Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 1085-1091

METABOLISM OF 7,12-DIMETHYLBENZ[a]ANTHRACENE AND ITS METHYL-HYDROXYLATED METABOLITES: FORMATION OF PHENOLIC METABOLITES AT THE 2-POSITIONS

Ming W. Chou, George D. Easton, and Shen K. Yang[1]

Department of Pharmacology, School of Medicine, Uniformed Services
University of the Health Sciences, Bethesda, Maryland 20014

Received May 4, 1979

SUMMARY: The 1- and 2-positions of 7,12-dimethylbenz[a]anthracene (DMBA) were thought not to be involved in biotransformation to 1,2-epoxide and 1,2-dihydrodiol because of steric hindrance from the 12-methyl group (Biochem. Biophys. Res. Commun. 85: 357-362, 1978). However, we have identified four 2-phenols as rat liver microsomal metabolites of DMBA and its methyl-hydroxylated metabolites, 7-hydroxymethyl-12-methylbenz[a]anthracene, 7-methyl-12-hydroxymethylbenz[a]-anthracene, and 7,12-dihydroxymethylbenz[a]anthracene. Our findings suggest that neither the 12-methyl group nor the 12-hydroxymethyl group blocks the microsomal oxygenations of the 1,2 positions of DMBA or its methyl-hydroxylated derivatives. The 2-phenols may be formed as nonenzymatic rearrangement products of the 1,2-epoxide intermediates, although their formations by a direct hydroxylation mechanism cannot be ruled out.

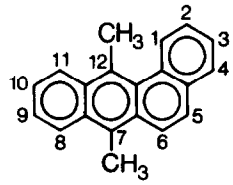
Recent evidence suggests that the potent carcinogen, 7,12-dimethylbenz-[a]anthracene (DMBA)[2], is metabolically activated at the 1,2,3,4-ring (1-10). DMBA-trans-3,4-dihydrodiol (5-8), 7-OHM-12-MBA-trans-3,4-dihydrodiol, 7-M-12-OHMBA-trans-3,4-dihydrodiol, and 7-12-diOHMBA-trans-3,4-dihydrodiol (6,7) have recently been found as metabolites of DMBA and its methyl-hydroxylated metabolites, 7-OHM-12-MBA, 7-M-12-OHMBA, and 7,12-diOHMBA. These 3,4-dihydrodiol metabolites possess higher mutagenic activity (8-10) and DNA-binding activity (6) than their parent hydrocarbons upon further metabolism. In addition to the 3,4-dihydrodiols, 3- and 4-phenols of DMBA and its methyl-hydroxylated derivatives have been reported as metabolites (11,12). However,

[1]To whom correspondence should be addressed.

[2]Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; 7-OHM-12-MBA, 7-hydroxymethyl-12-methylbenz[a]anthracene; 7-M-12-OHMBA, 7-methyl-12-hydroxymethylbenz-[a]anthracene; 7,12-diOHMBA, 7,12-dihydroxymethylbenz[a]anthracene; 2-OH-DMBA, 2-hydroxy-DMBA; 7-OHM-12-MBA-trans-3,4-dihydrodiol, trans-3,4-dihyhdroxy-3,4-dihydro-7-OHM-12-MBA. Other phenolic and dihydrodiol derivatives are similarly designated; HPLC, high pressure liquid chromatography; THF, tetrahydrofuran.

0006-291X/79/111085-07$01.00/0

enzymatic oxygenation products at the 1,2 positions have hitherto not been reported and it was thought that the 1,2 positions of DMBA were not available for metabolic oxidation because of steric hindrance from the 12-methyl group (10). We report the identification of 2-OH-DMBA, 2-OH-7-OHM-12-MBA, 2-OH-7-M-12-OHMBA, and 2-OH-7,12-diOHMBA as minor rat liver microsomal metabolites of DMBA and its methyl-hydroxylated metabolites. Our findings indicate that the presence of the 12-methyl group or the 12-hydroxymethyl group of DMBA suppresses rather than blocks completely metabolic oxidations at the 1,2 positions.



