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Di-epoxides of the three isomeric dicyclopenta-fused pyrenes: ultimate mutagenic active agents

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Abstract

To rationalize the high bacterial mutagenic response recently found for the (di-) cyclopenta-fused pyrene congeners, viz. cyclopenta[*cd*]-(**1**), dicyclopenta[*cd*,*mn*]-(**2**), dicyclopenta[*cd*,*fg*]-(**3**) and dicyclopenta[*cd*,*jk*]pyrene (**4**), in the presence of a metabolic activation mixture (S9-mix), their (di-)epoxides at the externally fused unsaturated five-membered rings were previously proposed as the ultimate mutagenic active forms. In this study, cyclopenta[*cd*]pyrene-3,4-epoxide (**5**) and the novel dicyclopenta[*cd*,*mn*]pyrene-1,2,4,5-di-epoxide (**6**), dicyclopenta[*cd*,*fg*]pyrene-5,6,7,8-di-epoxide (**7**) and dicyclopenta[*cd*,*jk*]pyrene-1,2,6,7-di-epoxide (**8**) were synthesised from **1** to **4**, respectively, and subsequently assayed for bacterial mutagenicity in the standard microsomal/histidine reverse mutation assay (Ames-assay with *Salmonella typhimurium* strain TA98). The di-epoxides **6**–**8** are present as a mixture of their *cis*- and *trans*-stereo-isomers in a close to 1:1 ratio (^{1}H NMR spectroscopy and ab initio IGLO/III//RHF/6-31G** calculations). The direct-acting mutagenic activity and the strong cytotoxicity exerted by **5**–**8** both in the absence or presence of an exogenous metabolic activation system (±S9-mix) demonstrate that the ultimate mutagenic active forms are the proposed (di-)epoxides of **1**–**4**.

Keywords: Bacterial mutagenicity; Direct-acting mutagens; *Salmonella typhimurium* TA98; Epoxidation; Ab initio RHF/6-31G**; IGLO/III//RHF/6-31G**; Semi-empirical AM1 calculations

1. Introduction

Cyclopenta-fused polycyclic aromatic hydrocarbons (CP-PAH) represent a sub-class of PAH and contain at least one externally fused unsaturated five-membered ring. CP-PAH are ubiquitous compounds in our environment, which are formed during incomplete combustion of organic matter, viz. fossil fuels [1,2]. CP-PAH generally draw attention due to their anomalous physico-chemical properties [3–6], but in particular due to their enhanced genotoxic behaviour, i.e. CP-PAH are bacterial and mammalian cell mutagens,

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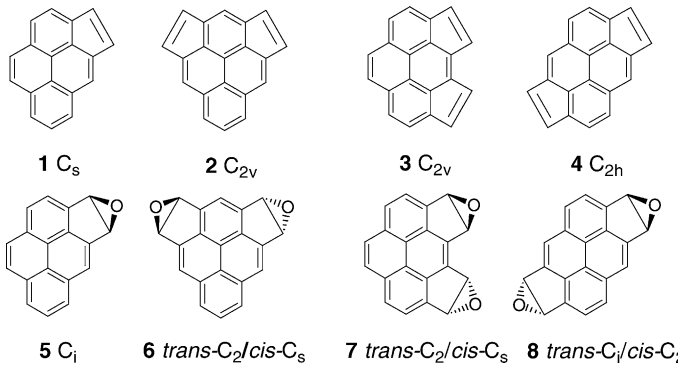

Fig. 1. The (di-)cyclopenta-fused pyrene congeners **1**–**4** and their corresponding (di-)epoxides at the cyclopenta moieties **5**–**8**, which are assayed for their bacterial mutagenic response following the standard protocol by Ames and coworkers (*S. typhimurium* TA98 ±S9-mix) [22,23]. Note that the di-epoxides **6**–**8** are presumably present as two distinct stereo-isomers, i.e. the *cis*-di-epoxides and *trans*-di-epoxides. Here only the *trans*-di-epoxide stereo-isomers of **6**–**8** are shown. The molecular point group for each stereo-isomer obtained after geometry optimization at either the ab initio RHF/6-31G** [37,38] or the semi-empirical AM1 level of theory [25] is shown.

tumorigens and (co-)carcinogens in contrast to their parent PAH [7–12].

Therefore, CP-PAH are recognized as important contributors to the mutagenicity and tumorigenicity associated with combustion exhausts; they pose a potential health hazard to humans [13,14]. One of the best-studied CP-PAH is cyclopenta[*cd*]pyrene (**1**, Fig. 1), which has been put forward as the main contributor to the total bacterial mutagenic response found for the non-polar fraction of combustion exhausts [15]. In addition, **1** has also been proposed as the primary contributor to the total mutagenicity in airborne particulate matter [16]. Nevertheless, the available amount of **1** does not account for the total mutagenic activity found. Hence, other still unidentified but biologically active PAH must be present. Potential candidates are the three isomeric dicyclopenta-fused pyrene congeners, i.e. dicyclopenta[*cd*,*mn*]-(**2**), dicyclopenta[*cd*,*fg*]-(**3**) and dicyclopenta[*cd*,*jk*]pyrene (**4**, Fig. 1), which were recently synthesized [4,17]. Their availability, subsequently, enabled their unequivocal identification as constituents of the non-polar fraction of combustion exhausts [18,19]. Compounds **2**–**4** have been also suggested to be formed as undesired products during the thermal removal of pyrene from contaminated soil [20].

