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Synthesis and Characterisation of *N*-Alkylated L-Valine Methylamide Products from Diol Epoxide Metabolites of Fluoranthene and Benzo[*a*]pyrene

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The reactive diol epoxides of fluoranthene and benzo[*a*]pyrene, (±)-*anti*-*c*-1,*c*-10b-epoxy-1,2,3,10b-tetrahydrofluoranthene-*r*-2,*t*-3-diol (**1**) and (±)-*anti*-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-*r*-7,*t*-8-diol (**4**), respectively, are known genotoxic agents and effective alkylators of nucleophilic sites in DNA and proteins. As models of *in vivo* *N*-alkylation at the *N*-termini of hemoglobin (Hb), *N*-L-valine methylamide (VMA) products of **1** and **4** were synthesised, *N*-(*r*-1,*c*-2,*t*-3-trihydroxy-1,2,3,10b-tetrahydrofluoranthen-*t*-10b-yl)-L-valine methylamide (**2a,b**) and *N*-(*r*-7,*t*-8,*t*-9-trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyren-*c*-10-yl)-L-valine methylamide (**5a,b**), respectively. For isolation of the reaction products from the alkylation of VMA two reversed-phase HPLC systems were developed. From each diol epoxide two diastereoisomeric products were isolated in separate fractions. The products were characterised by ^{1}H NMR spectroscopy as well as by thermospray (TSP) tandem quadrupole mass spectrometry (MS and MS/MS) and fluorescence. When analysed by TSP-MS/MS the quasi-molecular ions, $[M+H]^+$, have a neutral loss of either the VMA or the **1** and **4** adduct moieties, respectively. The major daughter ion from **5a,b** is the adduct moiety and the corresponding ion from **2a,b** is the VMA moiety. Minor daughter ions are, in both cases, $[(M-VMA)+H]^+$ ions with additional loss of H_2O or H_2O+CO. The higher the number of hydroxy groups in the **2a,b** ions, the more easily is the C10b-amino bond disrupted in collision-induced dissociation.

A number of polycyclic aromatic hydrocarbons (PAH), found in urban air pollution,[1] the diet,[2] certain occupational exposures[3] and tobacco smoke,[4] are known to induce mutagenic lesions through their reactive metabolites and hence they constitute a cancer risk.[5] Therefore, it is of importance to develop a method to monitor mutagenic exposure of humans to PAH. Two PAHs of particular interest are fluoranthene (FA) and benzo[*a*]pyrene (BaP). BaP is a classical carcinogen intensively studied and is a potent carcinogen in animal experiments.[5] FA is interesting because of its occurrence at relatively high concentrations in the environment.[6] FA is present at 40 times higher levels than BaP in diesel and gasoline exhausts (from engines without catalyst).[7] Dietary intake of PAH contains larger amounts of FA than of any other measured PAH, the intake of FA being six to eight times higher than that of BaP.[2]

FA is metabolised *in vivo* by cytochrome P450 isozymes (not yet identified) and epoxide hydrolase to mutagenic diol epoxides (DEs) where the enantiomers (±)-*anti*-*c*-1,*c*-10b-epoxy-1,2,3,10b-tetrahydrofluoranthene-*r*-2,*t*-3-diol (**1**) are more effective mutagens than other diol epoxides of FA, such as (±)-(*syn*)-2,3-diol 1,10b-epoxides and (±)-(*syn*/*anti*)-1,10b-diol 2,3-epoxides (bacterial HPRT mutation assay).[8] BaP is metabolised *in vivo* by cytochrome P-450 isozymes 1A and epoxide hydrolase to different mutagenic DEs where the + enantiomer of (±)-*anti*-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene-*r*-7,*t*-8-diol (**4**) is the isomer that gives the highest frequency of mutations in V79-cells (HPRT mutation assay)[9] and the highest levels of papillomas in mice.[10]

Though FA is a weak carcinogen it is as potent a mutagen as BaP.[11] Therefore, at low exposure levels, where induced mutation is a determinant of the cancer risk increment, FA, owing to its abundance in emissions from combustion, might be more important a contributor than BaP to the risk from PAH exposure.[11–14] At high doses in carcinogenicity tests, however, BaP is about 100 times more effective compared with FA,[15,16] evidently due to the promoter action of BaP.[11]

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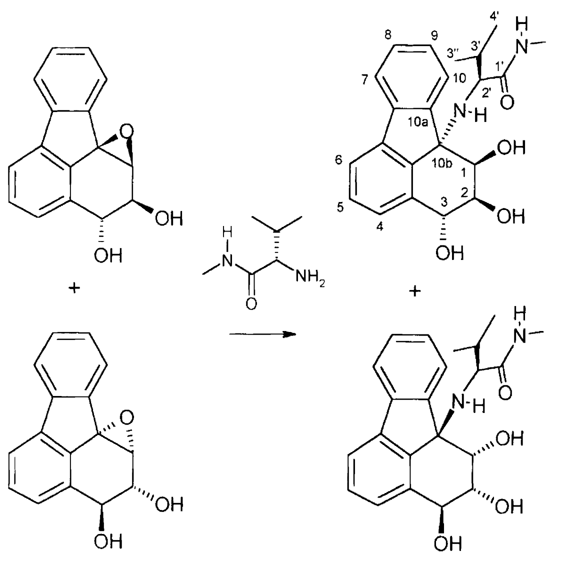
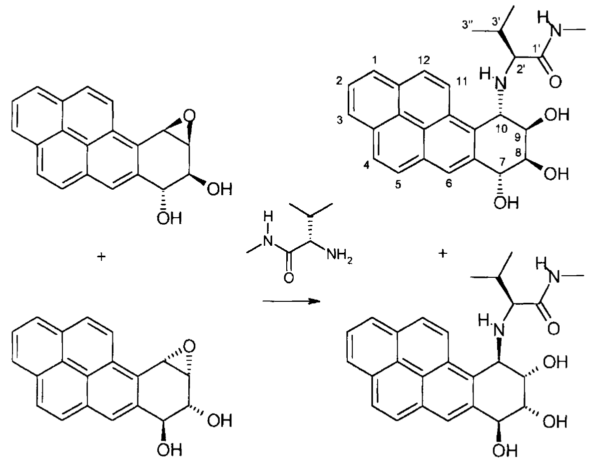
Exposure to mutagens can be monitored *in vivo* through reaction products (adducts) of their reactive metabolites with nucleophilic sites in hemoglobin (Hb).[17,18] Amino nitrogens (in valine, lysine or histidine) have been demonstrated as sites for alkylation in Hb by **4**.[19] Carboxy groups were judged to be the main target for alkylation of proteins by **4**.[20] Except for one of the aspartate adducts in human Hb,[20] the formed esters are, due to fast hydrolysis, less stable than the adducts to amino groups. For the establishment of a relationship between exposure dose and degree of alkylation it is essential that the adducts monitored have a degree of stability. Previously, adducts to the *N*-terminal valines in Hb have been shown to be useful as markers for exposure to low molecular weight compounds and the corresponding adducts from **1** and **4** might serve as markers for FA and BaP exposure, respectively.[17]

