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Identification of human and murine sulfotransferases able to activate hydroxylated metabolites of methyleugenol to mutagens in *Salmonella typhimurium* and detection of associated DNA adducts using UPLC–MS/MS methods

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Methyleugenol, a secondary metabolite present in many herbal spices, is carcinogenic in various tissues of mice and rats but negative in standard *in vitro* mutagenicity tests. Several observations indicate that hydroxylation followed by sulfation is an important activation pathway in the carcinogenicity and DNA adduct formation by methyleugenol and other alkenylbenzenes in animal models. However, sulfation is not taken into account in standard *in vitro* tests. Therefore, we have studied whether expression of murine or human sulfotransferases (SULTs) in the target strain, *Salmonella typhimurium* TA100, leads to the activation of hydroxylated metabolites of methyleugenol [(+)-1′-hydroxymethyleugenol, (−)-1′-hydroxymethyleugenol and (*E*)-3′-hydroxymethylisoeugenol]. Human SULT1A1 (a form expressed at high levels in many tissues) and SULT1C2 (expressed primarily in foetal tissues) activated all three compounds even at very low substrate concentrations. At higher concentrations, activation was also observed with human SULT1A2 and SULT1E1. Murine Sult1a1 required higher substrate concentrations than its human orthologue. Other SULT forms (human 1A3, 1C1, 1C3, 2A1 and 2B1b as well as murine 1d1) did not activate any methyleugenol metabolites studied. Furthermore, we developed isotope-dilution mass-spectrometric methods for the sensitive and specific detection of DNA adducts formed by methyleugenol metabolites. All three hydroxylated metabolites formed the same DNA adducts in *S. typhimurium* TA100-hSULT1A1: high levels of N^2-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine and modest levels of N^6-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyadenosine. Adduct levels correlated with the mutagenic effects induced. No adducts were formed by the test compounds in the SULT-deficient standard strain TA100. In conclusion, several methyleugenol metabolites are activated to DNA-reactive mutagens in *S. typhimurium* upon incorporation of appropriate sulfation capacity. We have identified human and murine SULT forms able to catalyse this activation. Methods were developed that may be utilised to analyse DNA samples from human tissues specifically for the possible presence of methyleugenol adducts.

Introduction

Methyleugenol is a secondary metabolite occurring in herbal spices, such as basil, pimento, laurel, anise and tarragon. Moreover, essential oils rich in methyleugenol are used for flavouring foods and cosmetics. The usage of pure methyleugenol for this purpose has been banned in some countries (e.g. the European Union) but not in others (e.g. USA). Methyleugenol induced tumours in various tissues of rats and mice (1,2). In particular, it is a potent hepatocarcinogen in both species. Methyleugenol and various other alkenylbenzenes form DNA adducts in liver and other tissues of rodent models, suggesting that they are genotoxic carcinogens (3–7). However, they are negative in the Ames test and other standard *in vitro* genotoxicity assays (2). In general, DNA adducts detected in animal tissues involved the binding of the 3′-C atom of the alkenylbenzenes to the exocyclic amino groups of purine bases (3–7). 1′-Hydroxylated metabolites administered to animals form the same DNA adducts, usually at higher levels than the parent alkenylbenzenes, and are carcinogenic, suggesting that they represent proximate genotoxicants/carcinogens. The sulfotransferase (SULT) family 1 inhibitor pentachlorophenol drastically reduced carcinogenicity and DNA adduct formation by safrole and 1′-hydroxysafrole in rat and mouse models (6–9). Likewise, tumorigenesis and DNA adduct formation by safrole was strongly reduced in brachymorphic mice, which are characterised by a reduced synthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the cofactor for SULTs (7). These various findings suggested that the metabolic pathways exemplified for methyleugenol in Figure 1 are important for biological effects of alkenylbenzenes *in vivo*. It is important to notice that standard *in vitro* mutagenicity tests are deficient in sulfo conjugation activity due to the absence of SULT enzymes in the target cells and the lack of cofactor in the usual activating system, liver S9 mix (10,11). Supplementation of the cofactor PAPS to the S9 mix is not a reliable remedy, as sulfo conjugates are charged molecules, which do not efficiently penetrate cell membranes (10,12).

We have expressed various human and rodent SULTs in Ames's *Salmonella typhimurium* strains (12,13). The objective of the present study was to find out whether hydroxylated metabolites of methyleugenol are activated to mutagens in this model. Whereas the role of sulfation is convincingly documented for genotoxic and carcinogenic activities of alkenylbenzenes in rodent models, the SULT forms involved have not yet been identified and it is not known whether human SULTs have similar activities. Therefore, recombinant bacteria were used in the present study to identify critical SULT forms. It has to be noted that 1′-hydroxymethyleugenol is a chiral molecule, existing as two enantiomers. Moreover, in addition to 1′-hydroxymethyleugenol, its isomer 3′-hydroxymethylisoeugenol (existing as E and Z isomers) may be activated by SULT (Figure 1). We have not found any information on the enantiomeric composition of metabolically formed 1′-hydroxymethyleugenol.

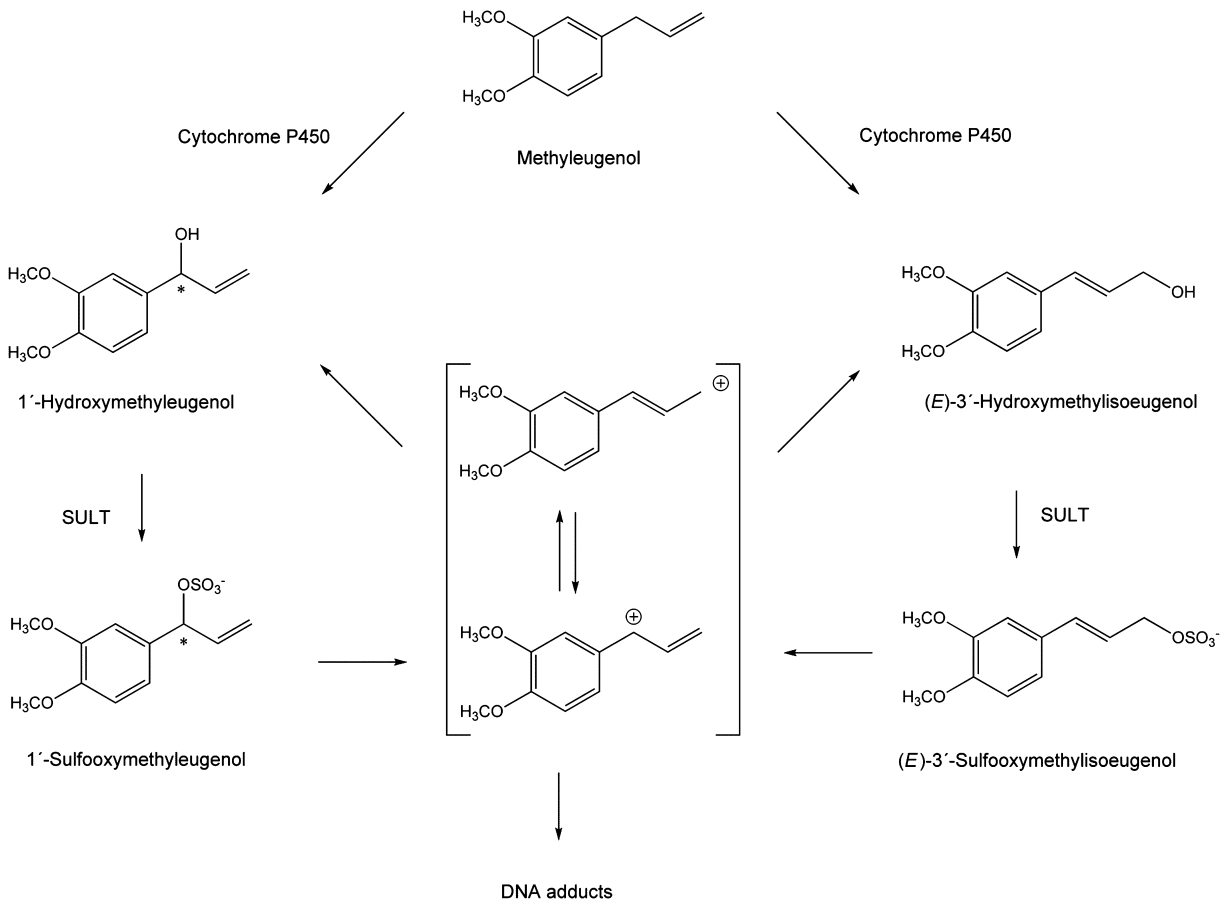

Fig. 1. Bioactivation pathways of methyleugenol leading to electrophilic sulfuric acid esters. Chiral centres are marked with an asterisk.