Structural formula of DMBA.

MATERIALS AND METHODS

Materials: DMBA was purchased from Eastman Chemical Co. and was purified by passing it through a silica gel (Bio-Sil A, 100-200 mesh, BioRad Laboratories) column with hexane as the eluting solvent. 7-OHM-12-MBA, 7-M-12-OHMBA, and 7,12-diOHMBA were synthesized according to a modified procedure (13) of Boyland and Sims (14). DMBA 1-, 2-, 3-, and 4-phenols were kindly provided by Professor Melvin S. Newman of The Ohio State University. The UV absorption and fluorescence spectra of these synthetic phenols were obtained from compounds that had been purified by HPLC. Liver microsomes were obtained from phenobarbital pretreated (i. p. injections of 75 mg/kg body weight on each of 3 days) male Sprague-Dawley rats weighing 80-100 g. Metabolites of DMBA, 7-OHM-12-MBA, 7-M-12-OHMBA, and 7,12-diOHMBA were obtained by in vitro incubation of each of the substrates with liver microsomes and cofactors at 37°C for 1 hr and prepared for HPLC isolation as previously described (6).

Isolation of Metabolites: A Spectra-Physics model 3500B liquid chromatograph was fitted with a DuPont 4.6 mm inside diameter x 25 cm Zorbax ODS (C_{18}) column. The column was eluted at ambient temperature with 50% (v/v) methanol in water for 10 min and followed by a 40-min linear gradient of 50% (v/v) methanol in water to 100% methanol at a solvent flow rate of 0.8 ml/min. Each of the chromatographic peaks which was combined from 10 to 20 chromatographic runs was further purified by an isocratic system on a DuPont 6.2 mm inside diameter x 25 cm Zorbax SIL (silica gel) column with varying ratios of THE/hexane/methanol as the eluting solvent. This latter HPLC system was used to confirm the purity of the metabolites and was also used to separate overlapping metabolites that were inseparable with the ODS column (6).

Physical Properties of the Metabolites: Ultraviolet-visible absorption spectra of the metabolites were measured in methanol on a Cary 118C spectrophotometer. Fluorescence spectra of the metabolites were measured in methanol and in 0.1

N NaOH on a Perkin-Elmer model 44A spectrofluorimeter. Mass spectral analysis of the metabolites was performed on a Finnigan 4000 gas chromatograph/mass spectrometer/data system by electron impact with a solid probe at 70 eV and 250°C ionizer temperature.

RESULTS AND DISCUSSION

The retention times of four 2-phenols and some other known metabolites on the reversed-phase HPLC are listed in Table I. The uv-vis absorption spectra and fluorescence spectra are shown in Fig. 1. The 2-OH-DMBA (m/e of M^+ at 272), 2-OH-7-M-12-OHMBA (m/e of M^+ at 288) and 2-OH-7-OHM-12-MBA (m/e of M^+ at 288) were found as minor metabolites when DMBA was used as substrate in the in vitro incubation. The inability to identify 2-OH-7,12-diOHMBA as an in vitro incubation product from DMBA may be due to the small extent of further metabolism of the minor metabolite, 7,12-diOHMBA, during the metabolism of DMBA. When 7-OHM-12-MBA was used as the substrate, 2-OH-7-OHM-12-MBA (m/e of M^+ at 288) and 2-OH-7,12-diOHMBA (m/e of M^+ at 304) were found as two of the