Studies of reaction kinetics and the degree of alkylation by PAH metabolites of the *N*-terminal valine residues in the α- and β-Hb chains require a substance that can be used as a model of the reactivity at the *N*-terminal amino nitrogens. L-Valine methylamide (VMA) has been used to compare the second-order alkylation rate constants for a number of alkylating agents.[21] There is good correspondence between the *N*-termini of the Hb-globin chains and VMA with respect to determinants of reaction rates such as pK_a and nucleophilic strength of the reactive sites. Reaction products of FA and BaP diol epoxides with VMA (the VMA-adducts **2a,b** and **5a,b**, respectively) were synthesized and the **a** and **b** diastereoisomeric products were isolated in separate fractions (Figs. 1 and 2). ^{1}H NMR spectra, especially coupling constants as determined for adjacent pairs of methine protons, furnished evidence for the proposed structures and configurations of each pair of adducts (**2a,b** and **5a,b**, respectively). For validation of the sensitive techniques for quantification, the VMA-adducts were used as models for *N*-terminal Hb-adducts (i.e., biomarkers of PAH). Characteristic quasi-molecular ions, mass fragmentation and neutral loss from the VMA-adducts were determined by means of MS and MS/MS and might be useful for identification as well as quantification of *N*-alkyl adducts, from **1** and **4**, in proteins, peptides and amino acids. The VMA-adducts were further studied by means of relative fluorescence responses in order to evaluate sensitivity for detection.



1 **2a,b**

Fig. 1. Reaction between (±)-*anti*-*c*-1,*c*-10b-epoxy-1,2,3,10b-tetrahydrofluoranthene-*r*-2,*t*-3-diol (**1**) and L-valine methylamide with the diastereoisomeric reaction products (**2a,b**). The products correspond to the 3*R*, 2*S*, 1*R*, 10b*S*, 2′*S* isomer (above) and the 3*S*, 2*R*, 1*S*, 10b*R*, 2′*S* isomer (below).



4 **5a,b**

Fig. 2. Reaction between (±)-*anti*-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-*r*-7,*t*-8-diol (**4**) and L-valine methylamide with the diastereoisomeric reaction products (**5a,b**). The products correspond to the 7*R*, 8*S*, 9*R*, 10*S*, 2′*S* isomer (above) and the 7*S*, 8*R*, 9*S*, 10*R*, 2′*S* isomer (below).

Results and discussion

Synthesis. An improved method for purifying VMA synthesized from L-valine methyl ester was developed compared with the purification according to Calleman *et al.*[21] After removal of L-valine (tetrahydrofuran filtration) followed by precipitation of the VMA hydrochloride before ion-exchange chromatography the isolated VMA was without impurities as observed in GC–MS, chemical ionisation in the positive ion mode (PCI) and HPLC (UV detection).

Owing to limited access of DEs the syntheses with VMA had to be carried out with very small quantities (1.5 mg). Therefore, conditions for optimization of the VMA product yields were developed, especially through counteraction of the hydrolysis of DEs. In the reactions *tert*-butyl alcohol was used as the solvent which, in contrast with water, does not react with the DEs. To

increase the slow reaction rate of **1** in *tert*-butyl alcohol addition of 50% water was essential.

Chromatography. The DEs **1** and **4** and their products in the reaction with VMA were analysed by means of HPLC (Figs. 3 and 4). For each of the two compounds, the hydrolysis product (**3** and **6**, respectively) and the products from the reaction with VMA (**2a**, **2b**, and **5a**, **5b**, respectively) were detected.

The HPLC system designed for **4** and its reaction products was composed of tetrahydrofuran (THF) and 2-methoxyethanol (Fig. 3). The presence of THF in water (pH $\geqslant$ 7.5) in the mobile phase lowered the hydrolysis rate of **4** considerably compared with aqueous medium. According to Whalen *et al.*,[22] dioxane has been shown to counteract hydrolysis of **4** and, like THF, dioxane is an aprotic solvent which prevents hydrogen-bond-induced catalysis. The resolving power in the HPLC analysis of the reaction products, from **1** and **4**, was further improved by the addition of 2-methoxyethanol.

Structure elucidation of **2a,b** *by NMR spectroscopy.* In studies of the products with cysteine (in Hb) and of hydrolysis products derived from **1**, the nucleophilic atoms have been shown to bind to carbon 10b[23,24] (Fig. 1). After *N*-alkylation of VMA by C_{10b} the position of the ring-opened oxirane oxygen can be directed *cis* (C_{10b}-*cis*) or *trans* (C_{10b}-*trans*) with respect to the amino group. From molecular models the theoretical dihedral angles between C_2-H and C_3-H from alkylation at C_{10b}-*cis* and C_{10b}-*trans* were calculated to be 153° and 145°, respectively. The corresponding angles between C_1-H and C_2-H were 23° and 46°, respectively. According to Karplus[25] the dihedral angles from C_{10b}-*cis* alkylation correspond to the coupling constant $J_{1,2}$ = 7–10 Hz and therefore the derived coupling constants ($J_{2,3}$ and $J_{1,2}$ = 4.0–4.4 Hz) are compatible with the dihedral angles from C_{10b}-*trans* alkylation (Fig. 4). This implies that **2a,b** are C_{10b}-*trans* products (Fig. 1).