However, we previously reported that hepatic microsomes from control and Aroclor 1254-treated rats form the (+) enantiomer of 1′-hydroxysafrole from safrole with an enantiomeric excess of 0.45 and 0.60, respectively (14). The E diasteromer of 3′-hydroxymethylisoeugenol is thermodynamically favoured compared to the Z form. Only the E diasteromer but not the Z isomer is formed in incubations of methyleugenol with microsomes from rat, human and bovine liver (A. T. Cartus, K. Herrmann, L. W. Weishaupt, K. H. Merz, W. Engst, H. R Glatt, D. Schrenk, manuscript in preparation). This raises the question whether the same or different SULT forms are involved in the activation of these isomeric phase-I metabolites of methyleugenol. We have investigated both enantiomers of 1′-hydroxymethyleugenol, but only the E diasteromer of 3′-hydroxymethylisoeugenol, as its Z does not appear to be formed metabolically. Finally, we devised mass spectrometric methods for identifying and quantifying DNA adducts in the test system.

Materials and methods

Chemicals

[$^{15}N_5$]-2′-Deoxyadenosine ($^{15}N_5$-dA) and [$^{15}N_5$]-2′-deoxyguanosine ($^{15}N_5$-dG) were obtained from Silantes (München, Germany). Solvents (HPLC-grade) used for gas chromatography, high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) were purchased from Carl Roth GmbH (Karlruhe, Germany). Herring sperm DNA, micrococcal nuclease (from *Staphylococcus aureus*) and alkaline phosphatase (from calf intestine) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Calf spleen phosphodiesterase type II was obtained from Merck (Darmstadt, Germany).

Nuclear magnetic resonance and gas chromatography analyses

^{1}H-nuclear magnetic resonance (NMR) analyses of (±)-1′-hydroxymethyleugenol, N^6-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyadenosine (N^6-MIE-dA) and N^2-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine (N^2-MIE-dG) dissolved in dimethyl sulfoxide (DMSO)-d_6 were performed with an NMR500-vnmrs500 spectrometer (Varian, Palo Alto, California) at 298 K. (*E*)-3′-Hydroxymethyleugenol dissolved in acetonitrile-d_3 was investigated with a 500 MHz Inova spectrometer (Varian) at 298 K. Gas chromatography–mass spectrometry operating in electron impact mode (GC-EI-MS) analyses were performed with a CP3800 gas chromatograph (Varian) equipped with a J&W DB-5MS Column (0.25 × 30 mm, 0.25 µm film; Agilent Technology, Waldbronn, Germany) and a Varian 320-MS detector (Varian) recording *m*/*z* of 100–400. The injection volume was 1 µl. The flow rate of the carrier gas, helium, was 1.2 ml/min. Gas temperature was increased from 50°C (hold for 1 min) to 250°C (hold for 4 min) at a rate of 5°C/min. All other parameters were taken from Jeurissen *et al.* (15), as used for the analysis of (±)-1′-hydroxyestragole.

Synthesis of hydroxylated metabolites of methyleugenol and (±)-1′-acetoxymethyleugenol

(±)-1′-Hydroxymethyleugenol was prepared from 3,4-dimethoxybenzaldehyde (Sigma–Aldrich) using the same principal methods as described by Borchert *et al.* (16) for the synthesis of (±)-1′-hydroxysafrole. The crude product (yield up to 93%) had a purity of at least 98%. Samples were purified by chromatography to give racemic material of >99% purity for mutagenicity testing and ^{1}H-NMR analyses. The ^{1}H-NMR spectrum agreed with that described by Cartus *et al.* (17). Other samples of the crude product were separated into the enantiomers by HPLC (Dionex, Idstein, Germany) equipped with two sequentially arranged columns (each 4.6 × 250 mm, 10 µm) containing Chiralcel OJ00CE-BG054 and OJ00CE-GI037 (Daicel Industries, Tokyo, Japan), respectively, and a UV-970 Intelligent ultraviolet (UV)/visible light detector (Jasco, Groß-Umstadt, Germany) recording absorption at 278 nm. Aliquots (100 µl) of the crude material, containing ∼5 mM 1′-hydroxymethyleugenol in *n*-heptane:ethanol (9:1), were injected. The same solvent mixture was used as isocratic eluent at a flow rate of 0.7 ml/min. One enantiomer [later identified as the (−) enantiomer] eluted at ∼50 min and the

other at ~55 min. After evaporation of the solvent, the purity of each enantiomer was >99% [no contamination with the other enantiomer or any other compounds detectable by HPLC–UV]. The polarity of the enantiomers was determined by circular dichroism spectroscopy using a Jasco-710 spectrometer (Jasco) at room temperature with a response time of 4 sec, a scanning speed of 50 nm/min and a wavelength range of 178–320 nm. Each enantiomer was diluted in *n*-hexane to a final concentration of 0.5 mM and added to a quartz cell of 1-mm optical path length.

The enantiomers [2 μg/μl in *n*-heptane:ethanol (9:1)] were further analysed by GC-EI-MS with the following result: (+)-1′-hydroxymethyleugenol: *m/z* (relative intensity, %): 139 (100), 151 (47), 165 (63), 176 (55), 194 (96); (−)-1′-hydroxymethyleugenol: *m/z* (relative intensity, %): 139 (100), 151 (47), 165 (96), 176 (71), 194 (93). These fragmentations correspond to those reported for (±)-1′-hydroxyestragole (18). The high purity of the enantiomers (>99%) previously determined by HPLC-UV was confirmed by this GC-EI-MS analysis.

(*E*)-3′-Hydroxymethylisoeugenol was prepared by isomerisation of (±)-1′-hydroxymethyleugenol using the same principal approach as described for the synthesis of (*E*)-3′-hydroxyisosafrole (16). The yield before purification was up to 46%. Purification and ^{1}H-NMR analysis were kindly performed by MicroCombiChem (Wiesbaden, Germany). Briefly, the crude product was dissolved in acetonitrile resulting in a concentration of nearly 50 mM (*E*)-3′-hydroxymethylisoeugenol. Aliquots of 250 μl were injected into an HPLC equipped with a 2700 Sample Manager, an Atlantis dC18 column (19 × 100 mm, 5 μm) and a 2487 Dual Absorbance detector (all from Waters, Eschborn, Germany) recording absorptions at 210 and 254 nm. A 7-min linear gradient was applied from 10% acetonitrile/0.1% formic acid (v/v) to 100% acetonitrile/0.1% formic acid (v/v). The flow was set at 18 ml/min. (*E*)-3′-Hydroxymethylisoeugenol eluted at 3.5 min. The ^{1}H-NMR spectrum agreed with that reported by Cartus *et al*. (17). The purity was >99% according to this ^{1}H-NMR analysis.

(±)-1′-Acetoxymethyleugenol was synthesised from (±)-1′-hydroxymethyleugenol using a protocol described for the acetylation of (±)-1′-hydroxyestragole (19). The yield was 80%. A sample of the crude product [2 μg/μl in *n*-heptane:ethanol (9:1)] was analysed by GC-EI-MS with the following result for the main product: *m/z* (relative intensity, %): 105 (100), 161 (33), 176 (77), 177 (16), 194 (43), 236 (14). These fragmentations correspond to those reported for (±)-1′-acetoxyestragole (18). The analysis indicated a purity of 82%. The crude intermediate was directly used for the synthesis of adduct standards. For mutagenicity testing (±)-1′-acetoxymethyleugenol was subjected to preparative HPLC yielding a purity of >99%.

Synthesis of 2′-deoxynucleoside adduct standards

We modified an approach that was previously used for the synthesis of adducts of estragole (19). Crude (±)-1′-acetoxymethyleugenol (200 μl), dissolved in 5 ml DMSO, was added to 45 ml of 10 mM 2′-deoxynucleoside (dA or dG) solution in 10 mM ammonium carbonate buffer (pH 7.6). After incubation at 37°C for 48 h, the product was isolated using two chromatographic steps. The initial step was conducted on a Prep LC 150 equipped with a SunFire C18 OBD column (19 × 150 mm, 5 μm) and a photodiode array detector 996 set to 263 nm for dG adducts or 273 nm for dA adducts (all equipment from Waters). A linear gradient of water acidified with 0.25% acetic acid (v/v) and 0.25% formic acid (v/v) (solvent A) and acetonitrile (solvent B) was used for elution at a flow rate of 15 ml/min. Solvent B was increased over a period of 30 min from 20 to 50% for elution of dA adducts and from 10 to 90% for elution of dG adducts. N^6-MIE-dA and N^2-MIE-dG eluted at 26 or 24 min, respectively. Identity and purity of the compounds were examined by UPLC-MS. The UV purity (>99%) was verified using the photodiode array detector signal at 273 nm (N^6-MIE-dA) or 263 nm (N^2-MIE-dG). After freeze-drying, the structures were confirmed by ^{1}H-NMR (DMSO-d_6, 500 MHz) analyses, as follows (numbering of positions in Figure 4).