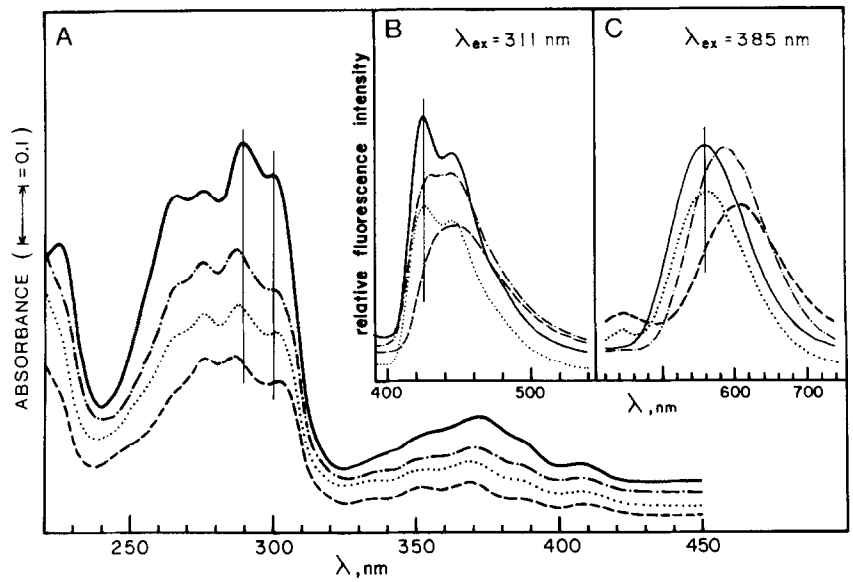


Figure 1 Spectroscopic properties of four 2-phenol metabolites. UV-vis absorption spectra were measured in methanol (A), uncorrected fluorescence spectra were measured in methanol (B), and in 0.1 N NaOH (C). Excitation wavelengths (λ_{ex}) are indicated in the figure. Differences in absorption and fluorescence maxima are contrasted by vertical lines. ———, 2-OH-DMBA; —·—·—, 2-OH-7-OHM-12-MBA; ....., 2-OH-7-M-12-OHMBA; — — —, 2-OH-7,12-diOHMBA.

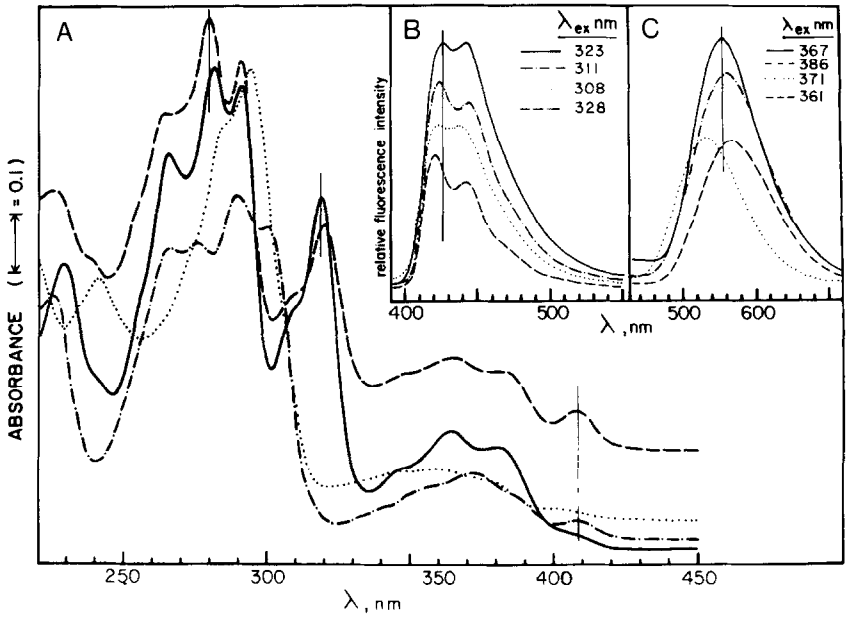


Figure 2 Spectroscopic properties of synthetic DMBA 1-, 2-, 3-, and 4-phenols. UV-vis absorption spectra were measured in methanol (A), uncorrected fluorescence spectra were measured in methanol (B) and in 0.1 N NaOH (C). Excitation wavelengths (λ_{ex}) are indicated in the figure. Differences in absorption and fluorescence maxima are contrasted by vertical lines. ———, 1-OH-DMBA; —·—·—, 2-OH-DMBA; ....., 3-OH-DMBA, — — —, 4-OH-DMBA.