Structure elucidation of **5a,b** *by NMR spectroscopy.* In a study carried out by Cheng *et al.*[26] the absolute configuration of the products of reaction between amino groups in purines and each of the two enantiomeres of **4** were determined by ^{1}H NMR spectroscopy. The position of the amino nitrogen was in all isomers located at carbon 10. The amino group was *trans* (C_{10}-*trans*) or *cis* (C_{10}-*cis*) with respect to the hydroxy group at carbon 9. The determinants of the C_{10}-*trans* configuration, according to Karplus,[25] were the $J_{7,8}$ and $J_{9,10}$ coupling constants, which, for all C_{10}-*trans* purine products, were 9.2–9.4 and 3.0–3.9 Hz, respectively (the corresponding coupling constants for C_{10}-*cis* were 3.2–4.2 and 4.3–5.4 Hz, respectively). To determine whether the amino group in **5a,b** was C_{10}-*trans* or C_{10}-*cis*, the $J_{7,8}$

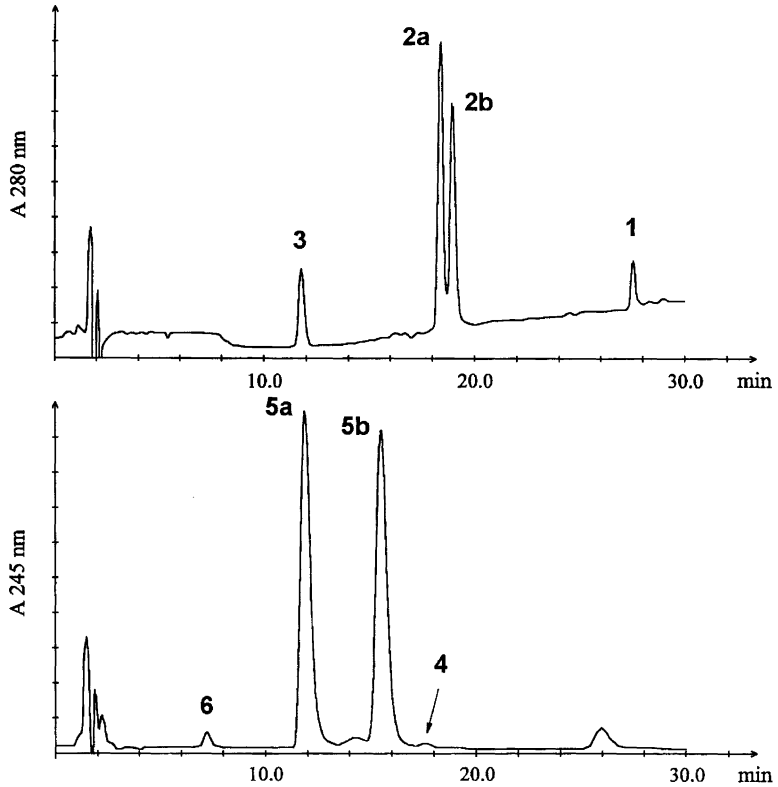


Fig. 3. The product distribution from the reaction of **1** with VMA after 43 h (upper trace). The two peaks **2a** and **2b** are the products of reaction with VMA. Product **3** is the hydrolysis product. The product distribution from reaction of **4** with VMA after 24 h (lower trace). The two peaks **5a** and **5b** are the products of reaction with VMA. Product **6** is the hydrolysis product.

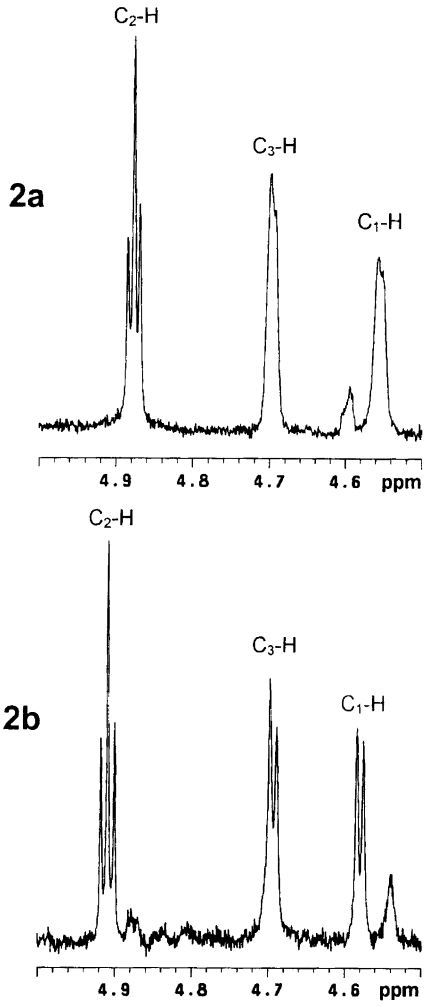


Fig. 4. The ^{1}H NMR shifts from the methine protons in the aliphatic ring chains of **2a** and **2b**.

and $J_{9,10}$ coupling constants were measured (Fig. 5). The coupling constants obtained from **5a,b** ($J_{7,8}$ = 8.6–9.3 and $J_{9,10}$ = 2.8–3.0 Hz, respectively) imply that both diastereoisomers are C_{10}-*trans* products (Fig. 2).

Analysis of **2a,b** *by means of thermospray mass spectrometry.* Both **2a** and **2b** gave rise to three major positively charged thermospray (TSP) ions: $[M+H]^+$ (*m/z* 383), $[(M-H_2O)+H]^+$ (*m/z* 365) and $[(M-H_2O-CO_2)+H]^+$ (*m/z* 321). The ions have three (*m/z* 383), two (*m/z* 365) and no (*m/z* 321) hydroxy groups, respectively. The daughter ions of *m/z* 383, **2a** (Fig. 6) and **2b**, are ions from either the VMA or the FA triol unit with a disrupted C10b–amino bond (Table 1). The major daughter ion of *m/z* 383 is $[(M+H)-(\text{FA triol})]^+$ (*m/z* 131) [FA triol is the neutral loss (M = 252) from an intact adducted moiety of **1**]. The minor daughter ions are the adduct moieties $[(M+H)-\text{VMA}]^+$ (*m/z* 253), $[(M+H)-\text{VMA}-H_2O]^+$ (*m/z* 235) and $[(M+H)-\text{VMA}-H_2O-CO]^+$ (*m/z* 207). The daughter ions of *m/z* 365, **2a** and **2b**, mainly give ions cleaved in the VMA moiety with a minor and a major ion from disrupted $C_{1'}$–$C_{2'}$ bonds and one minor ion from a disrupted amide bond (Table 1). To be totally fragmented the *m/z* 321 ion needs higher collision energy than the TSP ions *m/z* 383 and *m/z* 365. The ions with none or two instead of three hydroxy groups have more stable C10b–amino bonds and need higher energy for disruption. This bond is disrupted to an extent of 99% for *m/z* 383, 1–10% for *m/z* 365 and <1% for *m/z* 321 at the same argon collision energy (10 eV). In spectra from **2a** and **2b** the relative intensities of the daughter ions from *m/z* 365 differ. Furthermore, the ions *m/z* 207 and *m/z* 278 are present in the **2b** spectrum and practically absent in the **2a** spectrum (Table 1).