N^6-MIE-dA: δ (p.p.m.): 2.27 (m, 1H, H2′′′); 2.73 (m, 1H, H2′′′); 3.53 (m, 1H, H5′′′); 3.62 (m, 1H, H5′′′); 3.73 (s, 3H, OCH_3); 3.74 (s, 3H, OCH_3); 3.88 (dd, 1H, H4′′′, J = 4 Hz, J = 7 Hz); 4.26 (ps, 2H, H3′); 4.41 (m, 1H, H3′′′); 5.22 (dd, 1H, OH-5′′′, J = 5 Hz, J = 7 Hz); 5.30 (d, 1H, OH-3′′′, J = 4 Hz); 6.26 (dt, 1H, H2′, J = 16 Hz); 6.36 (dd, 1H, H1′′′, J = 6 Hz, J = 8 Hz); 6.44 (d, 1H, H1′, J = 16 Hz); 6.86 (m, 1H, H6 or H5); 6.88 (m, 1H, H5 or H6); 7.00 (d, 1H, H3, J = 2 Hz); 8.07 (bs, 1H, H6′′); 8.23 (bs, 1H, H2′′); 8.35 (s, 1H, H8′′).

N^2-MIE-dG: δ (p.p.m.): 2.19 (m, 1H, H2′′′); 2.64 (m, 1H, H2′′′); 3.51 (m, 1H, H5′′′); 3.56 (m, 1H, H5′′′); 3.74 (s, 3H, OCH_3); 3.76 (s, 3H, OCH_3); 3.81 (m, 1H, H4′′′); 4.07 (pt, 2H, H3′, J = 5 Hz); 4.37 (m, 1H, H3′′′); 4.91 (ps, 1H, OH-5′′′); 5.27 (d, 1H, OH-3′′′, J = 4 Hz); 6.17 (dd, 1H, H1′′′, J = 7 Hz, J = 8 Hz); 6.24 (dt, 1H, H2′, J = 16 Hz); 6.51 (d, 1H, H1′, J = 17 Hz); 6.64 (bs, 1H, H2′′); 6.88 (d, 1H, H6, J = 9 Hz); 6.92 (dd, 1H, H5, J = 2 Hz, J = 9 Hz); 7.04 (d, 1H, H3, J = 2 Hz); 7.87 (s, 1H, H8′′); 10.90 (bs, 1H, H1′′).

Isotope-labelled adducts were prepared in the same way at a 30 times smaller scale using $^{15}N_5$-dA and $^{15}N_5$-dG instead of their unlabelled congeners.

Bacterial strains and mutagenicity assay

Human and murine SULTs (reference type sequences) were expressed in *S. typhimurium* using the pKKneo vector, as described previously (12). To be consistent with our previous publications, we stayed with the original designation of SULTs. This is relevant for human SULT1C1 and SULT1C2, which have been renamed to SULT1C2 and SULT1C4 by Blanchard *et al*. (20). The names of the strains are composed of the name of the recipient strain (TA100 in the present study) and that of the expressed SULT form (with the prefixes h for human and m for murine forms). Human SULT protein levels in the recombinant strains were 0.9–5% of total cytosolic protein in the recombinant strains (12). The lowest and highest values were observed in strains TA100-hSULT1A1 and TA100-hSULT1A1*1Y. Both strains express the same protein. However, a number of synonymous mutations (not affecting the amino acid sequence) were introduced in hSULT1A1*1Y to enhance expression. mSult1a1 protein amounted to 4% of the cytosolic protein in TA100-mSult1a1. TA100-mSult1d1 gave a strong signal in the immunoblot, which, however, could not been quantified as no purified standard was available.

The bacteria were grown in Nutrient Broth No. 2 (Oxoid GmbH, Wesel, Germany) at 37°C with shaking for 8 h. Strain TA100 was grown in the absence of antibiotics. Neomycin (50 μg/ml) was added to the growth medium for the recombinant strains. The cultures were centrifuged, suspended in medium A (1.6 g/l Bacto Nutrient Broth and 5 g/l NaCl), adjusted turbidimetrically to a titre of 1–2 × 10^9 bacteria (colony-forming units)/ml and kept on ice. Shortly before use, they were centrifuged again and suspended at a 5-fold higher density in medium A. Mutagenicity was determined using a modified version of the liquid pre-incubation assay described by Maron and Ames (21). The bacterial suspension (100 μl) and the test compound (in 10 μl DMSO) were added sequentially to a glass tube containing 500 μl of 100 mM $MgSO_4$, pre-warmed to 37°C. After incubation for 60 min at 37°C, 2.0 ml of 45°C warm soft agar (5.5 mg/ml agar, 5.5 mg/ml NaCl, 50 μM biotin, 50 μM histidine, 50 μM tryptophane, 25 mM sodium phosphate buffer, pH 7.4) was added, and the mixture was poured onto a Petri dish containing 24 ml minimal agar (15 mg/ml agar in Vogel-Bonner E medium with 20 mg/ml glucose). After incubation for 3 days in the dark, the colonies (his^+ revertants) were counted. Incubations were carried out in triplicate or quadruplicate, except for the negative controls (usually twice as many plates), unless specified otherwise. Most compounds were tested in various strains on several occasions. In general, the results of the last experiment, with optimised dose levels, are presented. The result of an individual experiment was classified positive, if the number of revertants (mean value at any dose level) was increased at least 2-fold above the number of spontaneous revertant colonies with a plausible dose–response relationship. It was also classified positive if the increase was at least 1.5-fold and confirmed in repeated experiments. Specific mutagenicities (revertants per nanomole) were calculated from the slope of the initial part of the dose–response curve of the positive results. For negative results, a conservative limit of detection is given by dividing the number of spontaneous revertants by the highest dose that could be adequately tested (no obvious toxicity).

Analysis of adducts formed in herring sperm DNA and in bacteria

Herring sperm DNA (20 mg, dissolved in 8 ml water) and (±)-1′-acetoxymethyleugenol (0.1 μmol, dissolved in 2 ml DMSO) were incubated at 37°C for 1 h. Then, 1 ml of a sodium acetate solution (3 M, pH 5.2) was added. The DNA was recovered by precipitation with 16.5 ml ice-cold 2-ethoxyethanol and subsequent storage at −20°C for 1 h. Then, it was centrifuged at 5000 × *g* for 30 min, washed with 1 ml 70% ethanol, dried and dissolved in 1 ml water. The DNA concentration was determined spectrophotometrically with a Nanodrop ND-1000 spectrophotometer (peqlab Biotechnologie, Erlangen, Germany) measuring the absorbance at 260 nm.

Bacteria were exposed to the test compounds as in the mutagenicity assay. After the 60-min exposure period, they were harvested by centrifugation (10 min, 9000 × *g*, 4°C). The resulting bacterial pellet was washed by resuspension in 1 ml water and repeat of the centrifugation step. DNA was extracted from the bacteria as described previously (22) and isolated using a standard phenol–chloroform protocol (23).

An aliquot of DNA (12.5 μg dissolved in 56 μl water), internal standards (126 fmol [$^{15}N_5$]-N^6-MIE-dA and 156 fmol [$^{15}N_5$]-N^2-MIE-dG in 15 μl of 75% methanol:25% water), 16 μl buffer (100 mM sodium succinate buffer, pH 6.0, 50 mM $CaCl_2$) and 24 μl enzyme mixture (containing 2.7 U micrococcal nuclease and 53 mU calf spleen phosphodiesterase) were incubated at 37°C for 16 h. Then, 38 μl of Tris buffer (0.5 M, pH 10.9) and 3 μl alkaline phosphatase solution (3 U) were added and the incubation was continued over night.

Proteins were precipitated by addition of 500 µl ice-cold ethanol. After mixing and centrifugation (10 min, 19 000 × *g*, 4°C), the supernatant was collected followed by removal of the solvent under reduced pressure in a Speedvac. The residue was taken up in 50 µl of 75% methanol and centrifuged (10 min, 19 000 × *g*, 4°C). The supernatant was analysed using an Acquity UPLC connected sequentially to an Acquity photodiode array detector (irrelevant for the adduct analyses) and a Xevo TQ triple quadruple mass spectrometer (Waters). It was equipped with an Acquity UPLC BEH Phenyl column (2.1 × 100 mm, 1.7 µm). A gradient of water acidified with 0.25% acetic acid (v/v) and 0.25% formic acid (v/v) (solvent A) and acetonitrile acidified equally (solvent B) was used as mobile phase at a flow rate of 0.3 ml/min. After injection of 7.5 µl of the sample, chromatographic separation was achieved using a 5-min linear gradient from 20 to 80% solvent B. Mass spectrometric analyses of the adducts were performed in the positive ionisation mode using multiple reaction monitoring (MRM) for specific detection. The tuning parameters were set as follows: temperature of electrospray source: 110°C; desolvation temperature: 450°C; desolvation gas (nitrogen) flow: 950 l/h; collision gas (argon) flow: 0.23 ml/min (pressure: 3.5×10^{-3} mbar); dwell time per transition: 48 ms; capillary voltage: 0.9 kV. Three characteristic *m/z* transitions were used for each adduct (Table I). The cone voltage and collision energy were optimised for each transition with the IntelliStart tool of the MassLynx 4.1 software (Waters) and are listed in Table I.

