Oxidative metabolism, mutagenic and carcinogenic properties of some polycyclic aromatic hydrocarbons

Michał K. Łuczyński[1], Maciej Góra[2], Paweł Brzuzan[3], Jarosław Wilamowski[2], Bartłomiej Kozik[2]

[1] Department of Chemistry, University of Warmia and Mazury in Olsztyn, pl. Łódzki 4, 10-957 Olsztyn, e-mail: michalkl@uwm.edu.pl
[2] Department of Organic Chemistry, Jagiellonian University, ul. Ingardena 3, 30-060 Kraków
[3] Department of Environmental Biotechnology, University of Warmia and Mazury, ul. Słoneczna 45d, 10-712 Olsztyn

ABBREVIATIONS

B[*c*]P	benzo[*c*]phenanthrene	Hep2G	human hepatoma cell line
CP[*a*]P	cyclopenta[*a*]phenanthrene	THB[*c*]P	tetrahydrobenzo[*c*]phenanthrene
CP[*c*]P	cyclopenta[*c*]phenanthrene	THCH	tetrahydrochrysene

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants. The number of publications every year (Kurihara et al. 2005; Ledesma et al. 2000; Meyer et al. 2005; Oleszczuk and Baran 2005; Russo et al. 2005; Scicchitano 2005; Wolska et al. 2005) may facilitate the understanding of how many disciplines and subjects may be connected with PAHs.

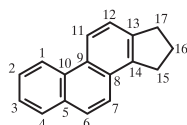
One of the most important applications of PAHs is their use in research on formation of cancer cells (Harvey 1991). In many cases mutagenic and carcinogenic activity of PAHs has been confirmed, and the occurrence of many of them is determined in routine environmental studies. Their level is determined in soil, drinking water, river, lake and marine sediments, and also in air analysis (Harvey 1997). As mussels are able to accumulate PAHs in their tissues, some mollusc species were used in persistent monitoring of the quality of water (Gewurtz et al. 2002).

Papers on anthropogenic sources of PAHs formation are well documented in literature, for example on medical waste plant (Lee et al. 2003) or combustion of coal tar (Ledesma et al. 2000). PAHs may also arise from non-anthropogenic sources, such as in soil as a result of degradation of organic matter (Pope et al. 2000; Thiele and Brummer 2002). A rising number of compounds classified as mutagens or carcinogens, along with restrictions in their emission, are subjected to attempts of the deactivation of PAHs existing in the environment. Among others, methods involving living organisms, such as some bacteria strains capable of neutralizing specific hydrocarbons, have become popular (Caldini et al. 1995). However, biological activity of these compounds is not restricted to

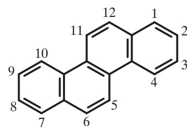
carcinogenicity; some of them are investigated for their anti--carcinogenic properties (Banik and Becker 2001; Becker and Banik 1998). Derivatives of tetrahydrochrysene are considered in research on selective estrogen receptors (Tedesco et al. 2001).

In this paper we present the actual state of knowledge concerning the correlation between the structure of cyclopenta[*a*]phenanthrene (**1**), cyclopenta[*c*]phenanthrene (**4**), chrysene (**2**) and benzo[*c*]phenanthrene (**3**) derivatives and their mutagenic and carcinogenic properties. The oxidative metabolism of these compounds is described with special attention to the paths leading to the most active products. The nomenclature and numbering of the carbon skeleton of polycyclic compounds follows the IUPAC rules (Nomenclature of Organic Chemistry 1979) as well as recommended exception from general rules, used in naming and numbering CP[*a*]P derivatives, which is based on steroid nomenclature (Scheme 1).

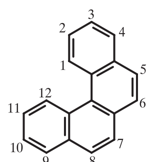
Scheme 1



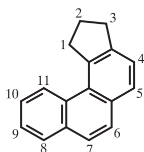
16, 17-dihydro-15*H*-cyclopenta[*a*]phenanthrene (**1**)



chrysene (**2**)



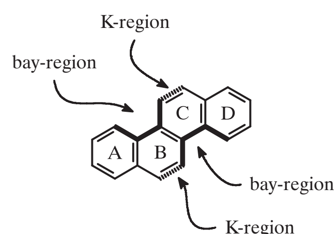
benzo[*c*]phenanthrene (**3**)

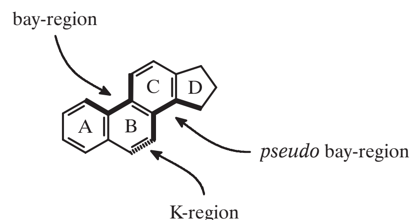


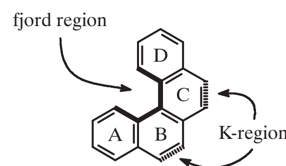
2, 3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (**4**)

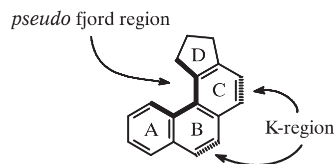
Explanation of terms linked to PAHs structure (such as bay--region, fjord-region, *pseudo* bay-region, *pseudo* fjord-region and K-region [Scheme 2]) seems useful for discussion on the structure-activity correlation of these compounds. Bay-region consists of three aromatic rings, connected in an angular manner, creating a recess which resembles a bay; a *pseudo* bay--region is composed of two aromatic rings and terminal aliphatic ring. Analogically, a fjord region consists of four aromatic rings. In case of *pseudo* fjord-region the terminal ring is saturated. All the mentioned structural elements have a specific impact on mutagenic and carcinogenic activity of PAH derivatives. As will be explained in the following sections, changes made in these fragments of molecule allow for studying the mechanisms of mutations and, in consequence, of cancer cells formation.

Scheme 2









METHODS FOR DETERMINING MUTAGENIC AND CARCINOGENIC PROPERTIES OF PAH DERIVATIVES

Some experiments on the PAHs genotoxic properties can be performed with the use of human-derived cells, which seems to be very important from the human medicine point of view. This subject is revised by Kransmüller and co-workers (2004). Other methods for estimation of different potentially dangerous properties of PAHs will be briefly reviewed; this should facilitate further understanding of the terms used in the text.