Analysis of **5a,b** *by means of thermospray mass spectrometry.* Both **5a** and **5b** have TSP ionisation spectra with dominant $[M+H]^+$ (*m/z* 433) ions (Table 2). The major daughter ion of *m/z* 433, **5a** (Fig. 7) and **5b**, is $[(M+H)-\text{VMA}]^+$ (*m/z* 303) and one minor daughter ion is $[(M+H)-(\text{BaP triol})]^+$ (*m/z* 131) [BaP triol is the neutral loss unit (M = 302) from the adduct moiety of **4**] (Table 2). All daughter ions of *m/z* 433 are ions from either the VMA or the BaP triol unit with a disrupted C10–amino bond (Table 2). In mass spectrometric studies by FAB (fast atom bombardment) of **4** bound to histidine,[27] the ions *m/z* 303, *m/z* 285 and *m/z* 257 appear which have been shown to be daughter ions from the adduct moiety. This moiety and its ions are released from adducts to secondary (imidazole) amino nitrogens and, as shown in this study, from adducts to primary (*N*-terminal) amino nitrogens.

Analysis by means of fluorescence spectrometry. The fluorescence response from the **5a** and **5b** isomers, respectively, are both five times lower than the response from **6**, measured at the **5a,b** excitation and emission maxima, 345 and 400 nm, respectively, and 30 times higher than the response from the **2a** and **2b** isomers, respectively, in a comparison between the responses at the excitation and emission maxima from each compound.

Conclusions

Each of the isolated oxirane ring-opened *trans* products **2a**, **2b**, **5a** and **5b** of racemic diol epoxides originates from one diol epoxide enantiomer. It has so far not been possible to allocate the isolated products to the (+) or (−) configurations. The chiral carbons in **2a,b** are C_3, C_2, C_1, C_{10b} and $C_{2'}$ (Fig. 1). The corresponding carbons in **5a,b** are C_7, C_8, C_9, C_{10} and $C_{2'}$ (Fig. 2). The absolute configurations of the **a** and **b** products (in **2** as well as **5**) are *R*, *S*, *R*, *S*, *S* and *S*, *R*, *S*, *R*, *S* (in the consecutive order of chiral carbons), respectively, or *vice versa.* It is thus the *S*-configuration at $C_{2'}$, i.e., the L-valine α-carbon that renders the two components of the pairs **2a,b** or **5a,b** diastereoisomeric, with different physical properties as revealed by ^{1}H NMR shifts and retention times in the HPLC chromatograms.

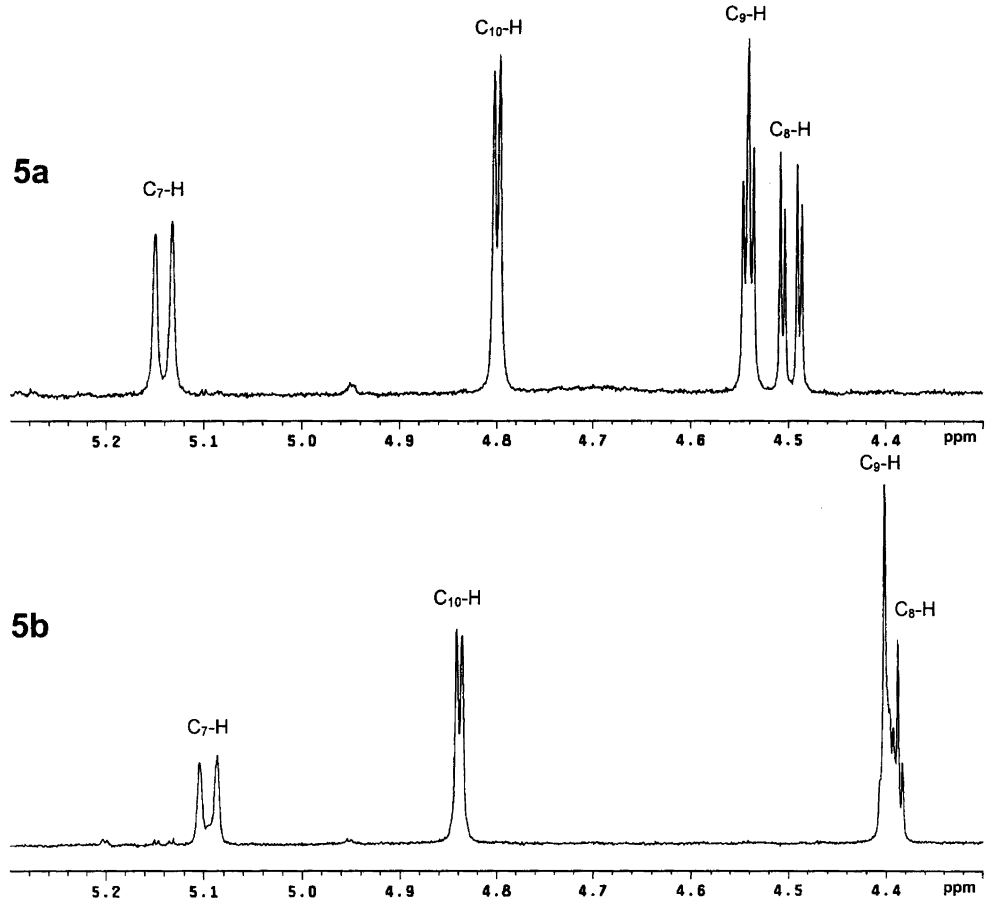


Fig. 5. The ^{1}H NMR shifts from the methine protons in the aliphatic ring chains of **5a** and **5b**.