Determination of the limits of detection and quantification of adducts N^6-MIE-dA and N^2-MIE-dG in DNA

Unexposed DNA (12.5 µg per sample) was digested to deoxynucleosides as described in the preceding section but without the addition of internal isotope-labelled adduct standards. Immediately before UPLC–tandem mass spectrometry (MS/MS) analysis, samples were spiked with 0 (blank), 2.5, 5, 10 or 50 fmol of each adduct, N^6-MIE-dA and N^2-MIE-dG. The limit of detection was defined as the adduct level giving a peak area of the quantifier that was the mean plus three times the standard deviation of the blank value from 10 separate digests of unexposed DNA. The limit of detection was defined as the adduct level giving a corresponding peak area that was the mean plus nine times the standard deviation of the blank value from 10 separate digests of unexposed DNA. Under the conditions used, limits of the detection amounted to 2 N^6-MIE-dA and 6 N^2-MIE-dG adducts per 10^8 nucleosides. The corresponding limits of quantification were 3 N^6-MIE-dA and 12 N^2-MIE-dG adducts per 10^8 nucleosides. Usage of higher amounts of DNA in the analyses could reduce these limits. However, this was not required for the present study.

Results and discussion

Mutagenicity in SULT expressing Salmonella strains

The benzylic ester (±)-1′-acetoxymethyleugenol is an uncharged analogue of (±)-1′-sulfooxymethyleugenol. Therefore, it may better penetrate cell membranes than the latter. It was prepared for the synthesis of adducts. In addition, we tested it for mutagenicity in standard *Salmonella* strains. It elevated the numbers of revertants from strains TA100 and TA104 up to 3-fold above the spontaneous levels, requiring doses of ∼100 nmol/plate (data not shown). These strains are primarily reverted by substitution mutations (21). No mutagenicity was detected with strain TA1538, a strain that detects frame-shift mutations (21). We previously had expressed human SULTs in TA1538 (13) and TA100 (12). Since TA100, but not TA1538, was responsive to (±)-1′-acetoxymethyleugenol, a set of TA100-derived strains expressing various SULTs was subsequently used for studying the mutagenicity of hydroxylated metabolites of methyleugenol. The results are summarised in Table II. Dose–response curves are presented in Figures 2 and 3.

Racemic 1′-hydroxymethyleugenol was available in much larger quantities than the individual enantiomers. It was used for testing high doses (Figure 2). At a dose of 3000 nmol, (±)-1′-hydroxymethyleugenol led to a reproducible but weak increase in the number of revertants with control strain TA100 (Figure 2A). A thousandth of this dose was sufficient to induce a comparable effect, when hSULT1A1 was expressed in the target strain (Figure 2B). This SULT form has been detected in most human tissues examined, the levels being particularly high in liver and small intestine (24). The (+) enantiomer appeared to be slightly more potent than the (−) enantiomer when hSULT1A1 was used for activation, as reflected by higher numbers of revertants at low concentrations and reduced numbers of revertants at high concentrations due to bacteriotoxicity (Figure 3B–C and data from repeat experiments). The mutagenic activity of both enantiomers was nearly 3-fold higher in strain TA100-hSULT1A1*1Y than in TA100-hSULT1A1. Both strains express the same SULT, but the expression level is ∼5.5-fold higher in TA100-hSULT1A1*1Y. The other human SULT form that efficiently activated 1′-hydroxymethyleugenol was hSULT1C2 (Figure 3F). This enzyme showed a more pronounced enantioselectivity for the (+) enantiomer than hSULT1A1 and any other SULT form studied. Expression of SULT1C2 appears to be low in adult human tissues but high in foetal lung and kidney (25). Murine Sult1a1 activated both enantiomers of 1′-hydroxymethyleugenol (Figure 3H). However, the activation was weaker than when the orthologous human enzyme was expressed at a lower level (TA100-hSULT1A1) or higher level (TA100-hSULT1A1*1Y) than mSult1a1 in TA100-mSult1a1.

(*E*)-3′-Hydroxymethylisoeugenol was not mutagenic in the parental strain TA100 but was only tested up to a maximal dose of 1000 nmol/plate (Figure 2A). It was activated by the same SULT forms as its positional isomer, 1′-hydroxymethyleugenol, but with some quantitative differences. (*E*)-3′-Hydroxymethylisoeugenol, unlike 1′-hydroxymethyleugenol, was equally well activated in strains TA100-hSULT1A1 and TA100-hSULT1A1*1Y, indicating that the enzyme level was not limiting. This finding suggests that (*E*)-3′-hydroxymethylisoeugenol is a better substrate for hSULT1A1 than are the 1′-hydroxymethyleugenol enantiomers. (*E*)-3′-Hydroxymethylisoeugenol showed higher mutagenic activity than 1′-hydroxymethyleugenol in TA100-hSULT1A2 (Figures 2C and 3D), whereas the situation was reverse in TA100-hSULT1C2 (Figure 3F).

Table I. Transitions used for MS/MS analysis of adducts N^6-MIE-dA and N^2-MIE-dG[a]

m/z transition	Comment	Cone voltage, V	Collision energy, eV
N^6-MIE-dA			
428.1 (433.1*) → 177.2	Loss of dA, quantifier	33	28
428.1 (433.1*) → 146.1	Loss of dA and one methoxy group	33	44
428.1 (433.1*) → 312.2 (317.2*)	Loss of deoxyribose	33	18
N^2-MIE-dG			
444.1 (449.1*) → 328.2 (333.2*)	Loss of deoxyribose, quantifier	22	11
444.1 (449.1*) → 164.1 (169.1*)	Loss of deoxyribose and phenylethyl group	22	27
444.1 (449.1*) → 177.1	Loss of dG	22	27

[a]Values for isotope-labelled standards are marked with an asterisk, if different from the unlabelled adduct.

Table II. Mutagenicity of hydroxylated metabolites of methyleugenol in the standard strain *Salmonella typhimurium* TA100 and in TA100-derived strains expressing various human and murine SULT forms

Expressed SULT	Revertants per nanomole[a]			
	(±)-1′-Hydroxymethyleugenol	(+)-1′-Hydroxymethyleugenol	(−)-1′-Hydroxymethyleugenol	(*E*)-3′-Hydroxymethylisoeugenol
None	0.02[b]	<0.5[b]	<0.5[b]	<0.1[b]
Human 1A1	18[b]	12[b]	8[b]	20[b]
Human 1A1 (*1Y, elevated expression)	—	35[b]	25[b]	20[b]
Human 1A2	0.6[b]	0.7[b]	0.4[b]	2[b]
Human 1A3	—	<0.5[b]	<0.5[b]	<0.5[b]
Human 1C1	—	<1	<1	<1
Human 1C2	—	12[b]	5[b]	1.4[b]
Human 1C3	—	<1	<1	<1
Human 1E1	—	1.5[b,c]	1[b,c]	<1[b]
Human 2A1	0.03[b]	<1	<1	<0.1[b]
Human 2B1b (*1Q, high expression)	—	<1	<1	<1
Murine 1a1	—	5[b]	2[b]	0.8[b]
Murine 1d1	<1	<1	<1	<1

—, Not tested.
[a]Estimated from the initial slope of the dose–response curves. As the linear part of the curves was small with the compounds tested, the values are not very accurate (factor of 1.5 for subjectivity and 2 for inter-experimental variation). For negative results, a conservative estimate of the limit of detection is given. This limit depended on the doses used with the given test compound. In particular, racemic 1′-hydroxymethyleugenol was available in larger quantities than its individual enantiomers.
[b]Representative curves are shown in Figures 2 and 3.
[c]Our standard criterion for a positive result (at least 1.5 increase in the number of revertants above the control level) was not met. However, the increases in the number of revertants were so consistent that some activation by the expressed SULT has to be inferred.