Nuclear abnormalities (NA)

Several types of variations from the usual erythrocyte nuclear morphology can be observed following exposure to a PAH contaminant. According to Carrasco et al. (1990), the nuclear abnormalities can be classified into five categories. *Micronuclei* are non-refractory structures, of the same color as the nucleus and with a round, almond or ovoid shape about one-fifth the size of the main nucleus; "*blebbed*" nuclei present a relatively small evagination (bleb) of the nuclear envelope which seems to contain euchromatin; "*lobed*" nuclei are those with evaginations larger than blebs described above; "*notched*" nuclei present an appreciable depth into a nucleus that does not contain nuclear material; and "*vacuolated*" *nuclei* are those with a well-defined hole devoid of any visible material. Whereas the model appeared to be sensitive in screening the mutagenic activity of chemical compounds under laboratory conditions (Pacheco and Santos 1996), the low and variable frequencies of the nuclear lesions scored from the field samples may present serious difficulty to the useful application of the test (e.g. Carrasco et al. 1990).

DNA strand breakage (SB) and comet assay

Sensitive measurements of DNA strand breakage can be achieved by a variety of methods, e.g. alkaline elution, alkaline unwinding or by single cell gel (comet) electrophoresis (reviewed by Mitchelmore and Chipman 1998). Singh and co-workers (1988) were the first to adapt a number of earlier methods to develop an assay of single cell gel electrophoresis under alkaline conditions to emphasize the occurrence of DNA strand breakage. The subsequent visualization of mobility of DNA fragments from the nucleus (the "comet" analysis) is a very sensitive and convenient way to detect strand breakage in individual cells. A range of genotoxic chemicals (both with and without the requirement for metabolic activation) gave positive effects in various cell types of vertebrate and invertebrate aquatic species (see, for example, Pavlica et al. 2000; Woźnicki et al. 2004).

Cytochrome P450

Polycyclic aromatic hydrocarbons stimulate expression of various members of the cytochrome P450 family of genes (*CYP*), particularly those of the *CYP1* family. The induction of hepatic *CYP1A* in fish by certain classes of chemicals has been suggested as an early warning system, a most sensitive biological response for assessing environmental contamination conditions (Arinç et al. 2000, and references therein). This has implications for human consumption of fish, as well as for the health status of aquatic organisms.

The cytochrome P450 detoxification system is, thus, an extensively studied enzyme system and has been found in bacteria, plants, and animals. It is involved in the metabolism of such compounds such as steroids, prostaglandins, eicosanoids, drugs, and xenobiotics (Nelson et al. 1996). Cytochrome P450, the terminal oxidase of monooxygenases, is mainly localized in the endoplasmic reticulum and in mitochondria of vertebrates. It catalyzes oxidation of a number of organic chemicals to more soluble metabolites, which can be further conjugated by Phase II enzymes and then excreted. Cytochrome *P450* genes are highly diverse, approximately 120 different subfamilies of cytochrome *P450* (*CYP*) genes have been identified and characterized by a wide range of xenobiotic-metabolizing functions. The most intensively studied cytochrome P450 protein is cytochrome P4501A, CYP1A (Stegeman et al. 2001).

Two genes (*CYP1A1* and *CYP1A2*) in mammals characterize well the *CYP1A* family. Both genes are coordinately regulated by the same aromatic hydrocarbon receptor (AhR) (see review by Hahn et al. 2005). It is known that CYP1A metabolizes several PAHs to reactive electrophilic metabolites that form DNA adducts and induce oxidative DNA damage. Thus, the increased synthesis of CYP1A may ultimately result in carcinogenicity (Burczynski and Pennig 2000). In fish, CYP1A seems to exist as a hybrid protein coded by a gene ancestral to both mammalian *CYP1A1* and *CYP1A2* forms, and the use of the name *CYP1A* rather than *CYP1A1* has been suggested (Stegeman 1995).

The mechanism of CYP1A induction has been examined in many teleost fish species, including rainbow trout (Cao et al. 2000). Its gene (*CYP1A*) is highly inducible by PAHs, polychlorinated biphenyls (PCB's), furans and dioxins. Early work demonstrating induction of CYP1A mRNA in fish in response to PAH used Northern blot analysis (Stegeman 1995). With advances in molecular biology techniques, determination of CYP1A mRNA levels by quantitative reverse transcription polymerase chain reaction (RT-PCR) has been added to biomonitoring studies (see for example Cao et al. 2000; Rees et al. 2003). A fairly new technology that emerged in the early 1990s is quantitative real-time PCR (Q-PCR). The Q-PCR reaction is monitored in real time by fluorescence either with the incorporation of the SYBR green dye that fluoresces only when it is intercalated into DNA or by a fluorescent probe that is complementary in sequence to the cDNA of interest (Higuchi et al. 1993). Recently Rees and Li (2004) have developed and evaluated a real-time quantitative PCR assay for measuring the induction of liver CYP1A mRNA in four salmonid species.

P53 protein

The *p53* tumor suppressor is an universal sensor of genotoxic stress that regulates the transcription of genes required for cell--cycle arrest and apoptosis in vertebrates as diverse as fish and humans (Soussi et al. 1990). Somatic mutations of the *p53* gene, particularly mutations within the DNA sequence-specific binding domain, are often associated with the loss of tumor suppressor activity and may also result in the gain of oncogenic function

(Greenblatt et al. 1994). Damage of this gene was reported in 50% of a total number of human tumors and in 60% cases of human lung tumors (Bennett et al. 1999; Greenblatt et al. 1994; Harris 1996). Sequencing of the *p53* gene and structural analysis of the p53 protein from several vertebrate species revealed 11 exons with five well-conserved functional domains (I-V) that are involved in DNA-protein interactions and stabilization of protein conformation (Krause et al. 1997). Analyses of mutational spectra from tumors in vertebrates have identified numerous mutational 'hot-spots' which are located primarily within conserved domains II-V and are involved in sequence-specific DNA. A large proportion of *p53* mutations involve G:C→A:T transitions and G:C→T:A transversions, alterations frequently associated with such mutagens as alkylating agents (Crook and Vousden 1992). The fish *p53* sequences have been used in mutational studies to fingerprint genotoxins in the aquatic environment (Bhaskaran et al. 1999).

