ELSEVIER

Mutation Research 465 (2000) 85–90

Genetic Toxicology and Environmental Mutagenesis

www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Bioactivation of the carcinogen 11-methoxy-16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene

Fenton S. Catterall [a], Maurice M. Coombs [b,*], Costas Ioannides [a], Kim Walton [a]

[a] *Molecular Toxicology Group, School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, UK*
[b] *Department of Chemistry, School of Physical Sciences, University of Surrey, Guildford, Surrey GU2 5XH, UK*

Received 24 August 1999; received in revised form 27 October 1999; accepted 28 October 1999

Abstract

The title compound is a more potent carcinogen than would be anticipated from its simple phenanthrene structure lacking further D-ring conjugation. In vitro it undergoes microsomal metabolism to yield as major metabolites its 15- and 17-alcohols and its 16,17-diol; other minor metabolites are also derived from attack at the 5-membered ring, but no evidence of aromatic oxidation is apparent. The title compound is a weak mutagen in the Ames' test with *Salmonella typhimurium* TA100, but only with microsomal bio-activation. The 17-ol and 16,17-diol are inactive, with or without biological activation. By contrast the 15-alcohol, a rather reactive compound, is a strong mutagen both in the presence and absence of the bio-activation system. This, therefore, may be the proximate carcinogen, and its structural analogy to the naturally occurring hepato-carcinogen safrole is noted.

Keywords: Bioactivation; Carcinogen; 11-Methoxy-16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene

1. Introduction

Carcinogenicity in the cyclopenta[*a*]phenanthrene series is induced by correct (electron releasing) bay-region substitution [1] and is enhanced by a conjugated endocyclic or exocyclic double bond at C-17 [2]. Thus, whereas neither the unsubstituted hydrocarbon 16,17-dihydro-15*H*-cyclopenta[*a*]-phenanthrene (**1a**) nor its 17-ketone derivative (15,16-dihydrocyclopenta[*a*]phenanthren-17-one, **2a**) is carcinogenic, the 11-methyl hydrocarbon (**1b**) is weakly active [3] whilst the 11-methyl-17-ketone (**2b**) is a strong carcinogen, similar in potency to the classical polycyclic hydrocarbon benzo[*a*]pyrene [4]. Moreover, like the latter it is bioactivated through metabolism to its *trans*-3,4-dihydro-3,4-dihydroxy-*anti*-1,2-epoxide which is the proximate carcinogen [5].

Not unexpectedly the 11-methoxy-17-ketone (**2c**) is also a moderately strong carcinogen [6], but here there is a difference because the 11-methoxy hydrocarbon (**1c**) is almost equally active [7]. This is all the more surprising because the 17-methyl derivative (**3**) of the latter did not give rise to tumours [8]. It therefore seemed interesting and important to examine this matter further, and the in vitro microsomal metabolism of the 11-methoxy hydrocarbon (**1c**) has now been studied with this in view.

* Corresponding author. Tel.: +44-1483-87-6827; fax: +44-1483-87-6851

1383-5718/00/$ - see front matter
PII: S1383-5718(99)00215-6

2. Materials and methods

2.1. Chemistry

2.1.1. Improved preparation of 16,17-dihydro-11-methoxy-15H-cyclopenta[a]phenanthrene (1c)

16,17-Dihydro-17-hydroxy-11-methoxy-15*H*-cyclopenta[*a*]phenanthrene (1.32 g, 5 mmole) was stirred with dry tetrahydrofuran (20 ml) protected from moisture in an ice bath. Pyridine-sulphur trioxide complex (1.2 g, 7.5 mmole) was added giving a cream suspension which thickened on being stirred in ice for 1.5 h, then at room temperature for an additional 1 h. The flask was again cooled in ice during the dropwise addition of a solution of lithium aluminium hydride (1.15 g, 3 mmole) in dry tetrahydrofuran, and stirring was continued at room temperature overnight giving a greyish-yellow suspension. Water (1.1 ml) was added cautiously followed by 15% aqueous sodium hydroxide (1.1 ml) and more water (3.3 ml), and finally ether (100 ml) was added to the yellow suspension. The off-white precipitate was filtered off, washed with more ether, and the combined ether solutions were washed with water, dried over magnesium sulphate, and evaporated to give an orange gum (1.21 g). This was extracted several times with boiling hexane (50 ml), decanting from the brown residue; evaporation of the hexane left a white crystalline mass (0.75 g) of the 11-methoxy hydrocarbon (**1c**) with UV and NMR spectra as already described [9].