minor products. Similarly, 2-OH-7-M-12-OHMBA (m/e of M^+ at 288) and 2-OH-7,12-diOHMBA (m/e of M^+ at 304) were detected as two of the minor metabolites of 7-M-12-OHMBA, and 2-OH-7,12-diOHMBA (m/e of M^+ at 304) was also found as a minor metabolite of 7,12-diOHMBA. In spite of an intensive search of the HPLC-isolated fractions, we have failed to obtain evidence for the presence of 1-phenol metabolites, although the synthetic 1-OH-DMBA is separable from DMBA 2-, 3-, 4-phenols and is stable in the reversed-phase HPLC system.

The identification of 2-OH-DMBA is based on the comparison of its uv-vis absorption and fluorescence spectra and HPLC retention time with those of the synthetic standard 2-OH-DMBA (Figs. 1 and 2) and the molecular weight from mass spectral analysis. Upon HPLC purification and confirmation of their molecular weights by mass spectral analysis, the phenolic acid-dehydration products of the DMBA 3,4-, 5,6-, 8,9-, and 10,11-dihydrodiols are distinctly

different from the DMBA 1-, 2-, 3-, and 4-phenols. Due to the lack of synthetic standards, 2-OH-7-OHM-12-MBA, 2-OH-7-M-12-OHMBA, and 2-OH-7,12-diOHMBA were identified by mass spectral analysis and by comparing their uv-vis absorption spectra with that of the synthetic 2-OH-DMBA (Fig. 1). Although the uv-vis absorption spectra are closely similar (Fig. 1A), the retention times on HPLC (Table I) and the fluorescence spectra of all four 2-phenols

TABLE I

RETENTION TIMES ON A REVERSED-PHASE HPLC SYSTEM AND MOLECULAR WEIGHTS OF SOME KNOWN METABOLITES[a]

Metabolite	Ret. time (min)	m/e of M^+	Metabolite	Ret. time (min)	m/e of M^+
7-M-12-OHMBA-trans-5,6-diol	4.6	306	2-OH-7-OHM-12-MBA	28.1	288
7,12-diOHMBA-trans-3,4-diol	5.2	322	3-OH-7-OHM-12-MBA	30.9	288
7,12-diOHMBA-trans-8,9-diol	5.6	322	4-OH-7-M-12-OHMBA	32.8	288
7-OHM-12-MBA-trans-5,6-diol	12.1	306	4-OH-7-OHM-12-MBA	33.7	288
4-OH-7,12-diOHMBA	13.0	304	3-OH-7-M-12-OHMBA	34.2	288
7-OHM-12-MBA-trans-8,9-diol	13.3	306	DMBA-trans-3,4-diol	37.8	290
2-OH-7,12-diOHMBA	17.8	304	2-OH-7-M-12-OHMBA	38.8	288
7-OHM-12-MBA-trans-3,4-diol	18.6	306	7-OHM-12-MBA	41.6	272
3-OH-7,12-diOHMBA	19.2	304	7-M-12-OHMBA	42.6	272
DMBA-trans-5,6-diol[b]	19.3	290	1-OH-DMBA[c]	44.3	272
7-M-12-OHMBA-trans-3,4-diol[b]	19.3	306	2-OH-DMBA	45.7	272
DMBA-trans-8,9-diol[b]	22.7	290	3-OH-DMBA	46.1	272
DMBA-trans-10,11-diol[b]	22.7	290	4-OH-DMBA	47.0	272
7,12-diOHMBA	24.6	288	DMBA	56.2	256

[a]Detailed characterization of metabolites other than 2-phenols will be presented in a later report (M. W. Chou and S. K. Yang, manuscript in preparation). Retention times varied slightly among different HPLC runs under the same conditions, but the elution orders remained unchanged.

[b]These metabolites were resolved on a DuPont Zorbax SIL column (6.2 mm x 25 cm) with 60% (v/v) THF in hexane at a flow rate of 1 ml/min.