We recently reported the bacterial mutagenic response of **2**–**4** in the bacterial microsome/histidine reverse mutation assay (*Salmonella typhimurium* strain TA98) [21] according to the standard protocol by Ames and coworkers [22,23]. The number and the topology of the cyclopenta moieties along the pyrene perimeter were found to markedly affect the mutagenic response of **2**–**4**. Interestingly, **2**–**4** exert a high mutagenic activity dependent on metabolic activation (+S9-mix). Furthermore, unexpectedly, **3** and to a lesser extent **2**, also induced direct-acting mutagenicity (−S9-mix). The importance of the olefinic bonds in the five-membered rings for bio-activation, presumably via di-epoxide formation, was determined by assessing the mutagenic response of the corresponding dihydro derivatives of **2**–**4** in which the five-membered rings contain a saturated instead of an olefinic carbon–carbon bond. These partially saturated CP-PAH were found to be non-mutagenic both in the absence or presence of exogenous metabolic activation mixture (±S9-mix) [21]. A typical feature of CP-PAH is their low protein content requirement in the exogenous metabolic activation mixture (S9-mix) to exert maximal mutagenic response, which is indicative of a one-step metabolic activation pathway [7,8,24], viz. one-step epoxidation of the five-membered ring olefinic bond. This was also observed for compounds **2**–**4** [21]. The results suggested that the ultimate mutagenic active forms in the presence of S9-mix are the di-epoxides **6**–**8** (Fig. 1). The experimental findings were further substantiated by semi-empirical Austin Model 1 (AM1) [25] quantum chemical calculations of the heats of formation (ΔH_f° in kcal/mol) of the mono-, *cis*-di- and *trans*-di-epoxides. The calculations revealed that the ease for di-epoxidation of **2**–**4** followed the order in mutagenic

potency of **2**–**4** (**2** > **4** > **3**), when the optimal microsomal protein concentration [S9-mix, 2% (v/v)] for maximal mutagenic response was used.

Here we report the synthesis and bacterial mutagenic response (standard protocol by Ames et al., *S. typhimurium* strain TA98 ±S9-mix 2% (v/v), i.e. 0.29 mg protein/plate) of the previously proposed ultimate mutagenic active forms of **2**–**4**, i.e. the di-epoxides dicyclopenta[*cd*,*mn*]pyrene-1,2,4,5-di-epoxide (**6**), dicyclopenta[*cd*,*fg*]pyrene-5,6,7,8-di-epoxide (**7**) and dicyclopenta[*cd*,*jk*]pyrene-1,2,6,7-di-epoxide (**8**). Mono-CP-PAH (**1**) and its corresponding mono-epoxide (**5**) were also assayed for comparison. The results provide strong evidence that the di-epoxides **6**, **7** and **8** represent the ultimate mutagenic active forms of **2**, **3** and **4**, respectively.

2. Materials and methods

2.1. Test compounds and chemicals

The (di-)epoxides at the externally fused five-membered ring(s) cyclopenta[*cd*]pyrene-3,4-epoxide (5, CAS n 73473-54-8), dicyclopenta[*cd*,*mn*]pyrene-1,2,4,5-di-epoxide (**6**), dicyclopenta[*cd*,*fg*]pyrene-5,6,7,8-di-epoxide (**7**) and dicyclopenta[*cd*,*jk*]pyrene-1,2,6,7-di-epoxide (**8**) were synthesized and characterized by ^{1}H NMR spectroscopy (see Section 2.3).

Benzo[*a*]pyrene (B[*a*]P, 98.8%, CAS n 50-32-8) and 1-nitropyrene (1-NP, 99%, CAS n 5522-43-0) were purchased from Sigma–Aldrich and used without further purification. Dimethyl sulfoxide (DMSO, 99.9%, CAS n 67-68-5) was purchased from Aldrich and NADP monosodium salt (98%, CAS n 1184-16-3), D-glucose-6-phosphate anhydrous (G-6-P, 99%, CAS n 56-73-5) and potassium monopersulphate triple salt (caroate, CAS n 37222-66-5) were purchased from Sigma.

2.2. Mutagenicity assays

Mutagenic response was assessed using the *S. typhimurium* histidine (His) reverse strain TA98, following the protocol by Ames and coworkers [22,23]. The tester strain TA98 has been extensively used in the literature for assaying the mutagenic activity of PAH. It has been established that the single use of one tester strain is sufficient to determine a positive response [26]. The assays were carried out either in the absence or in the presence of an exogenous metabolic activation system (±S9-mix). The S9-mix consists of rat-liver microsome preparations (S9-fraction) obtained from Aroclor-1254-treated male Wistar rats and NADPH-generating co-factors. The total protein content and the activity of the cytochrome P450 isoenzyme P450-1A in the S9-fraction were determined to be 29.15 mg/ml (Lowry method) [27] and 51.58 pmol/ml/min/mg protein (EROD method) [28], respectively.