Ames test

One of the methods for determining mutagenic activity of specific compound is a bacterial test developed by Ames and co--workers (1975). In this simple and quick (48 hours) test specifically mutated strains of *Salmonella typhimurium* are used. Mutants are not capable of synthesizing histidine and are unable to grow colonies on agar without this amino acid. TA 98 and TA 100 are the most popular strains used in research on mutagenic properties of activated PAHs. After the treatment of bacteria with promutagene with activating system (S9 fraction containing microsomal enzymes from rat liver), mutations in some bacterial cells are observed. In this way revertants are formed because action of mutagen "reverts" the inability of certain strains to synthesize histidine, and this, in turn, allows their growth. Counting colonies on a Petri plate and comparing the results with control plates (positive with known mutagen versus negative with no mutagen) mutagenic activity of a given compound can be estimated. There exist many modifications of the Ames test, as well as many new bacteria strains designed for this purpose (Traczewska 2002).

Experiments on rodents

Experiments on rodents are the only kind of investigation that allow for defining a compound as carcinogen. Such experiments are very expensive (several hundred experimental animals needed) and long-lasting (about 2 years) (Williams et al. 2000). In experiments on rodents (skin painting test) Iball index is used for quantitative estimation of carcinogenic activity of a specific compound (Coombs and Bhatt 1987).

$$\textbf{Iball index} = \frac{\textbf{percentage of animals with tumor*}}{\textbf{mean latent period in days}} \textbf{ x 100}$$

*based on the number of animals alive at the time of the apperance of the first tumor at that group.

Theoretical predictions

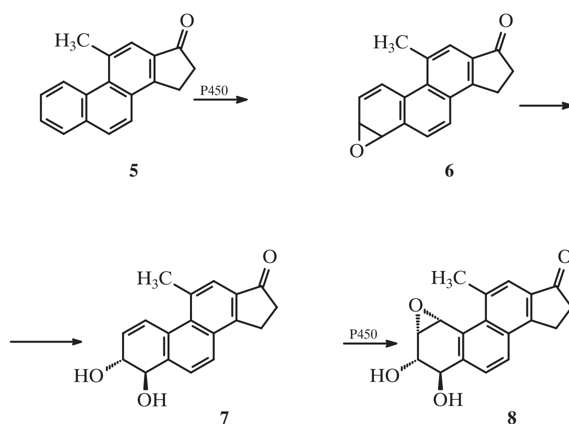
Apart from biological methods that allow for obtaining direct results on mutagenic or carcinogenic activity, there exist a number of computer databases and software for theoretical prediction of such properties. Such methods provide information that justifies further biological experiments. For example program DEREK [**D**eductive **E**stimation of **R**isk from **E**xisting **K**nowledge (after Long and Combes 1995)] is based on the correlation of the structure of the investigated compound with existing knowledge about the effects caused by similar structures. Structural data combined with data concerning potential metabolizing of similar structures by P450 enzymes leading to active arrangements (like bay-region diolepoxides) allow the prediction of potential mutagenic or carcinogenic activity.

METABOLIC ACTIVATION OF POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons themselves are not carcinogenic; their carcinogenic activity is generated on the metabolic pathway. Concerning the variability of oxidizing systems based on the family of CYP1 (P450), oxidation of different parts of a molecule is possible.

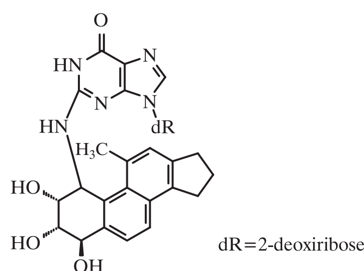
In the case of PAHs the correct mechanism of removing the potential toxin from the organism, leads through oxidation of K-region of a molecule (Jerina and Daly 1974). Such products are capable of binding to glucuronic acid or glutathione and in this form they are removed from the organism with other products of metabolism. K-region has the highest electron density, which makes it a place for almost defined and localized double bond. Usually the metabolism of a small percentage of molecules goes in a different way, and this can lead to mutagenic derivatives. The percentage of this inappropriate metabolic pathway of certain PAHs is a result of the specific structure of hydrocarbon. Interaction between the terminal aromatic ring of hydrocarbon with cytochrome P450 (Shimada et al. 2001) leads to epoxidation of aryl double bond in, for example, ring A of 11-methyl-CP[*a*]Ph--17-one (**5**). In the next step epoxide is hydrolyzed, and another bond is oxidized leading to diolepoxides or tetraols (Scheme 3).

Scheme 3



The way in which most PAHs metabolites interact with nucleosides is shown on Scheme 4. (Bachechi and Polcaro 1999) Electrodonating groups present in a bay region of a molecule accelerate hydrolysis of diolepoxides and increase their chance for covalent binding to DNA, eventually leading to mutation.

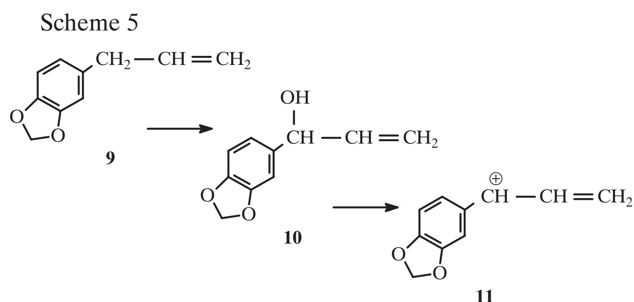
Scheme 4



As mentioned above, the metabolic path leading to mutagen or carcinogen goes through a state of epoxide, diol and finally diolepoxide (Scheme 3). However, depending on the activating medium used, a variety of products is obtained. For example, activation of ketone **5** with microsomal fraction of rat liver, mainly follows the way shown on scheme 3 (Abbott and Coombs 1981; Coombs et al. 1979). Metabolic profile of compound **5**, obtained by influence of Hep2G does not show the effect of the action of the P450 system that should lead to epoxidation of ring A of the molecule; instead, one of the main metabolites is 17-alcohol. This can be explained by the presence of specific reductase in this line that reacts with carbonyl function of compound **5**. The presence of other metabolites of ketone **5** is probably an effect of the action of aromatic hydrocarbon hydroxylase system (AHH) which is also present in this line. Examples of complexity of metabolism of 16,17-dihydro-11-methylCP[*a*]P depending on of the type of cells used in the experiment are described by Bhatt (1986). An interesting comparison of metabolic profile of benzo[*a*]pyrene, chrysene (**2**) and phenanthrene, activated with microsomal fraction from rat liver and catfish (*Ictalurus nebulosus*), has been described by Pangrekar and coworkers (1995). The main conclusion concerns a significantly lower degree of metabolizing PAHs by fish microsomes in comparison with the action of rat microsomes. Analogical experiments performed with chrysene (**2**) and 5-methylchrysene using microsomes of rat and rainbow trout (*Oncorhynchus mykiss*) showed a very similar degree of transformation of the studied hydrocarbons (Shappell et al. 2003).

