novel molecular structure (adduct) arising after covalent linkage of a mutagen with one or another portion of DNA molecule often leads to a mutation, which eventually may initiate cancer. Our results, compared to the data reported previously on the mutagenic activity of unsubstituted CP[*c*]Ph skeleton (Marrocchi et al. 1996), led us to hypothesise that a small part of the mutagenic potential of amino derivative is caused by the metabolism of A ring of a molecule, but the effect is additive with the stronger action of the metabolized amino group.

Neither of the compounds examined showed mutagenic activity without metabolic activation. The estimation of mutagenic effect was based on relative index, e.g. mutagenic rate (RM) as more reliable. On this basis it was proved that both compounds cause frame–shift as well as base-pair exchange types of mutation. It seems likely that both, ACP[*c*]Ph and

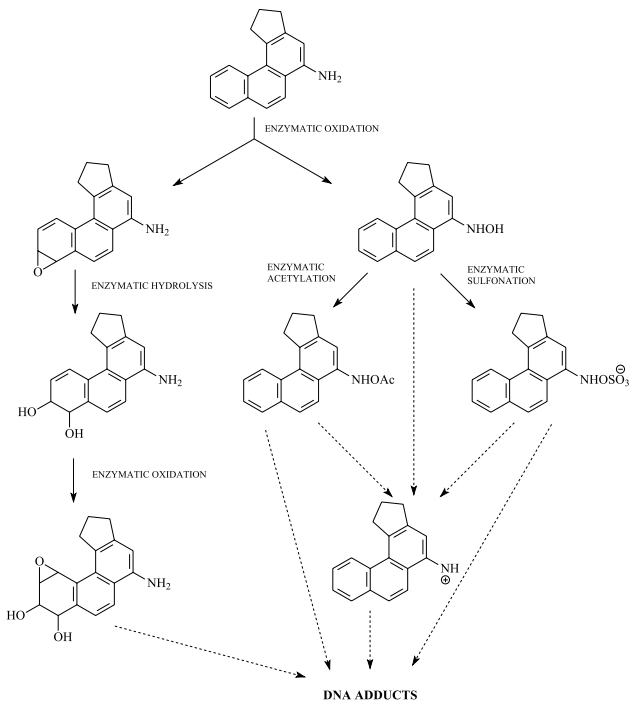

Figure 3. Possible mechanisms of metabolic activation of ACP[*c*]Ph. Both pathways described in the literature are possible. In case of AMCP[*c*]Ph the pathway leading to the active diol-epoxide is blocked by substitution with methoxy group in position 9 of CP[*c*]Ph skeleton.

AMCP[*c*]Ph, are more potent mutagens against TA98 than TA100 *Salmonella* strains. Furthermore, ACP[*c*]Ph was slightly more potent mutagen for either strains than AMCP[*c*]Ph. Finally, both compounds showed higher mutagenic activity than the unsubstituted CP[*c*]Ph, which is a moderate mutagen (Marocchi et al. 1996). We suggest that this may be due to the differences in pathways of the metabolic activation for ACP[*c*]Ph, presented in Figure 3. In the case of unsubstituted ACP[*c*]Ph, both epoxidation of ring A of the molecule and direct oxidation of the amino group to hydroxylamine is possible. The epoxidation step is blocked in the case of AMCP[*c*]Ph, because of the substitution of ring A with the methoxy group. However, other factors such as steric compatibility during the DNA base binding step may play a role (Xue and Warszawsky 2005).

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