The (di-)epoxides **5**–**8** were freshly dissolved in pro analyse DMSO (500 μg/ml) just before each assay and diluted as necessary for testing five different concentrations (0.0, 0.1, 0.5, 1.5 and 2.0 μg/plate, see Section 3. In all experiments triplicate plates were employed both in the absence (−S9-mix) and in the presence of a metabolic activation mixture [+S9-mix, 2% (v/v), 0.29 mg protein/plate] in at least two independent experiments. Well-established mutagens for strain TA98 were used as positive controls, i.e. the direct-acting mutagen 1-NP, 5.0 μg/plate (−S9-mix > 1000 revertants) [29] and dependent on metabolic activation B[*a*]P, 6.0 μg/plate (+S9-mix, 133.9 ± 20.5) [24]. Negative controls with only DMSO (−S9-mix, 10.2 ± 4.7; +S9-mix, 19.8 ± 6.5) and spontaneous His reversion revertants (−S9-mix, 13.3 ± 4.7; +S9-mix, 21.6 ± 6) for TA98 were included in each experiment. After incubation at 37 °C for 48 h, the His revertant colonies were counted manually. The results obtained are presented in dose–response curves of the mean numbers of His revertant colonies per dose of test compound on three different plates from two independent experiments; the spontaneous reversion is not subtracted. The standard deviation from each dose tested is shown as error bars in the dose–response curves (Figs. 2–5). The specific mutagenic activities, i.e. the numbers of His revertants induced per nmol of test compound (Table 2) are also calculated by least-squares regression from the initial ascending linear portion of the dose–response curves. See Appendix A for the actual data (mean His revertants ± S.D.) and correlation coefficients (r^2) derived from the calculation of the specific mutagenic activity. The following criteria [30,31] are considered to establish a positive mutagenic response: (i) threefold increase in the number of His revertants in plates with test compound as compared to the negative control (DMSO), (ii) ascending dose–response behaviour and

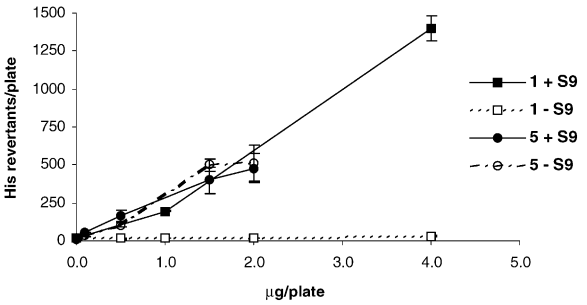

Fig. 2. Dose–response curve of cyclopenta[*cd*]pyrene (**1**) [■: +S9-mix 2% (v/v), □: −S9-mix] [21] and its corresponding cyclopenta[*cd*]pyrene-3,4-epoxide (**5**, Fig. 1 [●: +S9-mix 2% (v/v), ○: −S9-mix]. The standard protocol outlined by Ames and coworkers was followed using the *S. typhimurium* strain TA98 [22,23].

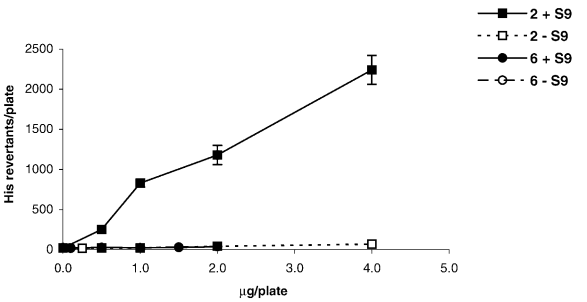

Fig. 3. Dose–response curve of dicyclopenta[*cd,mn*]pyrene (**2**) [■: +S9-mix 2% (v/v), □: −S9-mix] [21] and its corresponding dicyclopenta[*cd,mn*]pyrene-1,2,4,5-di-epoxide (**6**, Fig. 1 [●: +S9-mix 2% (v/v), ○: −S9-mix]. The standard protocol outlined by Ames and coworkers [22,23] was followed using the *S. typhimurium* strain TA98.

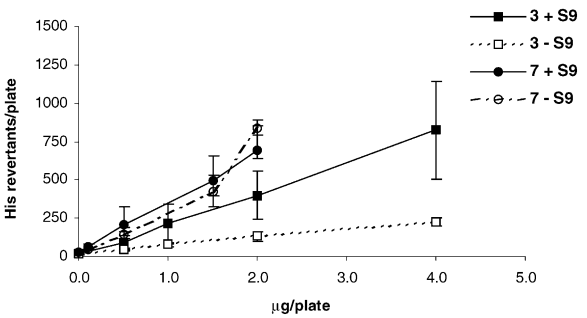

Fig. 4. Dose–response curve of dicyclopenta[*cd,fg*]pyrene (**3**) [■: +S9-mix 2% (v/v), □: −S9-mix] [21] and its corresponding dicyclopenta[*cd,fg*]pyrene-5,6,7,8-di-epoxide (**7**, Fig. 11[●: +S9-mix 2% (v/v), ○: −S9-mix]. The standard protocol outlined by Ames and coworkers [22,23] was followed using the *S. typhimurium* strain TA98.

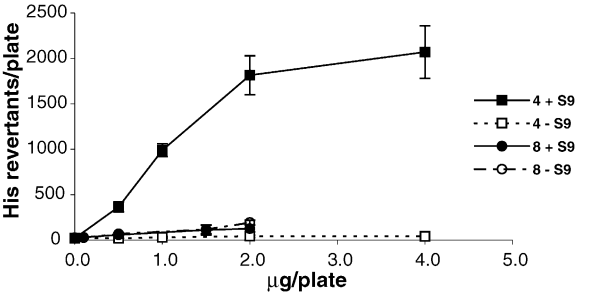

Fig. 5. Dose–response curve of dicyclopenta[*cd,jk*]pyrene (**4**) [■: +S9-mix 2% (v/v), □: −S9-mix] [21] and its corresponding dicyclopenta[*cd,jk*]pyrene-1,2,6,7-di-epoxide (**8**, Fig. 1 [●: +S9-mix 2% (v/v), ○: −S9-mix]. The standard protocol outlined by Ames and coworkers [22,23] was followed using the *S. typhimurium* strain TA98.