The process of oxidation of aromatic rings is not the only way to produce hazardous derivatives of PAHs. As described recently, there exists an alternative way, leading through enzymatic oxidation of alicyclic rings of CP[*a*]P (Catterall et al. 2000). 11-Methoxy-16,17-dihydro-15*H*-CP[*a*]P activated with microsomal fraction of rat liver (S9) produced a mixture of metabolites that were separated and identified by their comparison to the compounds obtained in a synthetic way (Coombs and Boyd 1998). The Ames test showed that only the 16,17-dihydro-15-hydroxy-11-methoxy-15*H*-CP-[*a*]P proved to be a mutagen. A way to explain this surprising fact is based on an analogy to safrole (**9**), which is a weak hepatocarcinogen, and in an attempt to find structural correlation between these two compounds. Inactivation of safrole goes through hydroxylation of benzylic position, formation of sulfuric ester and cleavage of C-O bond. Carbocation (**11**) obtained in this way is carcinogenous (Scheme 5).

Scheme 5



According to Coombs and Boyd (1998), "alternative" activation of the 16,17-dihydro-15-hydroxy-11-methoxy-15*H*-CP[*a*]P could go through analogical transformations.

Metabolism of PAHs substituted with amino group and metabolites of such compounds have not been investigated so far. Their activation process is described only theoretically, and there are two hypotheses: the classical one leading to diolepoxides and the other, based on direct oxidation of an amino group, leading to nitrenium ion $ArNH^+$(Ford and Griffin 1992; Ford and Herman 1992).

CORRELATION BETWEEN STRUCTURE AND MUTAGENIC OR CARCINOGENIC ACTIVITY OF POLYCYCLIC AROMATIC HYDROCARBONS

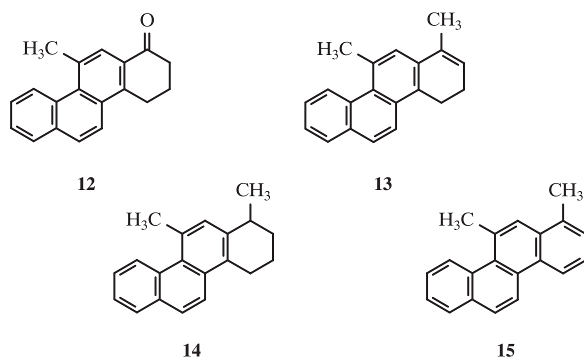
Relationships between the structure of PAHs and their mutagenic or carcinogenic activity may be of dual nature. One is related to the structure of the aromatic skeleton of the molecule. For instance, unsubstituted benzo[*a*]pyrene is one of the most potent procarcinogens examined. However, recent studies encompass not only the structure of aromatic fragment but, first of all, the substitution pattern of its skeleton. For example, 3-methylcholantrene exhibits even higher mutagenic properties than benzo[*a*]pyrene. Investigations concerning the relationship between the structure of an arrangement and its biological activity usually comprise the same aromatic skeleton, but vary in the location and character of substituents (Coombs and Bhatt 1987).

In structure-activity studies deformation of planarity of an aromatic molecule is one of the most important issues. Determination of this effect can be accomplished in two

ways: one of them is the introduction of a substituent into a bay region, and another is benzannelation of appropriate parts of a hydrocarbon. When the bay region is changed into the fjord region, the planarity of a molecule also becomes deformed. This change provides easier access to an epoxide ring of carcinogenic diolepoxide which, in consequence, facilitates its covalent binding to the DNA.

In the case of CP[*a*]P (**1**), a very significant increase in its harmful biological properties is observed when a double bond is introduced into an alicyclic ring, or when a hydrocarbon is transformed to ketone by oxidizing the aliphatic part of a molecule (Coombs and Bhatt 1987). An analogical relationship is observed in the case of THCH (Scheme 6).

Scheme 6



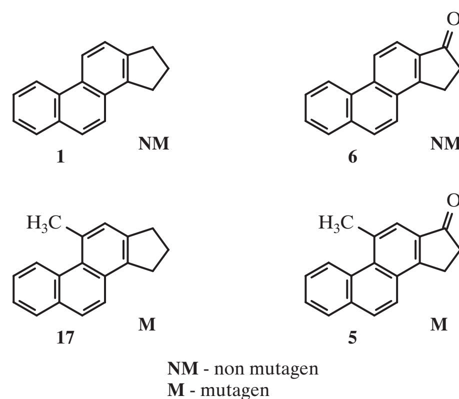
Ketone **12** is the most potent carcinogen, exceeding harmful properties of compounds **13**, **14**, and also dimethylchrysene **15** (Hecht et al. 1974).

As mentioned earlier, the bay region of a PAH molecule constitutes its most important part, and is responsible for its mutagenic activity. CP[*a*]P (**1**) provides a very good example for this theory – the unsubstituted hydrocarbon does not exhibit any significant activity while its derivative, substituted in position 11 with an electrodonating group (e.g. methyl), becomes a highly potent mutagen. A similar effect is induced by introduction of methoxyl or hydroxyl group. Recent studies on the effects of an amino group in position 11 of CP[*a*]P (**1**) agree well with the supposition that an activating group in a bay region of PAH increases its mutagenic properties (Catterall et al. 2001).