2.1.2. Preparation of the [methyl-^{14}C] labeled 11-methoxy hydrocarbon (1c)

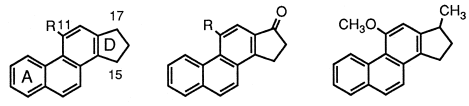
Using a gas line [^{14}C]methyl iodide (1 mCi) was condensed into a flask containing 15,16-dihydro-11-hydroxycyclopenta[*a*]phenanthren-17-one (248 mg), anhydrous potassium carbonate (207 mg), and dry acetonitrile (20 ml), and the mixture was stirred at ambient temperature with exclusion of moisture for 3 days. The solid was removed by filtration, washed with dichloromethane, and the combined solutions were evaporated to leave the [*methyl*-^{14}C]11-methoxy-17-ketone (**2c**) as a pale yellow solid (152 mg, 0.39 μCi/mg). This was converted into the [*methyl*-^{14}C]-11-methoxy hydrocarbon (**1c**) as described above. The final purification was by semi-preparative HPLC; it formed white crystals (77 mg, 0.36 μCi/mg) identical in all respects with the non-radioactive compound.

2.1.3. 16,17-Dihydro-15-hydroxy-11-methoxy-15H-cyclopenta[a]phenanthrene (4)

This compound was prepared by sodium tetrahydroborate reduction of the corresponding 15-ketone (Scheme 1) [9]. HPLC revealed that it exists as two epimers (RRT 0.75 and 0.83, RRT = relative retention time, relative to that of (**1c**) = 1.00) the ratio of which seemed to depend largely upon the temperature at which it had been previously kept. It formed white crystals which on being stored in a tightly stoppered glass tube at room temperature for some months became brown, and TLC disclosed the formation of a substantial amount of less polar impurities. Previously [9], difficulty had been experienced in obtaining correct combustion figures after purification by crystallisation; it was surprising to discover that sublimation at 100°C/0.1 Torr, giving a pure white crystalline solid, was a better method: found C, 81.6; H, 5.95. $C_{18}H_{16}O_2$ requires C, 81.8; H, 6.1%; λ_{max} ($\log_{10}$ ε) 215 (4.12), 228 (4.44), 253 (4.78), 273 (4.50), 294 (4.13), 305 (4.19), 325 (3.31), 341 (3.51), 357 (3.63) nm.

2.1.4. 16,17-Dihydro-11,15-dimethoxy-15H-cyclopenta[a]phenantrene (7)

16,17-Dihydro-11,15-dimethoxy-15H-cyclopenta-[a]phenantrene (**7**, Scheme 2) metabolite RRT 0.90, $C_{19}H_{18}O_2$: mass spectrum [mass/charge (% relative abundance)] 278 (17, molecular ion M +), 248 (52, M + $-OCH_2$), 247 (100, M + $-OCH_3$), 246 (27, M + $-OCH_3-H$), 233 (12, M + $-OCH_2-CH_3$), 215 (21, M + $-OCH_3-H-OCH_3$), 205 (10, M + $-OCH_2-CH_3-CO$); nmr ($CDCl_3$) δ 9.64 (1H, d, H-1), 7.96 (1H, d $J_{6,7}$ 9 Hz, H-7), 7.89 (1H, d $J_{3,4}$ 9 Hz, H-4), 7.80 (1H, d $J_{6,7}$ 9 Hz, H-6), 7.64–7.44

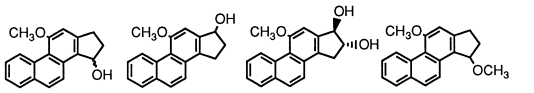


1a, R = H
b, R = CH_3
c, R = OCH_3

2a, R = H
b, R = CH_3
c, R = OCH_3

3

Scheme 1. Compounds mentioned in the text.