(iii) replication of results in at least two independent experiments. Note that the generally employed criterion of the three-fold increase rule is not sufficient to establish a positive response, since the negative controls differ for each test strain [32]. It should be noted that the bacterial mutagenic response of the CP-PAH **1**–**4** and their (di-)epoxides **5**–**8** was assayed using the same *S. typhimurium* TA98 frozen permanent, i.e. the method indicated in the Ames protocol to conserve bacterial strains and maintain their characteristics [22,23], and the same microsomal protein fraction (S9) for the exogenous metabolic activation mixture. The assays for **1**–**4** and **5**–**8** were conducted in December 2002 and April 2003, respectively.

2.3. Synthesis

Solvents were dried and purified using standard protocols. Commercial reagents were used as received. (Flash) Column chromatography was performed either on Merck kieselgel 60 silica (230-400 ASTM) or neutral aluminium oxide W200. Thin-layer chromatography (TLC) was carried out using TLC sheets (aluminium oxide 60 F_{254} neutral (type E) or TLC silica gel 60 F_{254}). ^{1}H-Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 300 or a Varian Unity Inova Spectrometer operating at 300.13 MHz and at 25 °C (solvent d_6-acetone). δ(^{1}H) chemical shift values are reported in ppm and are referenced to TMS. *J* values are given in Hz. For the ^{1}H NMR spectrum multiplicity is denoted as follows: s = singlet, d = doublet, t = triplet, dd = double doublet and m = multiplet. To

distinguish the coupling patterns of the two distinct *cis*- and *trans*-stereo-isomers of the di-epoxides of **2**–**4**, a Gaussian enhancement was performed with parameters LB = −1 and GB = 50%. Gas chromatography–mass spectrometry (GC–MS) analysis of the di-epoxides **6**–**8** was thwarted due to decomposition of **6**–**8** in de GC injector.

2.3.1. Synthesis of (di-)epoxides **5**–**8**

The epoxidation of the externally fused unsaturated five-membered rings was carried out using a solution of dimethyldioxirane in acetone as oxidation agent, which was prepared following literature procedures [33,34]. This method has been used previously for the synthesis of pure arene-oxides of CP-PAH, see for example [35,36]. Usually, 50–70 ml of 0.07–0.10 M dimethyldioxirane solutions in acetone were obtained. The dimethyldioxirane content was determined by the oxidation of methyl phenyl sulphide and monitoring the formation of methyl phenyl sulphoxide by capillary GC [33,34].

2.3.1.1. General procedure. The epoxidation was carried out in a 25-ml round-bottom flask at room temperature in the dark using ca. 0.05–0.1 mmol of the appropriate CP-PAH precursor, with either 2.5 mol equivalents (for **1**) or 5 mol equivalents (for **2**–**4**) of the freshly prepared dimethyldioxirane solution in acetone, in the presence of an equimolar amount of $NaHCO_3$.

Caution: Dimethyldioxirane is a volatile peroxide and must be synthesized and used with caution [33,34]. When the reaction mixture turned colourless, the complete conversion of the CP-PAH into its di-epoxide was established (^{1}H NMR spectroscopy). The reaction mixture was rapidly filtered and the filtrate was immediately concentrated in vacuo. The residue was dissolved in either dry acetone or dry CH_2Cl_2, dried over $MgSO_4$ and filtered. Concentration of the filtrate yielded analytically pure samples of the desired (di-)epoxides **5**–**8** in near quantitative yield, which were stored at −20 °C to prevent decomposition. The di-epoxides were unequivocally identified on the basis of their ^{1}H NMR spectral data, viz. the experimental δ(^{1}H) values and their integral ratios. Gaussian enhancement of the ^{1}H NMR spectra of di-epoxides **6**–**8** revealed that each di-epoxide was present as a mixture of two distinct stereo-isomers in a near to 1:1 ratio (integral ratios, cf. also Appendix B). Unfortunately, all attempts so far to separate these stereo-isomers were unsuccessful. The ^{1}H NMR assignments of **6**–**8** are supported by comparison with their experimental and computed δ(^{1}H) values (see Appendix B).

Prior to each bacterial mutagenicity assay the structural integrity of the mono-epoxide **5** and the di-epoxides **6**–**8** was assessed by ^{1}H NMR spectroscopy.

*2.3.2. Cyclopenta[cd]pyrene 3,4-epoxide (***5***)*

All spectral properties of **5** were in accordance with those previously reported [35].

*2.3.3. Dicyclopenta[cd,mn]pyrene-1,2,4,5-di-epoxide (***6***)*

Presumably, the *cis*-di-epoxide- and the *trans*-di-epoxide stereo-isomers of **6** are obtained (see Table 1 and Appendix B).

*2.3.4. Dicyclopenta[cd,fg]pyrene-5,6,7,8-di-epoxide (***7***)*

The assignment of the *cis*-di-epoxide- and the *trans*-di-epoxide stereo-isomers of **7** is based on a comparison of the experimentally observed and computed δ(^{1}H) values for their epoxide hydrogen atoms (see Table 1 and Appendix B).

*2.3.5. Dicyclopenta[cd,jk]pyrene-1,2,6,7-di-epoxide (***8***)*

Presumably, the *cis*-di-epoxide- and the *trans*-di-epoxide stereo-isomers of **8** are obtained (see Table 1 and Appendix B).