Cyclopenta[*a*]phenanthrene

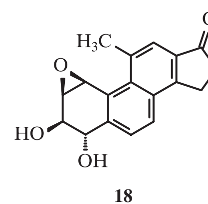
Monograph by Coombs and Bhatt (1987) brings a detailed description of the correlation between the substitution pattern of CP[*a*]P (**1**) and mutagenic and carcinogenic properties of a specific compound. However, this work does not address all of the possibilities, limiting the scope of substituents to methyl and alkoxy groups. The authors conclude that unsubstituted hydrocarbon (**1**) as well as its 17-keto derivative (**16**) is neither mutagenic nor carcinogenic. Nevertheless, substitution with an electrodonating group in position **11** increases their mutagenic and carcinogenic activity (Scheme 7).

Scheme 7



Detailed research of Baker et al. (1992), who compared biological activity of 11-methyl-17-ketone **5** and unsubstituted 17-ketone **16**, showed that only ketone **5** substituted in position 11 with methyl group exhibits carcinogenic activity. In addition, the micronuclei test did not show any significant activity of ketone **16**, while its 11-methyl derivative **5** has strong carcinogenous activity, comparable to that of benzo[*a*]pyrene. It should be noted, that both compounds give a positive response in the Ames test (Coombs et al. 1976). A suggested explanation is that the different structure of metabolites of both compounds vary in their mutagenic potential. An *in vitro* experiment showed, that ketone **5** is metabolized to trans-3R, 4R-dihydrodiol-anti-1,2-epoxide (**18**), while the metabolite of unsubstituted ketone **16** is of conformation syn (Hadfield et al. 1984) (Scheme 8).

Scheme 8

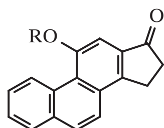


This explains the lack of response in the micronuclei test as well as in the *in vivo* experiment on mice, leading to the conclusion that syn-diolepoxide is not able to form stable DNA adducts. Another important position in this structure is the substitution of the C-7 carbon atom with the methyl group. This substitution may be considered as located in the so called *pseudo* bay region of a molecule. Biological activity of such compounds is significantly higher than that of unsubstituted ketone **16**, but not to such an extent as in the case of the previously described 11 carbon atom substitutions. Analogical relationship has been observed in the case of substitution with methoxy group (Coombs and Bhatt 1987).

According to the described metabolic pathway that leads to diolepoxides as a result of oxidation of A ring of CP[*a*]P (**1**), alkyl substituents in this ring should block this kind of activation and, therefore, prevent the mutagenic activity of a given compound (Coombs and Bhatt 1987). Lack of mutagenic activity of CP[*a*]P (**1**) substituted with the methyl group in position 6 is of interest because this substitution does not block oxidation of ring A. This is explained by the specific conformation of the by-product 3,4-dihydro-3,4-diol (Boyd et al. 1995a). In arrangements that exhibit mutagenic properties this conformation is described as *pseudo*-diequatorial. In the case of the discussed compound **5**, the conformation of diol is *pseudo*-diaxial which can prevent accommodation or even block the entry of this metabolite into the active site of cytochrome CYP1A1. This in turn would block further transformation of diol to the diolepoxide, that could be capable of interacting with DNA.

Another method leading to the increased mutagenic and carcinogenic activity of CP[*a*]P (**1**) is the introduction of double bond to the cyclopentane ring or substitution of C-17 carbon atom with methyl group. Also in this case, substitution in positions 11 or 7 increases mutagenic activity of a parent compound (Coombs et al. 1976). Authors (Coombs and Bhatt 1987) explained low biological activity of the compounds possessing the abovementioned substituents in other positions. The kind of substituent in biologically important position (7, 11) also influences mutagenic and carcinogenic activity of such compounds. Bhatt et al. (1982) reported results of investigations on the influence of the length of alkyl chain of ethers, derivatives of CP[*a*]P-11-ol, on their mutagenic activity. The authors emphasize the surprising activity of compounds having a methoxy or hydroxyl group, whereas the D-ring remained unchanged. In previous works, the authors suggested that only compound having a double bond, methyl group or carbonyl function in cyclopentane ring and electrodonating group in position 11, exhibit moderate to strong mutagenic or carcinogenic properties. For example, in earlier research (Bhatt et al. 1982) the authors examined 17-keto-11-ethers noting the following dependences: 17-keto-11-methoxy-CP[*a*]P **19a** was a strong initiator of carcionogenic process whereas ethyl ether **19b** was less active. Propyl **19c** and pentyl **19e** ethers did not show any activity. Surprisingly, 11-butoxy-17-ketone **19d** exhibited moderate mutagenic activity (Boyd et al. 1995b) (Scheme 9).

Scheme 9



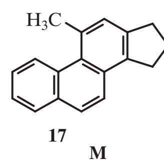
	R	Activity
19a	$-CH_3$	C
19b	$-C_2H_5$	C
19c	$-C_3H_7$	NC
19d	$-C_4H_9$	WC
19e	$-C_5H_{11}$	NC
19f	$-C_6H_{13}$	NC

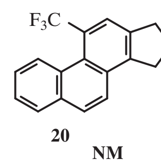
Carcinogenic activity
C – carcinogen
WC – weak carcinogen
NC – lack of carcinogenic activity

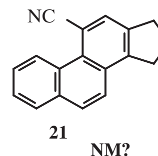
The main subject of Bhatt's work was to examine if there exists a correlation between evenness of carbon atoms in alkoxy substituents and carcinogenic activity of a specific compound. Data for hexyl ether of 17-ketone **19f** did not confirm this hypothesis. Similar results were obtained in the study on the influence of cyclopenta[*a*]phenanthrene-17-ketones substituted with alkoxy group in position 11 on activity of CYP1A1 and CYP1A2 systems (Boyd et al. 1995b). The strongest effect resulting in increased CYP1 and CYP1A2 apoproteins was observed in the case of ethoxy substituted CP[*a*]P-17-one **19b**, ketone substituted with methyl group **19a** showed a lower influence and the elongation of alkyl chain resulted in a lack of activity in this test. As it was proved the ketone bearing ethoxy group **19b** is unable to bind to the aromatic hydrocarbon receptor (Ar), so that the only compound giving positive response in the Ames test is 11-methoxy-17-ketone **19a**.