4, 15-ol, two epimers RRT 0.75 and 0.83 **5**, 17-ol RRT 0.78 **6**, 16,17-diol RRT 0.66 **7**, 11,15-dimethyl ether RRT 0.90

Scheme 2. Major metabolites of the carcinogenic 11-methoxy hydrocarbon (**1c**) separated by HPLC. The RRT values are their retention times relative to that of the unchanged hydrocarbon.

(2H, m, H-2,3), 7.34 (1H, s, H-12), 5.47 (1H, dd $J_{15,16}$ 2.5, 5.5, H-15), 4.13 (3H, s, 11-OCH_3), 3.41 (3H, s, 15-OCH_3), 3.32 (1H, m, H-16), 3.05 (1H, m, H-17), 2.41 (1H, m, H-16), 2.36 (1H, m, H-17) ppm from tetramethylsilane.

2.2. Incubation and isolation of metabolites

Male adult Wistar albino rats (180–200 g) were purchased from B & K Universal, Hull, West Yorkshire, UK. Induction of the CYP 1 family was achieved by administration of a single interperitoneal dose of Aroclor 1254 (500 mg/kg), dissolved in corn oil (200 mg/ml); the animals were sacrificed on the 5th day following administration. Livers were immediately excised, and the postmitochondrial (S9) fraction (for use as the activation system in the mutagenicity assays) and the microsomal fraction (for use in the metabolism studies) were prepared as previously described [10].

The incubation mixture contained phosphate buffer (0.1 M, pH 7.4, 6 ml), NADP (4 mM, 1 ml), glucose-6-phosphate (5 mM, 1 ml), glucose-6-phosphate dehydrogenase (20 units), and microsomes (25% w/v, 2 ml), all in a 250-ml conical flask open to the atmosphere and shaken in a water bath at 37°C. The reaction was started by addition of the substrate (**1c**) (1 mg in 100 μl of dimethylsulphoxide), and terminated at 30 min by cooling the flask in ice and adding ice-cold ethyl acetate (10 ml). Repeated extractions with six to eight lots of ethyl acetate were carried out without delay, the extracts were pooled, dried over magnesium sulphate, and evaporated to dryness below 40°C under reduced pressure. The residue was dissolved in ethanol (1–2 ml) and stored at room temperature to await HPLC separation.

This was carried out with a Waters 660 solvent programmer, 6000 A pumps, and UV detector set at 254 nm, on a Whatman Partisil 10 ODS-1 semi-preparative column with a linear gradient of 30% aqueous methanol changing to 100% methanol over 60 min at a flow rate of 2 ml/min. Aliquots of the separate fractions were assayed for radioactivity by scintillation counting and their UV spectra were recorded. Like peaks from several runs were pooled, evaporated to dryness at reduced pressure below 40°C, and the residue was dissolved in $CDCl_3$ for NMR analysis.

2.3. Mutagenicity tests

The mutagenicity of the 11-methoxy hydrocarbon (**1c**) and its metabolites (**4**)–(**6**) was determined using the Ames test in the presence and absence of a microsomal activation system using *Salmonella typhimurium* TA100 [11]. This strain was used because it was found to be more easily reverted by the title hydrocarbon than TA 98. The activation system contained 10% v/v of the S9 (25% w/v) liver preparation. All tests were repeated to ensure reproducibility.

3. Results

The preparation of 11-methoxy-16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene (**1c**) was improved by avoidance of strong acid which causes some elimination and dimension during catalytic hydrogenolysis of the 17-ketone [9]. Use was made of Cory and Achiwa's method [12], conversion of the 17-alcohol to its sulphate ester with the sulphur trioxide-pyridine complex followed by in situ reduction of the ester with lithium aluminium hydride, to give the pure hydrocarbon in a 60% yield. ^{14}C-labeling was required to allow quantitation of the hydrocarbon metabolites; this was readily achieved by methylation of the 17-keto-phenol corresponding to (**2c**) with ^{14}C-methyl iodide followed by deoxygenation of the [*methyl* ^{14}C]-11-methoxy-17-ketone as described.