3. Results

Cyclopenta[*cd*]pyrene-3,4-epoxide (**5**), dicyclopenta[*cd,mn*]pyrene-1,2,4,5-di-epoxide (**6**), dicyclopenta[*cd,fg*]pyrene-5,6,7,8-di-epoxide (**7**) and dicyclopenta[*cd,jk*]pyrene-1,2,6,7-di-epoxide (**8**) were synthesized from their corresponding CP-PAH **1**–**4** (Fig. 1) by treatment with a freshly prepared solution of dimethyldioxirane in acetone as a mild epoxidation agent [33,34,35] (cf. Section 2.3.1). It is noteworthy that ^{1}H NMR analysis of the reaction mixture revealed that di-epoxide formation from **2** to **4** occurs in two consecutive steps. ^{1}H NMR spectroscopy also showed that upon conversion of **2**–**4** into the corresponding

Table 1
^{1}H NMR spectral data for the di-epoxides **6**–**8** (Fig. 1) present as a mixture of two distinct (*cis* and *trans*) stereo-isomers in a near to 1:1 ratio (see Section 2.3 and Appendix B)

Compound	^{1}H NMR
Dicyclopenta[*cd*,*mn*]pyrene-1,2,4,5-di-epoxide (**6**)	*cis*/*trans*, 52%: δ_H (d_6-acetone): 8.38 (2H, d, *J* 7.7 Hz), 8.35 (1H, s), 8.29 (2H, s), 8.11 (1H, t, *J* 7.4 Hz), 5.17 (4H, AB system, δ_A 5.19 Hz and δ_B 5.18 J_{AB} 2.2 Hz). *trans*/*cis*, 48%: δ_H (d_6-acetone): 8.38 (2H, d, *J* 7.7 Hz), 8.34 (1H, s), 8.30 (2H, s), 8.11 (1H, t, *J* 7.4 Hz), 5.16 (4H, AB system, δ_A 5.19 Hz and δ_B 5.18 J_{AB} 2.2 Hz).
Dicyclopenta[*cd*,*fg*]pyrene-5,6,7,8-di-epoxide (**7**)	*cis*, 53%: δ_H (d_6-acetone): 8.17 (4H, AB system, δ_A 8.18 and δ_B 8.16 J_{AB} 7.8 Hz), 8.07 (2H, s), 5.31 (2H, d, *J* 2.2 Hz), 5.16 (2H, d, *J* 2.2 Hz). *trans*, 47%: δ_H (d_6-acetone): 8.16 (4H, AB system, δ_A 8.18 and δ_B 8.14 J_{AB} 7.7 Hz), 8.06 (2H, s), 5.37 (2H, d, *J* 2.5 Hz), 5.17 (2H, d, *J* 2.5 Hz).
Dicyclopenta[*cd*,*jk*]pyrene-1,2,6,7-di-epoxide (**8**)	*cis*/*trans*, 60%: δ_H (d_6-acetone): 8.30 (2H, s), 8.25 (4H, AB system, δ_A 8.28 and δ_B 8.22 J_{AB} 7.7 Hz), 5.14 (4H, AB system, δ_A 5.16 Hz and δ_B 5.14 J_{AB} 2.4 Hz). *trans*/*cis*, 40%: δ_H (d_6-acetone): 8.31 (2H, s), 8.25 (4H, AB system, δ_A 8.29 and δ_B 8.22 J_{AB} 7.7 Hz), 5.16 (4H, AB system, δ_A 5.17 and δ_B 5.15 J_{AB} 3.0 Hz).

di-epoxides **6**–**8**, in all cases the two stereo-isomers of the di-epoxides are obtained in a close to 1:1 ratio (Table 1). Taking into account the epoxidation mechanism, the two stereo-isomers presumably represent the *cis*- and *trans*-di-epoxides of **6**, **7** and **8**, respectively. Unfortunately, all attempts to separate both stereo-isomers were hitherto unsuccessful. As a consequence, the stereo-isomer mixtures of the di-epoxides **6**–**8** were used in the bacterial mutagenicity assays.

The bacterial mutagenic response of **5**–**8** was determined in the *S. typhimurium* strain TA98 Histidine (His) reverse mutation assay both in the absence and in the presence of the optimal concentration of S9-fraction in the S9-mix for maximal mutagenic response, i.e. 0.29 mg [2% (v/v)] protein/plate. In Figs. 2–5 the dose–response curves are shown for the direct-acting (−S9-mix) and metabolic activation-dependent mutagenicity (+S9-mix) for the (di-)epoxides **5**–**8**. The results are compared with those previously found for CP-PAH **1**–**4** in the same dose range (0.0–2.0 μg/plate) and under similar conditions [21]. The specific mutagenic activity of **1**–**8**, viz. the number of His revertants/nmol of test compound, is reported in Table 2. Note that the bacterial mutagenic response of the CP-PAH **1**–**4** [21] and their (di-)epoxides **5**–**8** was assayed using the same *S. typhimurium* TA98 frozen permanent and the same microsomal protein fraction (S9) for the exogenous metabolic activation mixture (see Section 2.2).

The determination of the mutagenic activity of the (di-)epoxides **5**–**8** in the concentration range employed for their parent CP-PAH **1**–**4** (0.0–20.0 μg/plate [21]) is hampered by the high toxicity observed for the (di-)epoxides as indicated by a lack of the background lawn in the plates [23] at concentrations higher than 2.0 μg/plate with and without S9-mix (data not shown). Compounds **5**–**8** are assayed with S9-mix 2% (v/v) (0.29 mg protein/plate) determined as optimal for maximal response in a previous study for the parent CP-PAH **1**–**4** [21]. It has been established for several CP-PAH that their mutagenic response is markedly dependent on the protein concentration in the S9-mix, and that the highest mutagenicity is expressed at low concentrations [7,8,24]. It is noteworthy that (di-)epoxides **5**, **7** and **8** exhibit a positive mutagenic response both with and without exogenous metabolic activation mixture (±S9-mix); they act as direct-acting mutagens. In the case of di-epoxide **6**, a high toxicity (±S9-mix) is already found in the low concentration range (vide infra). Hence, its mutagenic potency could not be determined. Interestingly, the mutagenic activity of the (di-)epoxide derivatives **5**, **7** and **8** does not increase in the presence of S9-mix.