Coombs (1999) correlated in an interesting way biological activity with chemical shift of **H**C-12 proton in case of 11 substituted CP[*a*]P. Confirming that the presence of an electrodonating group in position 11 of CP[*a*]P will affect increased mutagenic or carcinogenic activity, he assumed that this will also increase the electron density of the environment; in that case the **H**C-12 proton will be shielded and a signal will be shifted up to 7.39-7.97 ppm. In the case of biologically inactive compounds which have electron-accepting substituents in position 11, signal of **H**C-12 proton is shifted downfield to 8.38 (for -CN) – 8.43 (for-CF_3). As already described, substitution of cyclopenta[*a*]phenanthrene in position 11 with trifluoromethyl group leads to a loss of its carcinogenic activity (Boyd et al. 1995c). Substitution with nitrile function should also lead to such an effect; however such a study has not been performed yet (Scheme 10).

Scheme 10

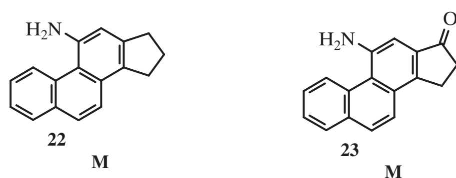






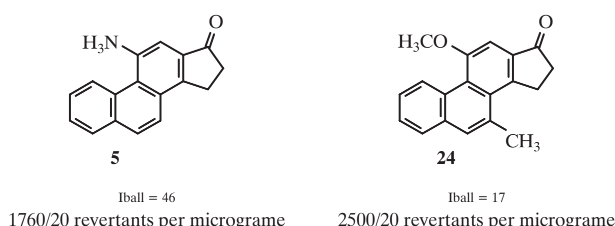
Preliminary investigations on the mutagenicity of CP[*a*]P substituted with amino function in this position using *Salmonella typhimurium* TA98 and TA100 strains showed moderate activity of such compound (Scheme 11). However there is still a need to study the mutagenic activity of this compound using strains YG 1023 and YG 1024, which are very sensitive to mutations caused by amino function.

Scheme 11



Some of the investigated compounds, for example two CP[*a*]P-17-ones **5** and **24** (Scheme 12), exhibit moderate or low mutagenic activity in the Ames test, while being strong carcinogens of high Iball index (Coombs et al. 1976). Compound **24** exhibits much higher mutagenic activity in bacterial assay than compound **5**; however **24** has much lower Iball index (17) than **5** (46).

Scheme 12



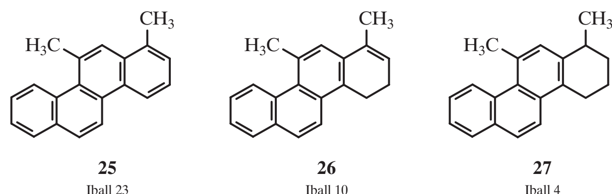
Iball = 46 | 1760/20 revertants per microgrameIball = 17 | 2500/20 revertants per micrograme

Another example of a moderate mutagen is 11,12-dimethyl--15,16-dihydroCP[*a*]P-17-one, with an Iball index = 30. Marrocchi et al. (2001) proposed a different approach in the modification of CP[*a*]P in order to study the influence of changes in CP[*a*]P skeleton on their mutagenic activity. The authors investigated the influence of benzannelations and introduction of carbonyl function into cyclopentane ring on mutagenic activity of CP[*a*]P (**1**) and CP[*c*]P. In the case of CP[*a*]P skeleton (**1**), it has been proven that benzannelations of A ring as well as benzannelations of D ring of this arrangement do not influence mutagenic properties of these compounds. The presence of carbonyl function in position 15 as well as in position 17 increases mutagenic activity of CP[*a*]P, as does the presence of a double bond in D ring of a molecule. Benzannelations decrease biological activity of 15- and 17 ketones; however they do not suppress it (Marrocchi et al. 2001).

Chrysene

Chrysene (**2**) is the subject of investigations of numerous research groups, working on the theory of chemical carcinogenesis. Data on mutagenic activity of eleven derivatives of chrysene using TA100 strain of *Salmonella typhimurium* has been presented by Coombs et al. (1976). Similarly to CP[*a*]P, an increase of mutagenic and carcinogenic activity of chrysene was observed while substituting the aromatic skeleton in position 5 (bay region) with a small electrodonating group. Among monomethyl chrysenes substitution in position 1 causes low mutagenic activity, but the compound becomes an initiator of the carcinogenic process. Substitution in positions 2, 3, 4 or 6 results in low carcinogenic activity, well correlated with mutagenicity of a specific derivative. Moderate carcinogenic activity of 5-methylchrysene agrees well with its mutagenic properties. Data on carcinogenic activity of the above mentioned compounds have been published by Hecht et al. (1974). Unsubstituted 1,2,3,4-tetrahydrochrysene-1-one exhibits mutagenic properties; however it does not show any carcinogenic activity. Substitution of this skeleton with methyl group in position 11 causes dramatic increase of carcinogenicity (Iball index = 45). This is comparable with analogical structure, 11-methyl-15,16-dihydro-CP[*a*]P-17-one (**5**), with the carcinogenicity index value of 46. An interesting case is a series of compounds having different saturation stages of ring A of chrysene skeleton. Keeping the same substitution pattern with two methyl groups in positions 1 and 11, it was shown that increasing hydrogenation of this ring decreases its carcinogenic potency (Scheme 13).