The most notable feature in the spectrum of metabolites from the 11-methoxy hydrocarbon (**1c**) disclosed by HPLC (Fig. 1) was the lack of polar compounds. In the case of the corresponding 17-ketones (**2a**) and (**2b**), the in vitro metabolism of which has been studied in detail (2,5), although the 15-ols

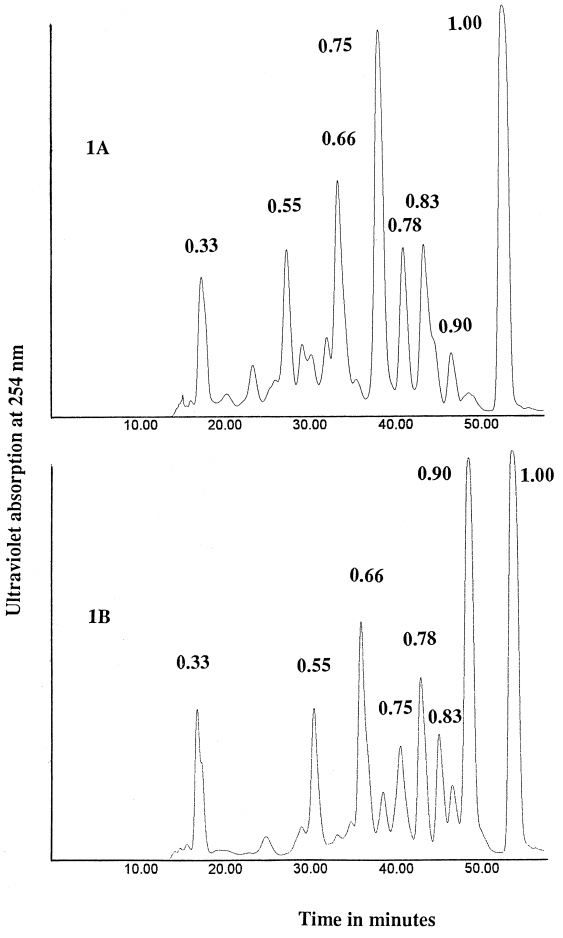


Fig. 1. Metabolites of the 11-methoxy hydrocarbon (**1c**) separated by HPLC: (A) within 2 h of extraction from the incubation mixture; (B) 26 h after 2 h of extraction from the incubation mixture (for details see the text). The numbers are the retention times relative to that of (**1c**) = 1.00. At 2 and 26 h the radioactivity associated with each fraction (as a percentage of the total radioactivity recovered) was, respectively, as follows: RRT 1.00 (unmetabolised substrate **1c**) 28.8, 29.1; RRT 0.90 (15-methoxy **7**) 1.5, 17.4; RRT 0.83 and 0.75 (15-ol epimers **4**) 21.7, 10.2; RRT 0.78 (17-ol **5**) 7.4, 7.3; RRT 0.66 (16,17-diol **6**) 9.0, 9.2; RRT 0.55 (D-ring diol?) 5.9, 5.6.

are major metabolites there is also substantial oxidation at ring A giving 1,2- and 3,4-dihydrodiols with further conversion into 3,4-dihydroxy-1,2-epoxides and tetrols. A preliminary study [13] of the microsomal metabolism of the unsubstituted hydrocarbon (**1a**) showed that bio-oxidation of this compound was largely confined to ring D, although some ring A involvement was also seen. It is thought that the very limited ring A oxidation may be the main reason for the low biological activity of these hydrocarbons as compared with their 17-keto derivatives. A study [14] of the mutagenicity of the *chemically* synthesised *trans*-3,4-dihydrodiols and *trans*-3,4-dihydro-3,4-dihydroxy-*syn* and *anti*-1,2-epoxides of the hydrocarbons (**1a**) and (**1b**) showed that mutagenicity increased markedly in the order: hydrocarbon $<$ 3,4-diol $\ll$ 3,4-diol-1,2-epoxide, with the latter powerfully active in the absence of biological activation. Thus, it seems probable that these hydrocarbons are activated in the same way as the corresponding 17-ketones.