CP-PAH **1** is a documented metabolic activation-dependent potent mutagen (specific mutagenic activity

Table 2
Specific mutagenic activities[a] (His revertants/nmol) and mutagenic potency[b] of **1**–**4** [21] and **5**–**8**

Compound	TA98 +S9-mix 2% (v/v)		TA98 −S9-mix	
	His revertants/nmol	Mutagenic potency[b]	His revertants/nmol	Mutagenic potency[b]
1[c]	63.5	+++	∼0.5	–
5	61.8	+++	61.0	+++
2[c]	163.3	+++	4.2	+
6	*,[d]	*,[d]	*,[d]	*,[d]
3[c]	48.0	+++	11.7	++
7	93.3	+++	101.1	+++
4[c]	82.0	+++	1.2	–
8	16.3	+++	23.9	+++

[a] Calculated by least-squares regression from the initial ascending linear portion of the dose–response curves (Figs. 2–5).
[b] Mutagenic potency: – negative, + weakly positive, ++ positive and +++ highly positive.
[c] For **1**–**4**, the dose range 0–20 μg/plate was used [21]. Note that the bacterial mutagenic response of the CP-PAH **1**–**4** and their (di-)epoxides **5**–**8** was assayed using the same *S. typhimurium* TA98 frozen permanent and the same microsomal protein fraction (S9) for the exogenous metabolic activation mixture, but in different periods (**1**–**4**: December 2002 and **5**–**8**: April 2003).
[d] Specific mutagenic activities could not be determined due to high toxicity, i.e. a lack of background lawn was observed.

of 63.5 His revertants/nmol [21], Table 2 and Fig. 2) [7] and this behaviour is re-confirmed in the present study. Its corresponding epoxide **5** exhibits a direct-acting specific mutagenic activity of 61.0 His revertants/nmol (Table 2 and Fig. 2) which accounts for the metabolic activation-dependent mutagenic response of **1**. In the presence of S9-mix, epoxide **5** exerts a similar mutagenic response (specific mutagenic activity, 61.8 His revertants/nmol).

In Fig. 3, the dose–response curves of compound **2** are compared to the response curves of its di-epoxide derivative **6** (±S9-mix). Note that although the dose-range tested is up to 2.0 μg/plate, even in this low concentration range **6** exhibits a high toxicity, i.e. lack of background lawn is observed. Hence, this hinders the assignment of the mutagenic potency of **6**.

In Fig. 4, the dose–response curves for compound **3** and its corresponding di-epoxide **7** are shown. CP-PAH **3** exhibits a high specific mutagenic activity with S9-mix, 48.0 His revertants/nmol, and it is also a direct-acting mutagen, 11.7 His revertants/nmol (Table 2). Its corresponding di-epoxide **7** exhibits a high direct-acting specific mutagenic response; without S9-mix the activity is almost 10-fold higher than for the parent CP-PAH **3** under the same conditions (101.1 His revertants/nmol, Table 2). In the presence of S9-mix, **7** also exhibits a high specific mutagenic activity (93.3 His revertants/nmol).

The dose–response curves for the di-epoxide **8** and its parent CP-PAH **4** are shown in Fig. 5. In the presence of S9-mix, **4** is more active as a mutagen than **8** (82.0 and 16.3 His revertants/nmol, respectively, Table 2). Without S9-mix, **8** exhibits a high mutagenic response in contrast to its parent **4** (−S9-mix, 23.9 His revertants/nmol).

4. Discussion

The use of acetone solutions of dimethyldioxirane as epoxidation system has been employed in the literature to obtain epoxides of CP-PAH in near quantitative yield [34]. In the present study, this method gives access to the synthesis of the novel di-epoxides **6**–**8**. In all cases, the di-epoxides are present as a mixture of presumably their *cis*- and *trans*-stereo-isomers (^{1}H NMR spectroscopy and ab initio IGLO/III//RHF/6-31G** calculations). Unfortunately, the separation of these stereo-isomers was hitherto unsuccessful due to the sensitive character of the di-epoxides. Their characterization and preferably also the isolation of the distinct stereo-isomers is of importance for future enzymatic metabolism studies on the formation of the ultimate active forms of the series of dicyclopenta-fused pyrene congeners **2**–**4**, which were shown to be highly biologically active (TA98 ±S9-mix) [21].

The (di-)cyclopenta-fused epoxides **5**, **7** and **8** are shown to be direct-acting mutagens (TA98 ±S9-mix). Hence, their role as ultimate mutagenic active forms of their parent compounds **1**, **3** and **4**, respectively, is confirmed. Di-epoxide **6** derived from **2**, which was previously found to possess the highest activity of the dicyclopenta-fused pyrene series tested (+S9-mix, 163.3 His revertants/nmol, Table 2), is too toxic under the present experimental conditions to determine its mutagenic response. A lack of background lawn is observed, and thus a small number of (revertant) colonies are scored. Since the corresponding derivative of **2**, partially hydrogenated at the cyclopenta moieties, is non-mutagenic both with and without metabolic activation mixture [21], we tentatively conclude that epoxide **6** is the mutagenic active form of CP-PAH **2**. Unfortunately, the mutagenic potency of **6** cannot be evaluated under the present conditions due to its high cytotoxicity.