Scheme 13

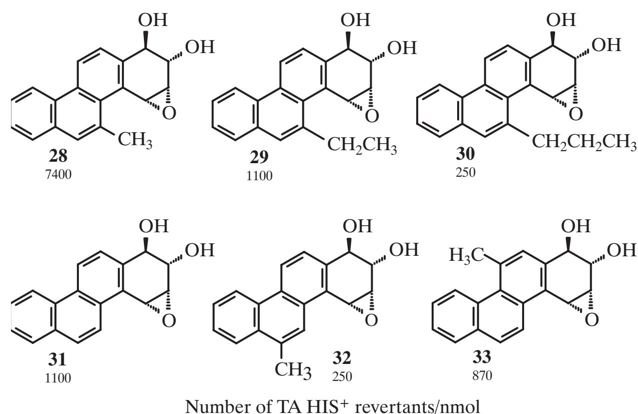


25 Iball 23 | **26** Iball 10 | **27** Iball 4

Among compounds **25** and **26** with Iball indexes of 23 and 10, respectively, no difference in their mutagenic activity was observed, although this value was much lower for the compound **27**. Kimri and Gayoso (1996) attempted theoretical prediction of carcinogenity of chrysene (**2**) substituted with methyl group. As a factor determining chrysene biological activity, the authors assumed susceptibility of C1 carbon atom to electrophilic attack. This process is very similar to the initial metabolic activation of chrysene (**2**) in living organisms, i. e. epoxidation of a double bond of the ring A. In order to simulate electrophilic attack the protonation of C1 carbon atom was considered to be a most simple reaction. The obtained data confirmed the hypothesis connecting carcinogenic activity of PAHs with existence of bay-region and substitution of bay--region with an electrodonating group. Amin and co-workers (1979) concerned a series of chrysenes substituted with methyl group in position 5, two methyl groups in positions 5 and 11, chrysene derivatives bearing groups such as: $-CH_2OAc$, $-CH_2OH$, $-CO_2CH_3$ in position 5 as well as its partially hydrogenated analogues. Besides 5-methylchrysene and 5,11-dimethyl derivative, none of the above compounds exhibited any particular mutagenic activity towards *Salmonella typhimurium* TA100 strain. These data suggest that oxidation of methyl group of 5-methylchrysene is not an important activating step determining mutagenic properties of 5-methylchrysene metabolites. The research was inspired by previous data gained by Amin's group, who showed that 5-(hydroxymethyl)chrysene, obtained in *in vitro* experiments performed with the use of rat liver homogenate, is one of the main metabolites of 5-methylchrysene.

In their other work, the authors discuss the influence of the length of alkyl chain in position 5 of chrysene on its mutagenic activity (Amin et al. 1988). Diolepoxides, having in the position 5 methyl, ethyl and *n*-propyl group were synthesized, which enabled direct estimation of mutagenic activity with no need for (*in vivo* or *in vitro*) enzymatic activation of the parent compound. The obtained diolepoxides were examined with the application of the Ames test using *S. typhimurium* TA100 strain, for which compound **28** with methyl group in position 5 showed highest mutagenic activity (Scheme 14).

Scheme 14



Number of TA HIS$^+$ revertants/nmol

Substitution with ethyl or propyl group in the bay-region of a molecule as well as substitution with methyl groups in positions 6 or 11 (compounds **32** and **33**) results in a significantly lower mutagenic activity of specific diolepoxide. Amin et al. (1988) hypothesize that the presence of substituent in position 5 pushes out epoxide ring from the plane of a molecule, making such an arrangement more capable of binding to the DNA. Therefore enlarging the volume of this substituent should enhance this effect. Preliminary experiments on the capability of such arrangements to covalently bind the DNA of calf thymus showed that its activity decreased with the increasing length of alkyl substituent. The authors suggest that methyl derivative of the discussed aromatic skeleton fulfils exactly the steric conditions required for reaction in hot spots of mutations in *S. typhimurium*. Another explanation could be the inclusion of a mutagen molecule in DNA structure, which usually precedes covalent binding (Smith et al. 2000). Deformation of planar structure caused by larger alkyl groups may be extensive enough to reduce the probability that such intercalation happens. Results of further *in vivo* experiments (Amin et al. 1991) comparing carcinogenic activity of some of the mentioned compounds agreed well with the data obtained earlier. The activity of anti-1,2-diol-3,4-epoxides of chrysene **31**, 5-methylchrysene **28**, 5-ethylchrysene **29** and 5-propylchrysene **30** was established on the basis of experimental formation of lung tumors in young mice exposed to these compounds. As expected, diolepoxide **28** derived from 5-methylchrysene showed the highest carcinogenic activity in those tests. Its activity was 15-30 times stronger than that of other compounds. Studies on the capability of PAHs to induce lung tumors based on mutations of *p53* suppressor gene, resulting from the binding of PAHs metabolites to its specific codons, confirmed the above dependencies (Smith et al. 2000). A detailed study of diolepoxide derived from 5,6-dimethylchrysene focused on its carcinogenic activity (Afshar et al. 1999) showed that introduction of a methyl group in bay-region of a molecule causes deformation of its planar structure. Deformed geometry of epoxide ring and planar structure of aromatic rings system facilitates binding of this metabolite to DNA and, in consequence, causes mutations leading to the formation of cancer cells.

Studies on metabolites of 3-hydroxychrysene provide an example of the influence of hydroxyl substituent on the biological activity of chrysene. One such metabolite, found during experiments with microsomal fraction of rat liver, was *trans*--1,2-dihydro-1,2,9-trihydroxychrysene (Hodgson et al. 1985). This triol, when treated with *m*-chloroperbenzoic acid, forms products which are capable of covalent binding to DNA *in vitro* (Phillips et al. 1986). *Anti*-triolepoxide showed the highest capability of interacting with DNA, which was 1.7 times stronger than that of *anti*-diolepoxide. About 10 times less activity was estimated for isomers *syn*, while derivatives having epoxide ring in K-region were even less reactive than syn isomers. As it was shown, the presence of hydroxyl group in position 3 of chrysene significantly increases its harmful biological activity.

Another substitution in chrysene skeleton is amino function. For instance, Catterall et al. (2001) describe a series of comparative Ames tests on specific *S. typhimurium* strains, performed for 2-aminochrysene and 5-aminochrysene. The latter exhibited much lower mutagenic activity than 2-aminochrysene. The authors conclude that the amino substituted PAHs undergo a different metabolic path. The theory of direct oxidation of amino group leading to nitrenium ion is discussed by Ford and Herman (1992). In case of this way of activation, the structure of aromatic skeleton of a molecule is extremely important. According to theoretical calculations and due to the fact that 5-aminochrysene amino group is connected to carbon atom, which in turn is directly connected to a tertiary carbon atom of the aromatic skeleton of molecule (Ford and Griffin, 1992), nitrenium ion which is generated in case of 5-aminochrysene, will be less stable than that from 2-aminochrysene.