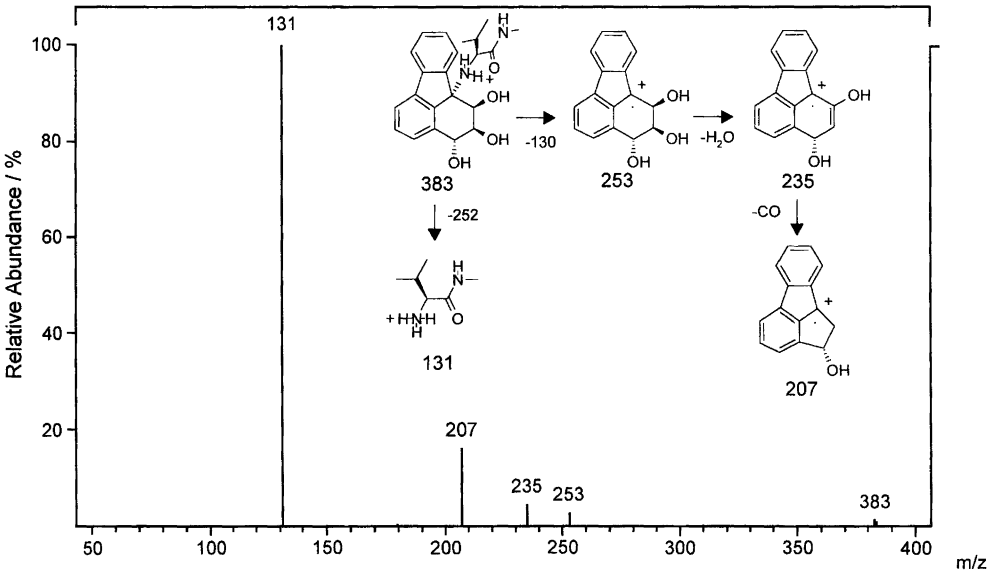


Fig. 6. Daughter ion spectrum from the **2a** $[M+H]^+$ ion (*m/z* 383), where the major daughter ion (*m/z* 131) corresponds to a neutral loss of the adduct moiety.

In HPLC analysis of *N*-terminal valine in Hb alkylated by **4** the use of fluorescence detection is sufficient for detecting the adduct at the pmol level (injected amount) and might be applied to *in vivo* dose monitoring. The *N*-terminal adducts from **1** are not useful for *in vivo* dose monitoring by means of fluorescence spectrometry, owing to weak fluorescence response.

A large part of **2a,b** is lost during the work-up proced-

Table 1. Daughter ions from the major TSP ions of **2a** and of **2b** with the corresponding neutral losses.

Daughter ions from $[M+H]^+$, *m/z* 383[a]	Daughter ions from $[(M+H)-H_2O]^+$, *m/z* 365[a]	Daughter ions from $[(M+H)-H_2O-CO_2]^+$, *m/z* 321[a]
m/z 253 (3%),[b] −[VMA]	*m/z* 334 (3%)[c] (15%),[d] $-[CH_3NH_2]$	*m/z* 262 (14%)[c] (5%),[d] $-[CH_3NHCOH]$
m/z 235 (3%)[c] (5%),[d] $-[VMA+H_2O]$	*m/z* 306 (100%),[b] $-[CH_3NHCOH]$	*m/z* 222 (49%)[c] (78%),[d] $-[CH_3NCO+CH_2CHCH_3]$
m/z 207 (8%)[c] (16%),[d] $-[VMA+H_2O+CO]$	*m/z* 278 (<1%)[c] (8%),[d] $-[CH_3NHCOH+CO]$	*m/z* 208 (100%),[b] $-[CH_3NHCOCHC(CH_3)_2]$
m/z 131 (100%),[b] −[FA triol moiety]	*m/z* 207 (<1%)[c] (16%),[d] −[VMA]	*m/z* 193 (22%)[c] (15%),[d] $-\{CH_3NHCOC[CH(CH_3)_2]NH\}$

[a]The major MS ions *m/z* 383, 365 and 321 with the relative intensities 90, 63 and 100%, respectively (from **2a** and **2b**), subjected to 10, 10 and 20 eV collision energy, respectively. [b]Relative intensity from **2a** and from **2b**. [c]Relative intensity from **2a**. [d]Relative intensity from **2b**.

Table 2. Daughter ions from the major TSP ion of **5a** and **5b** with the corresponding neutral losses.

Daughter ions from $[M+H]^+$, *m/z* 433[a]
m/z 303 (100%),[b] −[VMA]
m/z 285 (12%)[c] (30%),[d] $-[VMA+H_2O]$
m/z 257 (25%)[c] (28%),[d] $-[VMA+H_2O+CO]$
m/z 131 (19%)[c] (25%),[d] −[BaP triol moiety]

[a]The major MS ion *m/z* 433 subjected to 10 eV collision energy. [b]Relative intensity from **2a** and from **2b**. [c]Relative intensity from **2a**. [d]Relative intensity from **2b**.

ure, which may be due to loss of neighbouring hydroxy groups, which is also seen after TSP ionisation (Table 1) as the loss of H_2O and CO_2. The yield of **2a,b** was improved 2.5 times by avoiding evaporation (rotatory evaporator) of the ethyl acetate phase to dryness, prior to isolation by HPLC. This tendency towards degradation may also be a property of the adducts from **1** to *N*-terminal valine in Hb for which the VMA products are models. During the work-up procedure **5a,b**, in contrast with **2a,b**, are stable, and as long as the analysis is done by HPLC, degradation is not a problem.

Experimental

Instrumentation. The HPLC system was directed by means of an LKB 2152 controller and two LKB 2150 pumps (Bromma, Sweden) equipped with a Shimadzu SPD-2A UV detector or an RF-530 fluorescence detector (Kyoto, Japan). The samples were loaded and injected with injector 7125, Rheodyne (Cotati, CA, USA). The column for both analytical and preparative applications was Spherisorb C_{18}, 5 μm, 4.6 × 150 mm, Hichrom (Reading, Berks, UK).