Compound **7** exhibits a direct-acting specific mutagenic activity of 101.1 His revertants/nmol, and represents the most active compound of the epoxides tested in the present investigation. The specific mutagenic activity of **7** is more than twice that of its parent CP-PAH **3** in the presence of S9-mix (48.0 His revertants/nmol, Table 2). This suggests that the formation of the di-epoxide from **3** by the cytochrome P450 is at some point limited. Note that compound **3** itself is a direct-acting mutagen (−S9-mix, specific mutagenic activity of 11.7 His revertants/nmol) [21]. Hence, **3** represents the first identified contributor to the direct-acting mutagenic activity found in the non-polar fraction of combustion mixtures [21].

The metabolic activation-dependent mutagenic activity of **4** is partially confirmed to occur via its di-epoxide **8**, since the latter exhibits a specific direct mutagenic activity of 23.9 His revertants/nmol (Table 2). Probably, metabolic activation pathways other than the epoxidation of the cyclopenta moieties are operational. Note that while the parent CP-PAH **4** exerts a metabolic activation-dependent mutagenicity of 82.0 His revertants/nmol, its corresponding di-epoxide **8** in the presence of S9-mix exhibits a specific mutagenic activity of only 16.3 His revertants/nmol. Since the mutagenic activity for both **7** and **8** decreases in the presence of S9-mix, detoxification processes by the epoxide hydrolase present in the S9-mix are presumably operational.

The direct-acting mutagenic potency (−S9-mix) of the di-epoxides **6**–**8** (Table 2) follows the order **7** > **8** and **6**, the latter being highly toxic, which does not correspond to that previously found for their parent CP-PAH **2**–**4** in the presence of S9-mix 2% (v/v) (**2** > **4** > **3** [21]). Moreover, in our earlier study semi-empirical AM1 calculations of the heats of formation of the *cis*-di-epoxides and *trans*-di-epoxides **6**–**8** tend to rationalize the mutagenic potency order observed experimentally [21]. The formation of the di-epoxides may not be the major factor controlling the mutagenic response dependent on metabolic activation. Other factors may contribute, such as the shape of the compounds, i.e. the presence of two distinct cyclopenta-fused rings in the case of **2**–**4** as well as the two distinct di-epoxide stereo-isomers of **6**–**8**, which have to interact with the enzymes present in the S9-mix in order to be metabolized.

5. Conclusion

The synthesis of the novel di-epoxides at the unsaturated cyclopenta ring of **2**–**4** has been achieved and their mutagenic activity determined in the bacterial microsome/His reverse mutation assay (standard protocol by Ames et al., *S. typhimurium* strain TA98 ±S9-mix). All di-epoxides **6**–**8** act as direct-acting mutagens and show cytotoxicity. The results suggest that the proposed di-epoxides at the externally fused cyclopenta moiety **6**–**8** are the ultimate mutagenic active forms of the compounds **2**–**4**, which are ubiquitous in combustion exhausts. Moreover, the data once more demonstrate the effect of the topology of the cyclopenta moieties along the PAH perimeter with respect to the bacterial mutagenic activity of **2**–**4** and **6**–**8**. To what extent the distinct di-epoxide stereo-isomers of **6**–**8** will exhibit a different mutagenic response remains to be addressed. To gain insight in this question, separation of these stereo-isomers will be an important issue.

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Appendix A

Table A.1.

Appendix B

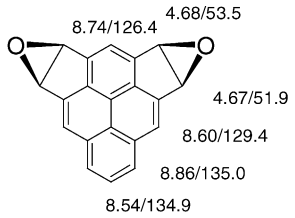
Computed IGLO/III//RHF/6-31G** $\delta(^1H)$ and salient $\delta(^{13}C)$ chemical shift values of **6**–**8**.

Additional support for the assignment of the two possible stereo-isomers of **6**–**8**, viz. their *cis*-di-epoxides and *trans*-di-epoxides obtained by di-epoxidation of CP-PAH **2**–**4**, respectively, their ^1H [$\delta(^1H)$] and ^{13}C NMR [$\delta(^{13}C)$] chemical shifts were computed using the direct version of the reliable ab initio individual gauge for localized orbitals (IGLO) method with the implemented basis set III [37,38] using ab initio RHF/6-31G** geometries of the *cis*- and *trans*-stereo-isomers of **6**–**8** optimized with GAMESS-UK [39]. All RHF/6-31G** optimized geometries were characterized as genuine minima by Hessian calculations; in all cases no imaginary vibrations were found. The ab initio IGLO/III//RHF/6-31G** computed $\delta(^1H)$ and $\delta(^{13}C)$ chemical shift values are referenced to the ^1H and ^{13}C NMR chemical shifts of tetramethylsilane (TMS) computed at the same level of theory (IGLO/III//RHF/6-31G**). The calculated distinct $\delta(^1H)$ and $\delta(^{13}C)$ values of the *cis*-di-epoxide and *trans*-di-epoxide stereo-isomers of **6**–**8** are presented in Fig. B.1. In agreement with previous results [40] IGLO/III generally reproduces the experimentally observed trends for $\delta(^1H)$ and $\delta(^{13}C)$ chemical shifts, albeit that the computed $\delta(^1H)$ and $\delta(^{13}C)$ values are commonly deshielded by ca. 0.5 and 2–4 ppm, respectively. In line with our proposed assignments using the experimental Gaussian-enhanced ^1H NMR spectra of **6**–**8** (Table 1) the computed $\delta(^1H)$ values of the respective *cis*-di-epoxide and *trans*-di-epoxide stereo-isomers are nearly identical. Only for **7** a clear-cut assignment of the two possible stereo-isomers is suggested (see Table 1). Note that for **6**–**8** the ab initio