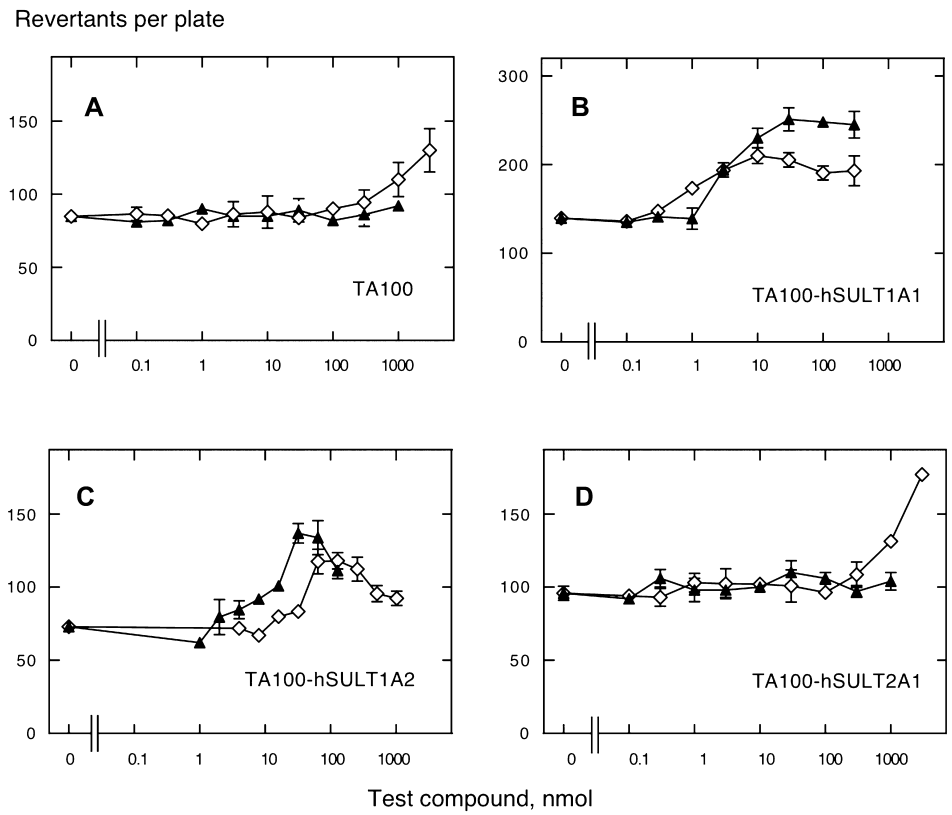

Fig. 2. Effect of expression of human SULTs in target cells on mutagenicity of (±)-1′-hydroxymethyleugenol and (*E*)-3′-hydroxymethylisoeugenol in the Ames test. Open rhomboids: (±)-1′-hydroxymethyleugenol; solid triangles: (*E*)-3′-hydroxymethylisoeugenol. Values are mean ± SE of three to four plates (substance-treated groups) or six to eight plates (negative control) from one experiment. SE is within the symbol if no error bar is visible.

It should be noted that the increases in the number of revertants induced by hydroxylated methyleugenol metabolites were rather weak in all strains, rarely exceeding the control value by a factor of >2. Therefore, we suspect that reactive sulfo conjugates formed from these compounds are relatively strongly bacteriotoxic, overshadowing mutagenic responses.

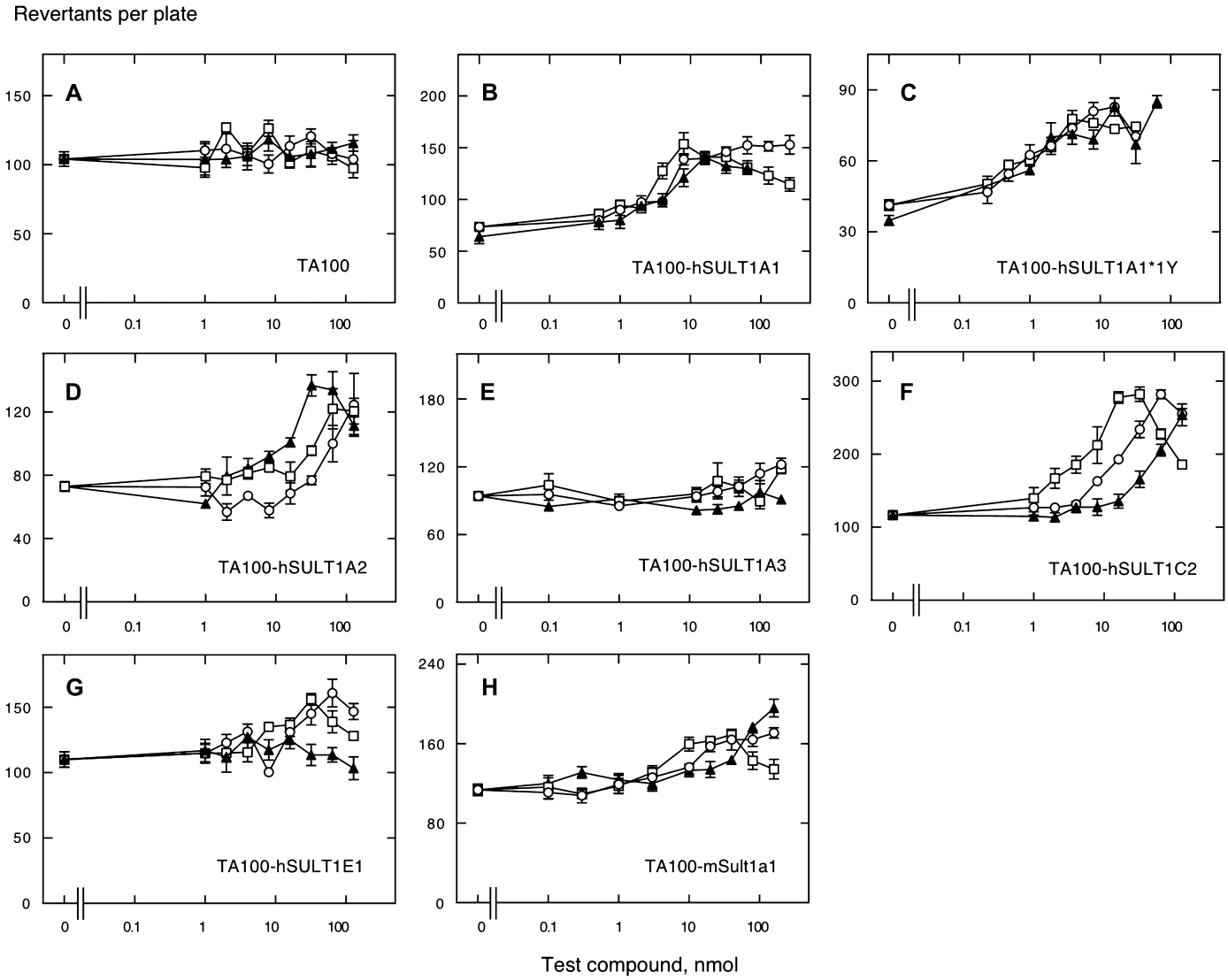

Fig. 3. Effect of expression of human and murine SULTs in target cells on mutagenicity of the enantiomers of 1′-hydroxymethyleugenol and (*E*)-3′-hydroxymethylisoeugenol in the Ames test. Open squares: (+)-1′-hydroxymethyleugenol; open circles: (−)-1′-hydroxymethyleugenol; solid triangles: (*E*)-3′-hydroxymethylisoeugenol. Values are mean ± SE of three to four plates (substance-treated groups) or six to eight plates (negative control) from one experiment. SE is within the symbol if no error bar is visible.

Characterisation of N^6-MIE-dA and N^2-MIE-dG

dA and dG adducts were prepared by incubation of the corresponding 2′-deoxynucleosides with (±)-1′-acetoxymethyleugenol. Mass spectra and structural formulas of the adducts as well as the putative structures of the fragments are presented in Figure 4. Additional fragments were observed when the collision energy was increased. The fragments *m*/z 164 and 148 have been observed with other dG and dA adducts involving bonding to exocyclic amino groups of nucleobases and appear to be characteristic for them (26,27). However, it was not possible to unambiguously identify the atoms in the deoxynucleoside and methyleugenol moieties involved in bonding. We have deduced this information from the ^{1}H-NMR spectra using the SDBS-RIO database (National Institute of Advanced Industrial Science and Technology, Japan). The following points are particularly informative: The signal for the exocyclic amine in dA (7.31 p.p.m.) was shifted to 8.07 in the dA adduct. This signal was broad (consequence of tautomerism with *N*-1 of purine) and small (one rather than two protons). The signals 6.26 and 6.44 (alkene group) showed a high coupling constant of 16 Hz, implying *trans* configuration (with lower coupling constants of 6–12 Hz being typical for *cis* configuration). Likewise, the signal for the exocyclic amine in dG (6.50 p.p.m.) was shifted to 6.64 in the dG adduct. This signal was broad (consequence of tautomerism with *N*-3 of purine) and small (one rather than two protons). Again, the signals 6.24 and 6.51 (alkene group) showed high coupling constants of 16 and 17 Hz, respectively, indicating *trans* configuration.

When dC was incubated with (±)-1′-acetoxymethyleugenol, several minor products were formed that may represent adducts. However, the levels of these products were too small for purification. dT did not appear to form adducts with (±)-1′-acetoxymethyleugenol.