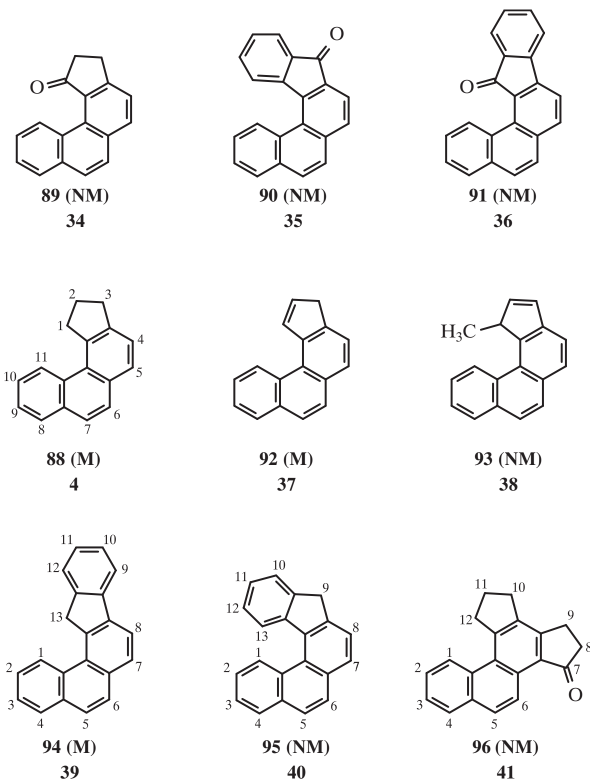
Cyclopenta[*c*]phenanthrene

At present there is a lack of model compounds enabling systematic investigations into biological activity of CP[*c*]P(**4**), which remains relatively unknown among well examined PAHs with a small number of condensed rings. The only research on its biological activity was performed by Taticchi group (Marrocchi et al. 1996, 2001). To our knowledge, synthesis and mutagenic activity against *S. typhimurium* of the compounds with methyl group, double bond and carbonyl function in cyclopentane ring as well as the influence of benzannelations of rings A and D of this skeleton have been described in only two publications (Marrocchi et al. 1996, 2001).

Among the compounds mentioned above, unsubstituted CP[*c*]P (**4**) exhibits the strongest mutagenic properties. The in-

troduction of carbonyl function in position 1 (**34**) causes the fading of mutagenic activity, and this has been explained as the excessive steric hindrance in *pseudo* fjord-region. The same explanation was given in the case of the compound (**38**), which has a double bond and methyl substituents at C-1 carbon atom. These compounds and their mutagenic properties are shown in Scheme 15.

Scheme 15

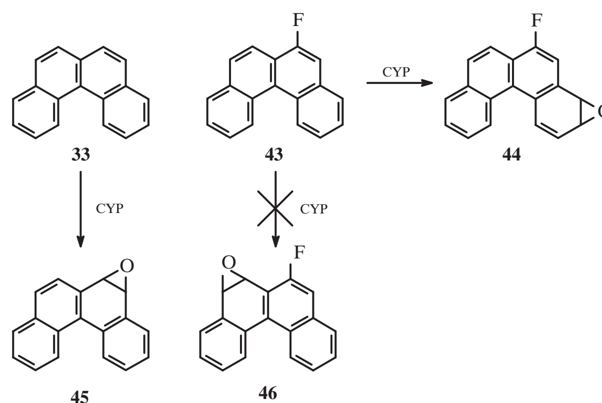


Among hydrocarbons consisting of five condensed rings, only compound **39** exhibits mutagenic properties; however, its activity is much lower than the activity of CP[*c*]P (Marrocchi et al. 1996).

Benzo[*c*]phenanthrene

B[*c*]P differs significantly from the carbon skeletons described above. Unsubstituted B[*c*]P (**3**) is such a strong carcinogen that it serves as a positive control in biological research. In most of the avaliable papers, the authors consider establishing new methods for determination of mutagenic or carcinogenic properties of PAHs with the use of B[*c*]P (**3**) (Buterin et al. 2000). Metabolites of B[*c*]P (**3**) are of great importance and interest; strong carcinogenic (Amin et al. 1995; Hecht et al. 1994; Seidel et al. 1995) and mutagenic (Glatt et al. 1991; Wood et al. 1984;) properties of products of their enzymatic oxidation were determined using different techniques, tests and cell lines or bacterial strains. In every case, anti-3,4-dihydroxy-1,2-epoxy-1,2,3,4-tetrahydroB[*c*]P exhibited the strongest activity. The metabolic pathway of B[*c*]P (**3**) was investigated in details with the use of genetically modified hamster V79 cells, which had three forms of rat cytochrome P450 and six forms of human cytochrome (Seidel et al. 1998). Studies comparing the carcinogenicity of metabolites of 5,6-dimethylchrysene and B[*c*]P proved that among diolepoxides formed from both compounds the metabolite of B[*c*]P exhibited the strongest carcinogenic activity (Amin et al. 1993). Investigations on the biological activity of 1,4-dimethyl-B[*c*]P and the synthesis, metabolism, and structure of its metabolites were performed by the group of Lakshman (Lakshman et al. 2000). In comparison to an unsubstituted hydrocarbon, the substitution of a fjord-region with methyl group significantly decreases carcinogenic activity of metabolites of this compound. As the metabolic pathway of B[*c*]P (**3**) leads mostly through the stadium of oxidation of K-region double bond of molecule, the effort to increase the carcinogenic activity of this compound was undertaken. The idea was to block this path by substituting C-6 carbon atom with fluorine (Jerina et al. 1982) (Scheme 16).

Scheme 16



The data obtained in this experiment showed the four-fold increase of carcinogenic activity of the compound substituted in this manner.

SUMMARY

This attempt to synthesize the present state of knowledge on the correlation between the structure of chosen PAHs and their mutagenic or carcinogenic activity shows that the dependencies cannot be treated univocally. Extreme complexity of the subject, typical for investigations on the boundary between chemistry and biology, inclines one to search for new ways of synthesis of new compounds, which would be of interest for biologists studying the problem of chemical carcinogenesis. On the other hand, there is a strong need for elaborating a standardized test that would explicitly define the carcinogenic properties of a specific compound. The use of a variety of methods for the determination of carcinogenic potency of chemical compounds combined with simultaneous differentiation of structure of examined compounds, sometimes leads to contradictory conclu-

sions. Correlation between mutagenic and carcinogenic activity of some compounds is also sometimes questionable. Modern molecular biology should allow for elaborating a method which would give a satisfactory and reliable answer to the fundamental question: is a specific compound carcinogenous for a human organism? And if so – to what extent?

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