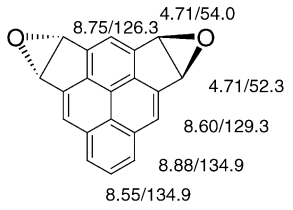
Table A.1
Mutagenic activity[a] of cyclopenta[*cd*]pyrene-3,4 (**5**), dicyclopenta[*cd,mn*]pyrene-1,2,4,5-di (**6**), dicyclopenta[*cd,fg*]pyrene-5,6,7,8-di (**7**) and dicyclopenta[*cd,jk*]pyrene-1,2,6,7-di-epoxide (**8**, Fig. 1) using the *S. typhimurium* strain TA98, with and without exogenous metabolic activation mixture (±S9-mix) following standard protocols [22,23]

Concentration (μg/plate)	**5**		**6**		**7**		**8**	
	+S9-mix 2% (v/v)	−S9-mix	+S9-mix 2% (v/v)	−S9-mix	+S9-mix 2% (v/v)	−S9-mix	+S9-mix 2% (v/v)	−S9-mix
0.0	20.5 ± 7.1	7.5 ± 2.6	26.3 ± 3.1[b]	22.8 ± 6.8[b]	26.3 ± 3.1	22.8 ± 6.8	26.3 ± 3.1	22.8 ± 6.8
0.1	53.5 ± 4.6	38.3 ± 10.9	24.3 ± 3.1[b]	16.8 ± 3.4[b]	61.3 ± 17.4	49.3 ± 6.3	30.3 ± 6.5	26.0 ± 4.2
0.5	164.0 ± 35.5	104.8 ± 12.6	27.5 ± 3.4[b]	26.5 ± 3.4[b]	209.1 ± 110.0	139.3 ± 21.3	57.8 ± 6.8	68.3 ± 13.4
1.5	400 ± 88.4	499.1 ± 45	25.1 ± 1.0[b]	23.1 ± 5.0[b]	496.5 ± 161.6	424.8 ± 102.5	112.2 ± 55.1	116.3 ± 47.1
2.0	476.7 ± 96.5	513.3 ± 119.8	26.3 ± 5.8[b]	32.8 ± 1.8[b]	690.7 ± 161.9	836.1 ± 49.1	126.5 ± 36.8	192.8 ± 25.1
His revertants/nmol (r^2)	61.8 (0.98)	61.0 (0.93)	–	–	93.2 (0.99)	101.1 (0.95)	16.3 (0.98)	23.90 (0.98)

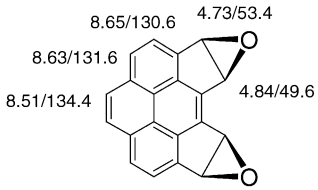
[a] Mutagenic response was determined as described in Section 2.2 [22,23]. Results shown are mean values ± S.D. (from triplicate plates for each dose in two independent experiments), and are given in His revertants per plate, without correction for spontaneous His revertants.
[b] Lack of background lawn; toxicity was observed.



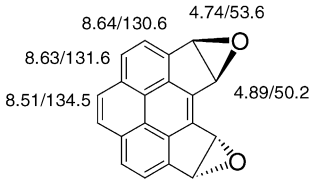
6 *cis*-C_s: ΔE 4.03 (4.17) [ΔH_f^o 4.40] kcal/mol



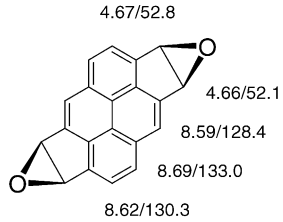
6 *trans*-C_2: ΔE 4.01 (4.10) [ΔH_f^o 4.20] kcal/mol



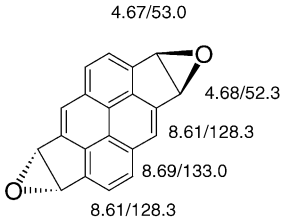
7 *cis*-C_s: ΔE 0.38 (0.54) [ΔH_f^o 0.40] kcal/mol



7 *trans*-C_2: ΔE 0.00 (0.00) [ΔH_f^o 0.00] kcal/mol



8 *cis*-C_2: ΔE 1.12 (1.26) [ΔH_f^o 1.50] kcal/mol



8 *trans*-C_i: ΔE 1.13 (1.24) [ΔH_f^o 1.40] kcal/mol

Fig. B.1. Computed IGLO/III//RHF/6-31G** δ(^{1}H) and δ(^{13}C) chemical shift values of the perimeter hydrogens and their carbon atoms of the *cis*-di-epoxide and *trans*-di-epoxide stereo-isomers of **6**–**8**. Δ*E* values (in kcal/mol) are calculated with respect to E_{tot}(IGLO/III//RHF/6-31G**: **7** *trans*-C_2) = −913.149302 hartree (E_{tot}(RHF/6-31G**: **7** *trans*-C_2) = −912.874097 hartree) and [ΔH_f°(AM1: **7** *trans*-C_2) = 114.5 kcal/mol] (see also [21]) [37,38]. All other computed quaternary δ(^{13}C) chemical shift values for **6**–**8** are available upon request from the corresponding author (LWJ).

Δ*E* values (both IGLO/III//RHF/6-31G** and RHF/6-31G**) and semi-empirical AM1 Δ*E* ($\Delta\Delta H_f^\circ$) values are very similar indicating that both stereo-isomers will be formed in a near to 1:1 ratio (see also Section 2.3).

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