In the present study, having all the ring D ketones and secondary alcohols of (**1c**) available and fully characterised from a recent chemical synthesis [9] greatly assisted identification of the major metabolites of this 11-methoxy hydrocarbon. A comparison of the relative retention times and UV spectra of these peaks (Fig. 1A) with those of the synthetic alcohols indicated that these were the 15-ol (**4**), the 17-ol (**5**) and the 16,17-diol (**6**).

By repeating this incubation several times and pooling the major peaks of like RRT enough of each was obtained to compare their NMR spectra with those of the synthetic alcohols, and in this way the identities of the 15-ol, 17-ol and *trans*-16,17-diol were positively confirmed. UV spectra of RRT 0.55 and the other less abundant peaks showed that they were all derived from ring D (RRT 0.33 was an artefact since it was not radioactive). When the ethanolic solution of metabolites was left for some

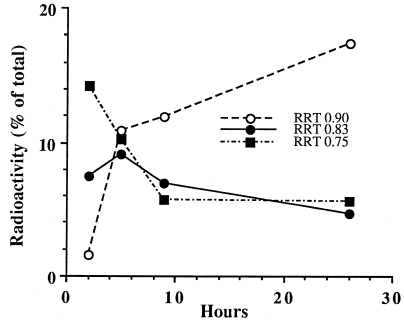


Fig. 2. Radioactivity in peaks at RRT 0.90, 0.83 and 0.75 (see Fig. 1) as a function of time of storage of the ethanolic metabolite mixture before injection on to the HPLC column.

time before injection into the HPLC column it was noticed that the amount of the 15-ol epimers diminished while the small peak at RRT 0.90 (Fig. 1A) increased. This was studied systematically over 26 h (Fig. 2), at the end of which RRT 0.90 was the most abundant metabolite (Fig. 1B).

The new metabolite RRT 0.90 had a UV spectrum almost identical with that of the 15-ol from which it formed; its structure was readily established from its NMR and mass spectra as 16,17-dihydro-11,15-dimethoxy-15*H*-cyclopenta[*a*]phenanthrene (**7**). It is not obvious how methylation of the 15-ol can take place in the ethanolic solution of the metabolite mixture, but the fact that it does occur emphasises the reactivity of this alcohol.

The importance of this 15-ol became more clear when these compounds were tested in the Ames test with *Salmonella typhimurium* TA100 (Fig. 3). With a microsomal activation system from Aroclor 1254-induced rats the 11-methoxy hydrocarbon (**1c**) was weakly mutagenic, in keeping with its known carcinogenicity, but no mutagenic response was evident in the absence of the activating system. The 17-ol was inactive with or without the microsomal system as was the 16,17-diol, whereas the 15-ol (**4**) was a strong mutagen both in the absence or presence of the activation system. The fact that of all the major metabolites the 15-ol is a potent direct acting mutagen makes it a likely candidate as the proximate carcinogen.

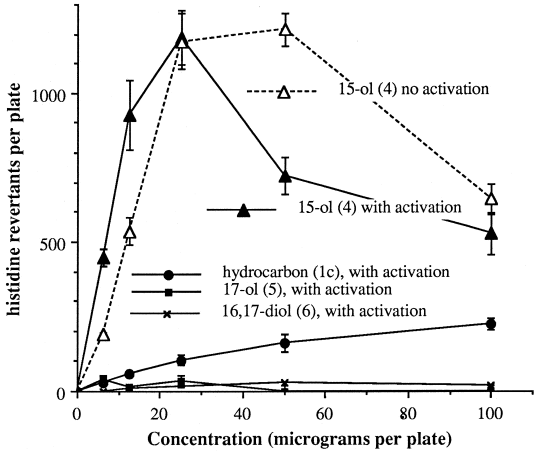
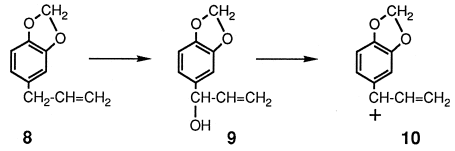