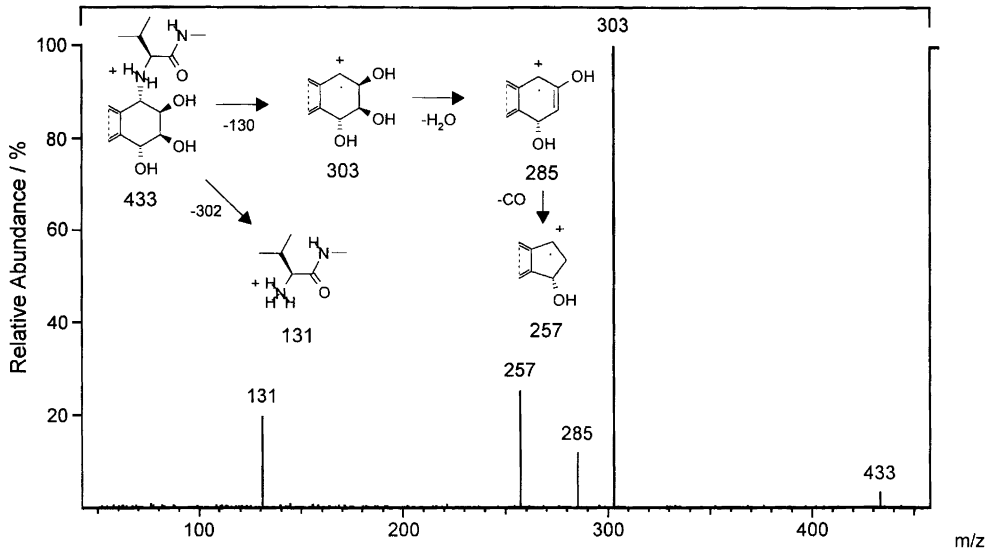


Fig. 7. Daughter ion spectrum from the **5a** $[M+H]^+$ ion (*m/z* 433), where the major daughter ion (*m/z* 303) corresponds to a neutral loss of the VMA moiety.

The UV spectra were recorded on a Shimadzu UV-160 spectrophotometer (Kyoto, Japan). The fluorescence spectra were recorded on a Hitachi F-4000 fluorescence spectrophotometer (Tokyo, Japan) equipped with a xenon lamp as the power source.

Proton NMR spectra of the PAH products were recorded for samples in acetone-d_6 with a 5 mm triple resonance PFG probe at 500 MHz on a Varian Unity Inova spectrometer equipped with a Sun Ultra workstation. Sufficient signal-to-noise ratios were obtained with 1000 scans for the fluoranthenyl compounds and 100 scans for the benzo[*a*]pyrenyl compounds. The dihedral angles of **2a** and **2b** were estimated by calculating the formation enthalpy minima with CS MOPAC Std software (MNDO function), CambridgeSoft Corporation (Cambridge, MA, USA).

Thermospray ionisation spectra of the PAH compounds were obtained by means of a Finnigan TSQ 700 triple quadrupole instrument (San José, CA, USA) and direct injection (20 µl). A mobile phase with 0.05 M ammonium acetate was used as an ionisation buffer. The flow was 1.0 ml min^{-1}, the applied discharge potential 1 kV and the electron energy 600 eV. Source-block and vaporisation temperatures were 220 °C and 108 °C, respectively. Argon was used as the collision gas at 1 mTorr in the collision chamber and an ion collision energy of 10 eV. The mass spectrometric characterisation of VMA was carried out by GC–MS, chemical ionisation in the positive ion mode (PCI), on a Finnegan 4500 mass spectrometer equipped with a Varian 3400 GC. Methane was used as the reagent gas at an ion source pressure of 0.35 Torr and an ionisation energy of 90 eV. The operating parameters for GC were as follows: helium carrier gas and a DB-17ht (30 m, 0.32 mm i.d. 0.15 µm film thickness) fused silica capillary column, J&W Scientific, Inc. (Folsom, CA, USA).

Chemicals. All chemicals were of analytical grade. Acetic acid, ethyl acetate, copper(II) nitrate trihydrate, hydrochloric acid, methanol, 2-methoxyethanol, methylamine (40%, w/w), ninhydrin, propanol, *tert*-butyl alcohol and tetrahydrofuran were obtained from Merck (Darmstadt, Germany). Dowex 50-X4 cation exchange resin, acetone-d_6, triethylamine and HCl–L-valine methyl ester were purchased from Sigma (St. Louis, MO, USA). Ammonia was purchased from Riede-de-Haën (Seelze, Germany). (±)-*anti*-*c*-1,*c*-10b-epoxy-1,2,3,10b-tetrahydro-fluoranthene-*r*-2,*t*-3-diol (**1**) and (±)-*anti*-*t*-9,*t*-10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene-*r*-7,*t*-8-diol (**4**) were obtained from the NCI Chemical Carcinogen Repository, Midwest Research Institute (Kansas City, MO, USA).

L-Valine methylamide. L-Valine methylamide (VMA) was synthesized according to Calleman *et al.*[21] 15 g (89 mmol) of HCl–L-valine methyl ester were dissolved in 42 ml of aqueous methylamine (40%, w/w). The mixture was stirred at ambient temperature until all methyl ester had been consumed, i.e., 15 h. The solvents were evaporated off from the reaction mixture in a rotatory evaporator at 70 °C to give a white syrup. This syrup was suspended in 150 ml THF at 60 °C after which the residue (L-valine) was removed by filtration and washed with 100 ml THF. The presence and the purity of VMA in the pooled eluates (250 ml) were checked by means of TLC (propanol–water, 7:3, SiO_2)[21] and no ninhydrin-positive compounds other than VMA were seen.