Adduct formation in cell-free (herring sperm) DNA

Herring sperm DNA was incubated with or without (blank) (±)-1′-acetoxymethyleugenol, digested to deoxynucleosides

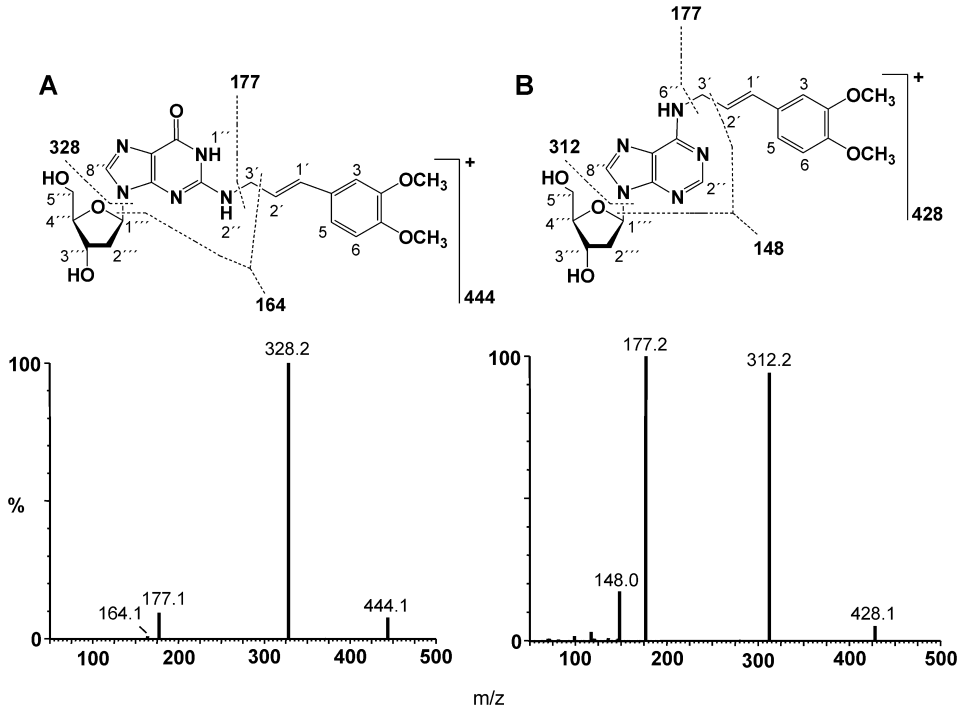

Fig. 4. Structural formulas of N^2-MIE-dG (left) and N^6-MIE-dA (right) with fragmentation patterns observed by positive electrospray MS/MS collision-induced dissociation. Product ion spectra of the protonated molecule of dA and dG were recorded under the same fragmentation conditions as used for adduct quantification. The adducts were generated by incubating the corresponding deoxynucleosides with (±)-1′-acetoxymethyleugenol. Their structures were corroborated by ^{1}H-NMR analyses (see main text), using the numbering system indicated in the structural formulas.

and analysed by UPLC–MS/MS in the MRM mode for the presence of adducts with the various nucleosides (Figure 5). A product with the retention time (2.39 min) and all three *m/z* transitions of N^2-MIE-dG in the same ratio as observed for the authentic standard was detected at high levels (Figure 5, traces A1–A3). Weak signals for two transitions (indicative of the loss of deoxyribose with or without concomitant loss of the phenylethyl group) were seen at a retention time of 2.05 min. They may represent a second dG adduct, e.g. involving bonding via the 1′-position of methyleugenol. Most likely, it reflects a minor adduct in DNA, as its signals were not only weaker than those of N^2-MIE-dG in the MRM scan but also in selected ion recording (SIR) for the mass of the positively ionised intact adduct and UV (photodiode array) scans. The situation was different when dG, rather than DNA, was incubated with (±)-1'-acetoxymethyleugenol. Then, N^2-MIE-dG as well as this uncharacterised product were formed at high levels.

Likewise, two different dA adducts appeared to be formed in the DNA (Figure 5B), although at lower levels than the dG adducts. The dominating signal (occurring at 2.68 min) corresponded to N^6-MIE-dA with all three *m/z* transitions in the expected ratio. The same signals were observed at nearly 10 times lower levels at 3.00 min retention time. This second signal was also weak in SIR and UV scans. The formation of this product was enhanced when dA, rather than DNA, was incubated with (±)-1′-acetoxymethyleugenol, similarly as described in the preceding section for the second dG adduct.

There was some evidence for the formation of a dC adduct at very low levels (Figure 5, traces C1–2, 1.41 min retention time). The MRM analyses for dT adducts (*m/z* 419.1 → 303.2, loss of deoxyribose, Figure 5, trace D1) indicated adduct candidates at 0.86 and 0.98 min. However, the former signal also occurred in unexposed DNA and a second transition (corresponding to loss of dT) was missing in both cases (Figure 5, trace D2). On the other hand, a compound with this second transition (corresponding to loss of deoxyribose) was observed at 1.32 min. However, these signals, which might, but do not have to, reflect dT adducts, were very weak. In conclusion, N^2-MIE-dG was the dominating adduct in DNA. In addition, the presence of N^6-MIE-dA could be demonstrated unambiguously.

DNA adduct formation in S. typhimurium under the conditions of the mutagenicity assay

In the initial experiment, strain TA100-hSULT1A1*1Y was treated with hydroxylated methyleugenol metabolites at a low concentration (6.6 μM, equivalent to a dose of 4 nmol/plate in the mutagenicity assay). Compared to the solvent control, the number of revertants was increased 1.5- to 1.7-fold by the three isomeric test substances (Table III). Levels of N^2-MIE-dG adducts were similar for all three isomers. They were 21- to 31-fold above the limit of quantification. All three *m/z* transitions were readily detectable, as shown for TA100-hSULT1A1*1Y bacteria treated with (+)-1′-hydroxymethyleugenol in Figure 6. N^6-MIE-dA adducts were also detected. However, their levels only were 1.5–3% of those of the dG adduct (Table III). No adducts were detected in control strain TA100 exposed to the same dose of the test compounds and in unexposed bacteria of either strain.

In the next experiment, strain TA100-hSULT1A1*1Y was treated with varying doses of (+)-1′-hydroxymethyleugenol. Adduct levels increased continuously with the dose, but the increase was hypolinear (Figure 7), similar to the dose–response curve for mutagenicity (Figure 3C). The reasons for this deviation from linearity are not known. They may involve saturation of SULT, inactivation of SULT by reactive sulfo conjugates or bacteriotoxicity, leading to compromised synthesis of the cofactor PAPS for example.

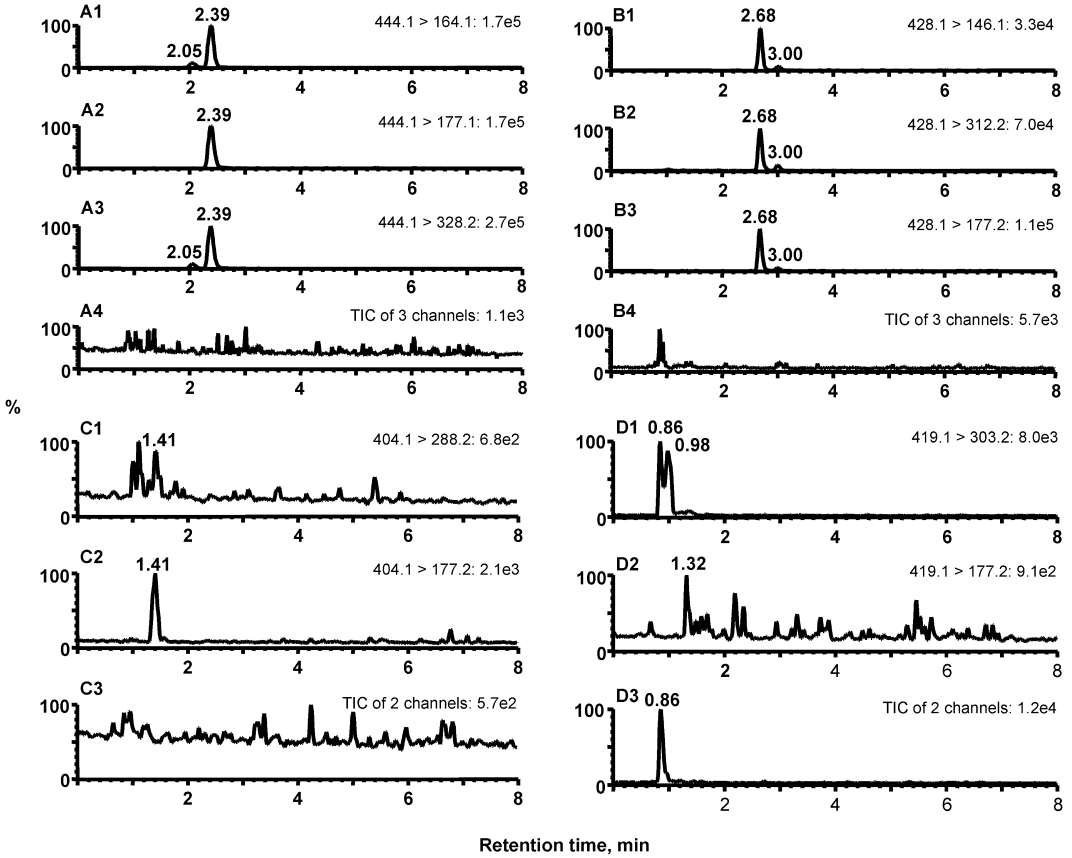

Fig. 5. Search for adducts with the various deoxynucleosides in herring sperm DNA incubated with (±)-1′-acetoxymethyleugenol. After digestion, DNA was analysed by UPLC–MS/MS in the MRM mode. The *m/z* transitions given in Table I were used for detection of dG adducts (panel A) and dA adducts (panel B). Analogous fragmentations (loss of deoxyribose and dN) were inferred for possible dC adducts (panel C) and dT adducts (panel D). Note that the signal intensities were high and moderate for the dG and dA adducts, respectively. Equivalent signals at the same retention times were produced by the chemically synthesised standards. In contrast, the signals of possible dC and dT adduct were very weak. The lowest trace in each panel represents untreated control DNA (total ion current of all channels recorded for the corresponding adducts).