[c]This is a synthetic standard. It has not been found as a metabolite.

measured in methanol and in 0.1 N NaOH are different (Fig. 1B and 1C). The shifts in fluorescence maxima of the 2-phenols in alkaline solution (Fig. 1C) are characteristic of phenolic polycyclic aromatic hydrocarbons. The shifts in fluorescence maxima are the most pronounced when the 12-methyl group of DMBA is hydroxylated (Fig. 1B and 1C).

Phenolic products of DMBA and its methyl-hydroxylated derivatives are most likely formed from the epoxide intermediates, although they may be formed by direct hydroxylation via an insertion mechanism (15). If the four 2-phenols were indeed formed from 1,2-epoxide intermediates, our results would indicate that all methyl groups and ring carbons numbered 1 to 6 and 8 to 11 of DMBA are involved in metabolic oxygenations. Trans-1,2-dihydrodiols have not been found as metabolites of either DMBA or its methyl-hydroxylated derivatives. This may be due to the fact that the 1,2 positions are minor enzymic oxygenation sites and the 1,2-epoxide intermediates may be unstable and mostly rearrange nonenzymatically to 2-phenols. Both of these situations may be caused by the steric hindrance from the 12-methyl and the 12-hydroxymethyl groups. Although the metabolism at the 1,2 positions of DMBA is suppressed by the presence of the 12-methyl group, the 1,2 positions of the four trans-3,4-dihydrodiol metabolites of DMBA (5-7,9) are, however, most likely involved in metabolic oxygenations to form 3,4-diol-1,2-epoxides which are believed to be the ultimate carcinogenic metabolites of DMBA (1-10).

REFERENCES

1. Moschel, R. C., Baird, W. M., and Dipple, A. (1977) Biochem. Biophys. Res. Commun. 76, 1092-1098.
2. Vigny, P., Duquesne, M., Coulomb, H., Tierney, B., Grover, P. L., and Sims, P. (1977) FEBS Lett. 82, 278-282.
3. Ivanovic, V., Geacintov, N. E., Jeffrey, A. M., Fu, P. P., Harvey, R. G., and Weinstein, I. B. (1978) Cancer Lett. 4, 131-140.
4. Dipple, A., Tomaszewski, J. E., Moschel, R. C., Bigger, C. A. H., Nebzydoski, J. A., and Egan, M. (1979) Cancer Res., 39, 1154-1158.
5. Tierney, B., Hewer, A., MacNicoll, A. D., Gervasi, P. G., Rattle, H., Walsh, C., Grover, P. L., and Sims, P. (1978) Chem.-Biol. Interactions, 23, 243-257.
6. Chou, M. W., and Yang, S. K. (1978) Proc. Natl. Acad. Sci., USA, 75, 5466-5470.
7. Yang, S. K., Chou, M. W., and Roller, P. P. (1979) J. Am. Chem. Soc., 101, 237-239.

8. Malaveille, C., Bartsch, H., Tierney, B., Grover, P. L., and Sims, P. (1978) Biochem. Biophys. Res. Commun. 83, 1468-1473.
9. Huberman, E., Chou, M. W., and Yang, S. K. (1979) Proc. Natl. Acad. Sci., USA, 76, 862-866.
10. Marquardt, H., Baker, S., Tierney, B., Grover, P. L., and Sims, P. (1978) Biochem. Biophys. Res. Commun. 85, 357-262.
11. Sims, P., and Grover, P. L. (1974) Adv. Cancer Res. 20, 165-274.
12. Yang, S. K., and Dower, W. V. (1975) Proc. Natl. Acad. Sci., USA, 72, 2061-2605.
13. Fu, P. P., and Yang, S. K. (1979) J. Labeled Compds. Radiopharmaceuticals, in press.
14. Boyland, E., and Sims, P. (1965) Biochem. J. 95, 780-787.
15. Jerina, D. M., and Daly, J. W. (1974) Science 185, 573-582.