Fig. 3. Mutagenic potential of 11-methoxy-16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene (**1c**) and its 15-hydroxy (**4**), 17-hydroxy (**5**) and 16,17-dihydroxy (**6**) metabolites. Mutagenicity was evaluated using the Ames test using *S. typhimurium* TA100, in the presence or absence of an activating system derived from the liver of Aroclor 1254-treated rats. Results are presented as mean ±S.D. of triplicates; the spontaneous reversion rate of 99±6 has already been subtracted. The methoxy hydrocarbon (**1c**), the 17-ol (**5**) and the 16,17-diol (**6**) were all inactive in the absence of microsomal activation (data not shown). The steeper decline in the number of revertants with increasing dose for the 15-ol with activation indicates that there is a deactivation system also present in the microsomal preparation.

4. Discussion

Several chemical differences were noted between the 11-methoxy and 11-methyl hydrocarbons and ascribed to enhanced electron release from the methoxy group in the former [9]. C-15 is attached *para* to the methoxy group in the aromatic ring and hence acquires electron density from the latter by mesomeric electron shift, so that C-15 is an especially electron rich centre. This no doubt accounts for the lability of the C(15)–O bond; C-17 is attached *meta* to the methoxy group and therefore cannot acquire electron density by this mechanism. Biologically the result is that the 17-ol (**5**) is not mutagenic, whereas the isomeric 15-ol (**4**) is a strong direct acting mutagen. Our attention was first drawn to this compound (**4**) when we found that it exists as a pair of epimers, readily interconverting under the influence of factors such as temperature, presumably by carbon–oxygen bond cleavage and reformation. This compound also seemed to be unusually reactive, decomposing slowly on being kept under ambient conditions with the formation of less polar materials, which however do not include the 15-methyl ether (**7**). The presence of the 15-ol as a major metabolite was expected because biological 15-hydroxylation is commonly found with other cyclopenta[*a*]phenanthrenes [2]. The observation that (**4**) was converted spontaneously at an appreciable rate into this methyl ether (**7**) when the metabolite mixture was allowed to stand at room temperature before chromatographic separation was unexpected; it suggests that a reactive methylating agent (methyl folate?) is co-extracted from the microsomal system by the ethyl acetate. However, irrespective of the mechanism, the fact that it occurs points again to the reactivity of the 15-ol. The role of this alcohol became apparent



Scheme 3. Bio-activation of safrole (**8**). The ultimate carcinogen (**10**) is formed by loss of OH-from (**9**) leaving, on the benzylic carbon atom, a positive charge which is partially stabilised by the adjacent conjugated system.

when, of the major metabolites (**4**)–(**6**), it alone was found to be a strong direct acting mutagen, whereas the parent 11-methoxy hydrocarbon (**1c**) was less active and then only after bio-activation.

This situation bears a strong structural analogy to the weak hepatocarcinogen safrole (1-allyl-3,4-methylenedioxybenzene, **8**) which is known to be metabolised to its 1′-hydroxy derivative (**9**) [15] (Scheme 3). It is thought that bio-activation of the latter (**9**) occurs through conversion to its sulphate ester with cleavage of the C–O bond to give the ultimate carcinogen (**10**) [16]. In the present case it is reasonable to conclude that the 15-ol (**4**) itself is the active compound. This unexpected mode of activation of a polycyclic aromatic compound is thus the direct result of strong electron release from the *para* 11-methoxy group, already implicated in certain unusual chemical reactions of (**1c**) [9]. It may serve to explain the higher than expected carcinogenicity of this compound although of course this explanation needs confirmation by an independent in vivo study of the comparative carcinogenic potentials of the 11-methoxy hydrocarbon and its 15-ol.