To precipitate VMA HCl gas was bubbled through the pooled THF eluate for 5 min with vigorous stirring, and HCl–VMA slowly started to crystallise. The THF was decanted and the salt was washed with ethyl acetate and dried *in vacuo*. To remove HCl, 5.0 g HCl–VMA was dissolved in 6.0 ml water and applied to a 1.5 × 30 cm Dowex 50-X4 column in the H^+-form. The protonated VMA was retained on the column and the deprotonated form was eluted with 0.5 M NH_3. Upon evaporation to dryness at 70 °C the pooled ninhydrin-positive fractions from the eluate, tested according to Brenner *et al.*,[28] yielded VMA as a white transparent syrup. Yield (HCl–VMA): 8.6 g (52 mmol, 58%). GC/MS-PCI (VMA): [IP 90 eV; *m/z* (% rel. int.)]: 71 (100, $[(M-CH_3NHCO)+H]^+$), 131 (70, $[M+H]^+$), 159 (3, $[M+C_2H_5]^+$), 171 (2, $[M+C_3H_5]^+$).

N-*(r-1,c-2,t-3-trihydroxy-1,2,3,10b-tetrahydrofluoranthen-t-10b-yl)-L-valine methylamide* (**2a,b**). A 0.3 M solution of VMA in 2.5 ml *tert*-butyl alcohol–water, 1:1 (v/v), was prepared and 1.5 mg (5.9 µmol) of the DEs **1** was added. The reaction mixture was held at 70 °C under argon. The rate of reaction was studied by means of HPLC. Aliquots of 10 µl were diluted 12 times in water and injected on to the HPLC column. After 35 h <3% of **1** remained. Before isolation of **2a** and **2b** by means of HPLC it was necessary to enrich the products by extraction of VMA. The reaction solution was mixed with 0.5 ml 1.0 M HCl and extracted with 2.5 ml of ethyl acetate. The organic phase was washed with 3.0 ml 1.0 mM HCl and finally with 2.0 ml water. To prevent decomposition of **2a,b** 1.0 ml water was added to the ethyl acetate and the solution was evaporated until 0.5 ml remained. The products were isolated by means of HPLC, as described below (Fig. 3). Total yield (best recovery): 0.80 mg (2.1 µmol, 36%) estimated from the UV extinction coefficient of **1** dissolved in THF (provided by the manufacturer) $\varepsilon_{276}=6.2\times10^3\ M^{-1}\ cm^{-1}$. ^1H NMR (**2a**): δ 0.62 (d, 3 H), 0.72 (d, 3 H), 1.56 (m, 1 H), 3.30 (s, 3 H), 3.82 (s, 1 H), 4.55 (d, C_1-H), 4.70 (d, C_3-H), 4.87 (dd, C_2-H, $J_{1,2}$ and $J_{2,3}=4.0$ Hz), 7.10–7.70 (7 H). (**2b**): δ 0.52 (d, 3 H), 0.61 (d, 3 H), 1.41 (m, 1 H), 3.30 (s, 3 H), 3.78 (s, 1 H), 4.58 (d, C_1-H, $J_{1,2}=4.4$ Hz), 4.69 (d, C_3-H, $J_{2,3}=4.4$ Hz), 4.91 (dd, C_2-H), 7.10–7.70 (7 H). UV (**2a,b**) (abs. tetrahydrofuran) 230 (sh), 273 and 307 nm. F (**2a**) (EM. 303 nm, exc. tetrahydrofuran) 260 and 275 nm, (EXC. 276 nm, em. tetrahydrofuran) 303, 326 (sh) and 396 nm. F (**2b**) (EM.

308 nm, exc. tetrahydrofuran) 260 and 279 nm, (EXC. 279 nm, em. tetrahydrofuran) 308, 326 (sh) and 396 nm.

N-(r-7,t-8,t-9-trihydroxy-7,8,9,10-tetrahydrobenzo[a]-pyren-c-10-yl)L-valine methylamide (**5a,b**). A 0.3 M solution of VMA in 1.5 ml of *tert*-butyl alcohol was prepared and 1.5 mg (5.0 μmol) **4** was added. The reaction was performed at 70 °C. The rate of reaction was followed by means of HPLC. Aliquots of 10 μl were diluted 12 times in water and injected on to the HPLC column. After 24 h the reaction had gone to completion. Before isolation of **5a,b** by means of HPLC the reaction solution was mixed with 3.0 ml 0.1 M HCl and the VMA was extracted as above and finally two products were isolated by means of HPLC, as described below. Total yield: 1.9 mg (4.5 μmol, 91%) estimated by UV absorbance[29] $\varepsilon_{345} = 35 \times 10^3\ M^{-1}\ cm^{-1}$. ^1H NMR (**5a**): δ 0.91 (d, 3 H), 0.93 (d, 3 H), 1.71 (m, 1 H), 3.00 (s, 3 H), 3.29 (d, 1 H), 4.50 (dd, C_8-H, $J_{8,9} = 2.3$ Hz), 4.54 (dd, C_9-H), 4.80 (d, C_{10}-H, $J_{9,10} = 3.0$ Hz), 5.14 (d, 1 C_7-H, $J_{7,8} = 8.6$ Hz), 8.00–8.65 (8 H). (**5b**): δ 0.93 (d, 3 H), 0.94 (d, 3 H), 1.91 (m, 1 H), 3.29 (d, 1 H), 4.40 (dd, C_8-H, $J_{8,9} = 2.4$ Hz), 4.40 (dd, C_9-H), 4.84 (d, C_{10}-H, $J_{9,10} = 2.8$ Hz), 5.10 (d, 1 C_7-H, $J_{7,8} = 9.2$ Hz), 8.00–8.55 (8 H). UV (**5a,b**) (abs. tetrahydrofuran) 249, 269, 280, 329 and 345 nm. F (**5a,b**) (EM. 400 nm, exc. tetrahydrofuran) 316 (sh), 329 and 345 nm, (EXC. 345 nm, em. tetrahydrofuran) 384 (sh), 400 and 420 (sh) nm.

The hydrolysis products from **1** and **4** (**3** and **6**, respectively) were synthezised according to Day *et al.*[23] and Naylor *et al.*,[30] respectively.

High performance liquid chromatography of the products from **1**. Analytical amounts (100 μl) were eluted with [1 mM triethylamine (TEA) in water]/(methanol–2-methoxyethanol, 3:1), in the ratio 85:15 for 5 min, then linearly to 30:70 over 25 min. The corresponding program for the system used for isolation of **2a,b** was 85:15 for 5 min, then linearly to 40:60 over 30 min and then 30:70 over 10 min. The flow was 1.0 ml min^{-1} and the products were detected at 280 nm (UV maximum of **3**). The **2a,b** extract (500 μl) was injected on to the HPLC column and 1 ml fractions were collected. The two groups of fractions containing the products were collected and again purified by HPLC. The pure fractions were then evaporated and either dissolved in THF (for the MS analysis) or, after pretreatment with methanol-d_4, **4**, in acetone-d_6 (for the NMR analysis).