Table III. Levels of DNA adducts formed by hydroxylated metabolites of methyleugenol in *Salmonella typhimurium* strains under the conditions of the mutagenicity assay

Treatment	TA100			TA100-hSULT1A1*1Y		
	Revertants per plate[a]	Adducts per 10^8 nucleosides[b]		Revertants per plate[a]	Adducts per 10^8 nucleosides[b]	
		N^6-MIE-dA	N^2-MIE-dG		N^6-MIE-dA	N^2-MIE-dG
Negative (solvent) control	113 ± 5	—	—	35 ± 3	—	—
(−)-1′-Hydroxymethyleugenol, 4 nmol	117 ± 3[ns]	—	—	60 ± 4***	4 ± 0	259 ± 30
(+)-1′-Hydroxymethyleugenol, 4 nmol	101 ± 9[ns]	—	—	54 ± 3***	6 ± 2	336 ± 32
(*E*)-3′-Hydroxymethylisoeugenol, 4 nmol	111 ± 5[ns]	—	—	58 ± 4***	11 ± 2	372 ± 58

—, below limit of detection (2 N^6-MIE-dA and 6 N^2-MIE-dG adducts per 10^8 nucleosides).
[a]Mean ± SE of six plates (negative control) or five plates (treatment groups).
[b]Mean ± SE of three incubations (each scaled up 10-fold over that of the mutagenicity assay).
Student's *t*-test compared to negative control: ns, $P > 0.2$; ***, $P < 0.001$.

Conclusions

We have shown in the present study that methyleugenol metabolites are mutagenic in the Ames test even at relatively low concentrations if appropriate activating enzymes, SULTs, are expressed in the target cells. Among the human enzymes, SULT1A1 showed the highest activity, followed by SULT1C2. SULT1A1 is the most abundant SULT form in the human organism. In contrast, expression of SULT1C2, up to date merely detected on the RNA level, has been observed only in few adult tissues (25). Its expression appears to be higher in various foetal tissues (25). To the best of our knowledge, it has not been studied whether alkenylbenzenes and their hydroxylated metabolites pass the placenta and reach foetal tissues. If this were the case, subsequent sulfation by foetal SULT forms might induce teratogenic and transplacental carcinogenic effects. However, taken together, our findings suggest that SULT1A1 is the most important SULT form involved in the activation of methyleugenol in the human organism, at least after birth. The orthologous murine form, mSult1a1, was somewhat less efficient in the activation of methyleugenol

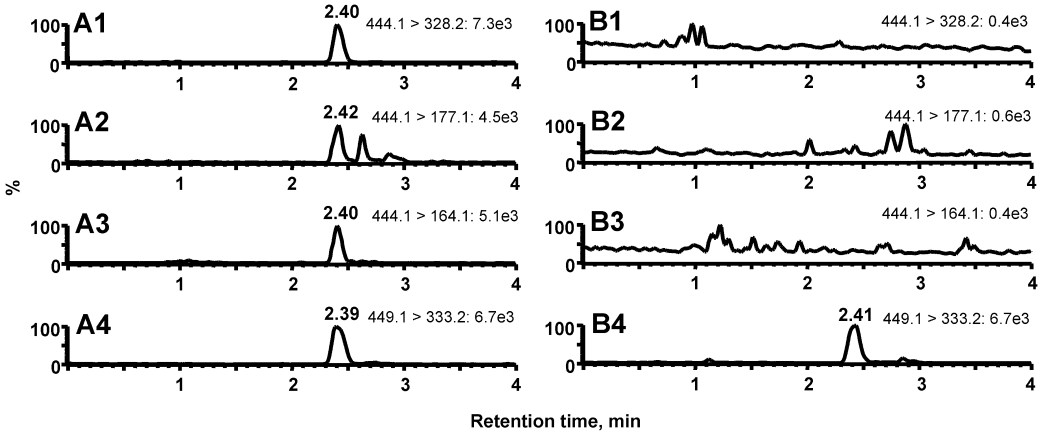

Fig. 6. Unambiguous detection of N^2-MIE-dG adducts in *Salmonella typhimurium* TA100-hSULT1A1*1Y treated with (+)-1′-hydroxymethyleugenol under the conditions of the mutagenicity assay. Strains TA100-hSULT1A1*1Y (panel A) and TA100 (panel B) were exposed to 4 nmol (+)-1′-hydroxymethyleugenol per incubation (610 µl). DNA was spiked with isotope-labelled internal standards, digested to deoxynucleosides and subjected to UPLC–MS/MS analyses in the MRM mode. Traces A1–3 and B1–3 represent characteristic *m/z* transitions of the analyte (see Table I). Traces A4 and B4 represent the transition of the quantifier for the internal standard.

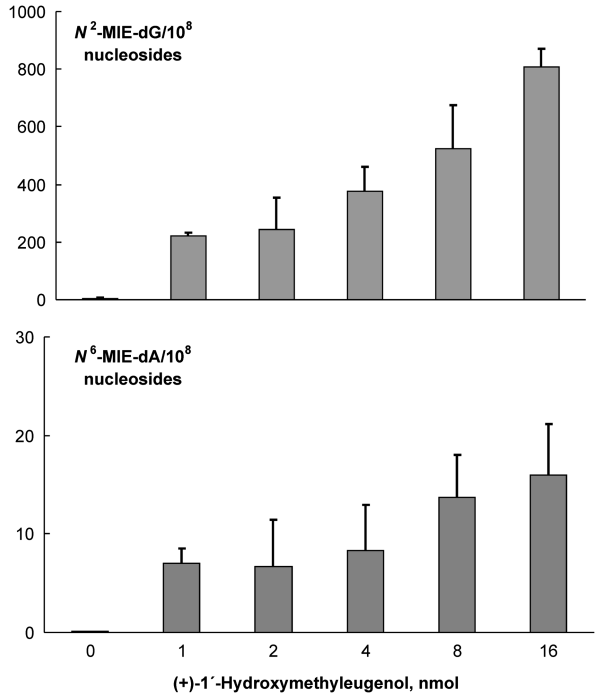

Fig. 7. Dose–response curve for DNA adducts formed by (+)-1′-hydroxymethyleugenol in *Salmonella typhimurium* TA100-hSULT1A1*1Y under the conditions of the mutagenicity assay. Upper panel: N^2-MIE-dG adducts; lower panel: N^6-MIE-dA adducts. Values are mean ± SE of three incubations. Mutagenicity results are presented in Figure 3C.

metabolites. It is highly expressed in liver, gut and lungs (28). One of these tissues, the liver, is the major target tissue for methyleugenol-induced tumorigenesis in the mouse (1,2). The liver expresses high levels of cytochromes P450, required for conversion of methyleugenol to its hydroxylated metabolites, which in turn may be further activated in liver by local SULT enzymes, including Sult1a1.

All three hydroxylated methyleugenol metabolites studied, (−)-1′-hydroxymethyleugenol, (+)-1′-hydroxymethyleugenol and (*E*)-3′-hydroxymethylisoeugenol were activated to mutagens by SULTs with moderate quantitative differences. Whereas (*E*)-3′-hydroxymethylisoeugenol was equally mutagenic in both strains expressing hSULT1A1 at different levels, the effects of both enantiomeres of 1′-hydroxymethyleugenol were enhanced with the increased expression level, suggesting that (*E*)-3′-hydroxymethyleugenol was the better substrate. This is not particularly surprising as its hydroxyl group is located at the end of a relatively flexible side chain, which may facilitate interaction with the active centres of enzymes. In contrast, the 1′-hydroxyl group is somewhat sterically hindered. Whereas (±)-1′-hydroxymethyleugenol has demonstrated higher carcinogenic activity in mouse liver than methyleugenol (1), (*E*)-3′-hydroxymethylisoeugenol has not yet been studied for carcinogenicity. However, the corresponding metabolite of another alkenylbenzene, safrole, has been investigated for various activities. Although 1′-hydroxysafrole and 3′-hydroxyisosafrole formed similar levels of adducts with nucleic acids in the presence of PAPS-fortified murine hepatic cytosol, 3′-hydroxyisosafrole was much less carcinogenic and formed much lower levels of adducts in mouse liver *in vivo* than 1′-hydroxysafrole (29). The difference was explained by the rapid biotransformation of 3′-hydroxyisosafrole in the mouse *in vivo* to the corresponding carboxylic acid. It remains to be studied whether the same sequestration occurs in humans and with 3′-hydroxylated metabolites of other alkenylbenzenes, such as methyleugenol.