Acknowledgements

We wish to thank Dr. G.W. Boyd for the mass spectrum of (**7**) and Mr. J. Bloxsidge for the NMR spectra of the metabolites.

References

[1] G.W. Boyd, M.M. Coombs, W.M. Baird, The presence of a trifluoromethyl rather than a methyl substituent in the bay-region greatly decreases the DNA-binding and tumour-initiating activity of the cyclopenta[*a*]phenanthrene-17-ones, Carcinogenesis 16 (1995) 2543–2547.

[2] M.M. Coombs, T.S. Bhatt, 1987. Cyclopenta[*a*]phenanthrenes; Polycyclic Aromatic Compounds Structurally Related to Steroids. Cambridge Univ. Press, Cambridge.

[3] A. Butenandt, H. Dannenberg, Untersuchungen über die krebserzengende Wirksamkeit der Methylhomologen des 1,2-Cyclopentaphenanthrenes, Arch. Geschwulstforsch. 6 (1953) 1–7.

[4] M.M. Coombs, T.S. Bhatt, S. Young, The carcinogenicity of 15,16-dihydro-11-methylcyclopenta[*a*]phenanthren-17-one, Br. J. Cancer 40 (1979) 914–921.

[5] M.M. Coombs, T.S. Bhatt, A.-M. Kissonerghis, J.A. Allen, C.W. Vose, Identification of the proximate and ultimate forms of the carcinogen 15,16-dihydro-11-methylcyclopenta[*a*]phenanthren-17-one, Cancer Res. 39 (1979) 4160–4165.

[6] T.S. Bhatt, S.T. Hadfield, M.M. Coombs, Carcinogenicity and mutagenicity of some alkoxy cyclopenta[*a*]phenanthrenes: effect of obstructing the bay region, Carcinogenesis 3 (1982) 677–680.

[7] T.S. Bhatt, Synthesis and biological activity of some further cyclopenta[*a*]phenanthrenes, Carcinogenesis 9 (1988) 1669–1672.

[8] M.M. Coombs, C.J. Croft, Carcinogenic cyclopenta[*a*]-phenanthrenes, Prog. Exp. Tumor Res. 11 (1969) 69–71.

[9] M.M. Coombs, G.W. Boyd, 1998. Potentially carcinogenic cyclopenta[*a*]phenanthrenes: Part 12. Synthesis of metabolites of the carcinogen 16,17-dihydro-11-methoxycyclopenta[*a*]phenanthrene, J. Chem. Res. (S), 692-3; (M), 2901–2911.

[10] C. Ioannides, D.V. Parke, Mechanism of induction of hepatic drug metabolising enzymes by a series of barbiturates, J. Pharm. Pharmacol. 27 (1975) 739–749.

[11] D.M. Maron, B.N. Ames, Revised methods for the *Salmonella* mutagenicity test, Mutat. Res. 113 (1983) 173–215.

[12] E.J. Cory, K. Achiwa, A method for the deoxygenation of allylic and benzylic alcohols, J. Org. Chem. 34 (1969) 3667–3668.

[13] M.M. Coombs, C. Spruce, Novel one-step synthesis and hepatic microsomal metabolism of 16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene, Polycyclic Aromat. Comp. 11 (1996) 275–282.

[14] C. Papaparaskeva-Petrides, C. Ioannides, G.W. Boyd, R.J. Young, R.G. Harvey, M.M. Coombs, The mutagenicity of chemically synthesised metabolites of 16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene and its carcinogenic 11-methyl homologue, Mutagenesis 8 (1993) 307–310.

[15] E.C. Miller, A.B. Swanson, D.H. Phillips, T.L. Fletcher, A. Liem, J.A. Miller, Structure-activity studies of carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole, Cancer Res. 43 (1983) 1124–1134.

[16] E.W. Boberg, E.C. Miller, J.A. Miller, A. Poland, A. Liem, Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfo-oxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver, Cancer Res. 43 (1983) 5163–5173.