High performance liquid chromatography of the products from **4**. The mobile phase used for analysis (100 μl injections) and isolation of **5a,b** was (1 mM TEA in water)/(THF–2-methoxyethanol, 3:1), in the ratio 70:30. The flow was 1.0 ml min^{-1} and the products were detected at 245 nm (UV maximum of **6**). Prior to isolation, the enriched residue containing **5a,b** was dissolved in 500 μl of THF–water, 3:7 (v/v). The sample was then injected on to the HPLC column and 1 ml fractions were collected. The two groups of fractions containing **5a** and **5b** were collected and again purified by HPLC. Then the pure fractions were evaporated and either dissolved in THF (for the MS analysis) or, after pretreatment with methanol-d_4, in acetone-d_6 (for the NMR analysis).

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References

1. Boström, C. E., Almén, J., Steen, B. and Westerholm, R. *Environ. Health Perspect. 102 (Suppl. 4)* (1994) 39.
2. De Vos, R. H., van Dokkum, W., Schouten, A. and de Jong-Berkhout, P. *Food Chem. Toxicol. 28* (1990) 263.
3. Dell'Omo, M. and Lauwerys, R. R. *Critic. Rev. Toxicol. 23* (1993) 111.
4. US Surgeon General's Office. *Smoking and Health. A Report of the Surgeon General*, US Dept. of Health, Education and Welfare, Rockville MD 1979.
5. International Agency for Research on Cancer. *IARC Monographs. On the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Polynuclear Aromatic Compounds, Part 1, Chemical, Environmental and Experimental Data*, IARC, Lyon 1983, Vol. 32.
6. Yang, Y. and Baumann, W. *Analyst 120* (1995) 243.
7. Scheutzle, D. and Frazier, J. A. In: Ishinishi, N., Koizumi, A., McClellan, R. O. and Stöber, W., Eds., *Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust*, Elsevier Science, Amsterdam 1986, pp. 41–63.
8. Babson, J. R., Russo-Rodrigues, S. E., Wattley, R. V., Bergstein, P. L., Rastetter, W. H., Liber, H. L., Andon, B. M., Thilly, W. G. and Wogan, G. N. *Toxicol. Appl. Pharmacol. 85* (1986) 355.
9. Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Thakker, D. R., Jerina, D. M. and Conney, A. H. *Biochem. Biophys. Res. Commun. 77* (1977) 1389.
10. Slaga, T. J., Bracken, W. J., Gleason, G., Levin, W., Yagi, H., Jerina, D. M. and Conney A. H. *Cancer Res. 39* (1979) 67.
11. Vaca, C., Törnqvist, M., Rannug, U., Lindahl-Kiessling, K., Ahnström, G. and Ehrenberg, L. *Arch. Toxicol. 66* (1992) 538.
12. Sjögren, M., Ehrenberg, L. and Rannug, U. *Mutat. Res. 358* (1996) 97.
13. Törnqvist, M. and Ehrenberg, L. *Environ. Health Perspect. 102 (Suppl. 4)* (1994) 173.
14. Barfknecht, T. R., Hites, R. A., Cavalier, E. L. and Thilly, W. G. In: Lewtas, J., Ed., *Toxicological Effects of Emissions from Diesel Engines*, Elsevier, New York 1982, p. 277.
15. Van Duuren, B. L. and Goldschmidt, B. M. *J. Natl. Cancer Inst. 56* (1976) 1237.
16. Busby, W. F. Jr., Goldman, M. E., Newberne, P. M. and Wogan, G. N. *Carcinogenesis 5* (1984) 1311.
17. Törnqvist, M. In: Everse, J., Vandegriff, K. D. and Winslow, R. W., Eds., *Methods in Enzymology*, Academic Press, New York 1994, Vol. 231, p. 650.
18. Ehrenberg, L. and Osterman-Golkar, S. *Teratogen. Carcinogen. Mutagen. 1* (1980) 105.

19. Naylor, S., Gan, L.-S., Day, B. W., Skipper, P. L. and Tannenbaum, S. R. *Biomed. Chromatogr. 3* (1989) 266.
20. Skipper, P. L., Naylor, S., Gan, L.-S., Day, B. W., Pastorelli, R., and Tannenbaum, S. R. *Chem. Res. Toxicol. 2* (1989) 280.
21. Poirier, V. and Calleman, C. J. *Acta Chem. Scand., Ser. B 37* (1983) 817.
22. Whalen, D. L., Montemarano, J. A., Thakker, D. R., Yagi, H. and Jerina, D. M. *J. Am. Chem. Soc. 99* (1977) 5522.
23. Day, B. W., Sahali, Y., Hutchins, D. A., Wildschütte, M., Pastorelli, R., Nguyen, T. T., Naylor, S., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. *Chem. Res. Toxicol. 5* (1992) 779.
24. Hutchins, D. A., Skipper, P. L., Naylor, S. and Tannenbaum, S. R. *Cancer Res. 48* (1988) 4756.
25. Karplus, M. *J. Am. Chem. Soc. 85* (1963) 2870.
26. Cheng, C. C., Hilton, B. D., Roman, J. M. and Dipple, A. *Chem. Res. Toxicol. 2* (1989) 334.
27. Zaia, J. and Biemann, K. *J. Am. Soc. Mass Spectrom. 5* (1994) 649.
28. Brenner, M. and Niederwieser, A. *Experientia 16* (1960) 378.
29. Moore, P. D., Koreeda, M., Wislocki, P. G., Levin, W., Conney, A. H., Yagi, H., Jerina, D. M. In: Jerina, D. M., Ed., *Drug Metabolism Concepts*, American Chemical Society, Washington DC 1977, pp. 127–154.
30. Naylor, S., Gan, L.-S., Day, B. W., Pastorelli, R., Skipper, P. L. and Tannenbaum, S. R. *Chem. Res. Toxicol. 3* (1990) 111.

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