Finally, we devised a mass spectrometric method for the specific detection of DNA adducts formed by methyleugenol. The usage of isotope-labelled internal standards allows accurate quantification. All three hydroxylated methyleugenol metabolites studied formed the same DNA adducts, high levels of N^2-MIE-dG and ∼50 times lower levels of N^6-MIE-dA in SULT-expressing *S. typhimurium* under the conditions of the mutagenicity assay. The sensitivity of the method is demonstrated by the observation that the level of N^2-MIE-dG adducts were 21- to 31-fold above the limit of quantification at doses of the test compounds leading to 1.5- to 1.7-fold increases in the number of revertants. Since the method is specific for these particular adducts, it will be useful when studying methyleugenol DNA adducts in tissue and blood samples of human subjects potentially exposed to many different genotoxicants.

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References

1. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A. and Miller, J. A. (1983) Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.*, **43**, 1124–1134.
2. NTP (2000) Toxicology and carcinogenesis studies of methyleugenol. *Natl Toxicol. Program Tech. Rep. Ser.*, **491**, 1–420.
3. Phillips, D. H., Miller, J. A., Miller, E. C. and Adams, B. (1981) Structures of the DNA adducts formed in mouse liver after administration of the proximate hepatocarcinogen 1′-hydroxyestragole. *Cancer Res.*, **41**, 176–186.
4. Phillips, D. H., Miller, J. A., Miller, E. C. and Adams, B. (1981) N^2 atom of guanine and N^6 atom of adenine residues as sites for covalent binding of metabolically activated 1′-hydroxysafrole to mouse liver DNA *in vivo*. *Cancer Res.*, **41**, 2664–2671.
5. Wiseman, R. W., Fennell, T. R., Miller, J. A. and Miller, E. C. (1985) Further characterization of DNA adducts formed by electrophilic esters of the hepatocarcinogens 1′-hydroxysafrole and 1′-hydroxyestragole *in vitro* and in mouse liver *in vivo*, including new adducts at *C*-8 and *N*-7 of guanine residues. *Cancer Res.*, **45**, 3096–3105.
6. Randerath, K., Haglund, R. E., Phillips, D. H. and Reddy, M. V. (1984) ^{32}P-Post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes: I. Adult female CD-1 mice. *Carcinogenesis*, **5**, 1613–1622.
7. Boberg, E. W., Miller, E. C., Miller, J. A., Poland, A. and Liem, A. (1983) Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1′-sulfooxysafrole is the major ultimate electrophilic and carcinogenic metabolite of 1′-hydroxysafrole in mouse liver. *Cancer Res.*, **43**, 5163–5173.
8. Boberg, E. W., Liem, A., Miller, E. C. and Miller, J. A. (1987) Inhibition by pentachlorophenol of the initiating and promoting activities of 1′-hydroxysafrole for the formation of enzyme-altered foci and tumors in rat liver. *Carcinogenesis*, **8**, 531–539.
9. Daimon, H., Sawada, S., Asakura, S. and Sagami, F. (1997) Inhibition of sulfotransferase affecting *in vivo* genotoxicity and DNA adducts induced by safrole in rat liver. *Teratog. Carcinog. Mutagen.*, **17**, 327–337.
10. Glatt, H. R. (2000) Sulfotransferases in the bioactivation of xenobiotics. *Chem. Biol. Interact.*, **129**, 141–170.
11. Glatt, H. R. (2005) Activation and inactivation of carcinogens by human sulfotransferases. In Pacifici, G. M. and Coughtrie, M. W. H. (eds), *Human Sulphotransferases*, Taylor & Francis, London, pp. 281–306.
12. Glatt, H. R., Schneider, H., Murkovic, M., Monien, B. H. and Meinl, W. (2012) Hydroxymethyl-substituted furans: mutagenicity in *Salmonella typhimurium* strains engineered for expression of various human and rodent sulphotransferases. *Mutagenesis*, **27**, 41–48.
13. Meinl, W., Pabel, U., Osterloh-Quiroz, M., Hengstler, J. G. and Glatt, H. R. (2006) Human sulfotransferases are involved in the activation of aristolochic acids and are expressed in renal target tissue. *Int. J. Cancer*, **118**, 1090–1097.
14. Landsiedel, R., Andrae, U., Kuhlow, A., Scholtyssek, M., Engst, W. and Glatt, H. R. (2000) Enantioselective metabolic activation of safrole. In Eisenbrand, G., Dayan, A. D., Elias, P. S., Grunow, W. and Schlatter, J. (eds), *Carcinogenic/Anticarcinogenic Factors in Food*, Wiley-VCH Verlag, Weinheim, Germany, pp. 464–467.
15. Jeurissen, S. M., Bogaards, J. J., Boersma, M. G. *et al.* (2006) Human cytochrome P450 enzymes of importance for the bioactivation of methyleugenol to the proximate carcinogen 1′-hydroxymethyleugenol. *Chem. Res. Toxicol.*, **19**, 111–116.
16. Borchert, P., Wislocki, P. G., Miller, J. A. and Miller, E. C. (1973) The metabolism of the naturally occurring hepatocarcinogen safrole to 1′-hydroxysafrole and the electrophilic reactivity of 1′-acetoxysafrole. *Cancer Res.*, **33**, 575–589.
17. Cartus, A. T., Merz, K. H. and Schrenk, D. (2011) Metabolism of methylisoeugenol in liver microsomes of human, rat, and bovine origin. *Drug Metab. Dispos.*, **39**, 1727–1733.
18. Paini, A., Punt, A., Viton, F., Scholz, G., Delatour, T., Marin-Kuan, M., Schilter, B., van Bladeren, P. J. and Rietjens, I. M. C. M. (2010) A physiologically based biodynamic (PBBD) model for estragole DNA binding in rat liver based on *in vitro* kinetic data and estragole DNA adduct formation in primary hepatocytes. *Toxicol. Appl. Pharmacol.*, **245**, 57–66.
19. Punt, A., Delatour, T., Scholz, G., Schilter, B., van Bladeren, P. J. and Rietjens, I. M. (2007) Tandem mass spectrometry analysis of N^2-(*trans*-isoestragol-3′-yl)-2′-deoxyguanosine as a strategy to study species differences in sulfotransferase conversion of the proximate carcinogen 1′-hydroxyestragole. *Chem. Res. Toxicol.*, **20**, 991–998.
20. Blanchard, R. L., Freimuth, R. R., Buck, J., Weinshilboum, R. M. and Coughtrie, M. H. (2004) A proposed nomenclature system for the cytosolic sulfotransferase (SULT) superfamily. *Pharmacogenetics*, **14**, 199–211.
21. Maron, D. M. and Ames, B. N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*, **113**, 173–215.
22. Baasanjav-Gerber, C., Hollnagel, H. M., Brauchmann, J., Iori, R. and Glatt, H. R. (2011) Detection of genotoxicants in Brassicales using endogenous DNA as a surrogate target and adducts determined by ^{32}P-postlabelling as an experimental end point. *Mutagenesis*, **26**, 407–413.
23. Gupta, R. C. (1993) ^{32}P-Postlabelling analysis of bulky aromatic adducts. *IARC Sci. Publ.*, 11–23.
24. Glatt, H. R., Boeing, H., Engelke, C. E. H., Kuhlow, L. M. A., Pabel, U., Pomplun, D., Teubner, W. and Meinl, W. (2001) Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects. *Mutat. Res.*, **482**, 27–40.
25. Sakakibara, Y., Yanagisawa, K., Katafuchi, J., Ringer, D. P., Takami, Y., Nakayama, T., Suiko, M. and Liu, M. C. (1998) Molecular cloning, expression, and characterization of novel human SULT1C sulfotransferases that catalyze the sulfonation of *N*-hydroxy-2-acetylaminofluorene. *J. Biol. Chem.*, **273**, 33929–33935.
26. Regal, K. A., Laws, G. M., Yuan, C., Yost, G. S. and Skiles, G. L. (2001) Detection and characterization of DNA adducts of 3-methylindole. *Chem. Res. Toxicol.*, **14**, 1014–1024.
27. Monien, B. H., Herrmann, K., Meinl, W. and Glatt, H. R. (2011) Furfuryl alcohol: mutagenicity and DNA adducts in Salmonella typhimurium strains expressing human sulfotransferase 1A1 and formation of the same adducts in mouse tissues *in vivo*. *Carcinogenesis*, **32**, 1533–1539.
28. Alnouti, Y. and Klaassen, C. D. (2006) Tissue distribution and ontogeny of sulfotransferase enzymes in mice. *Toxicol. Sci.*, **93**, 242–255.
29. Boberg, E. W., Miller, E. C. and Miller, J. A. (1986) The metabolic sulfonation and side-chain oxidation of 3′-hydroxyisosafrole in the mouse and its inactivity as a hepatocarcinogen relative to 1′-hydroxysafrole. *Chem. Biol. Interact.*, **59**, 73–97.