New Urinary Metabolites Formed from Ring-Oxidized Metabolic Intermediates of Styrene

Igor Linhart,*,† Jaroslav Mráz,‡ Jan Scharff,† Jan Krouželka,† Šárka Dušková,‡ Hana Nohová,‡ and Ľudmila Vodičková‡

Department of Organic Chemistry, Faculty of Chemical Technology, Institute of Chemical Technology, Prague, Technická 1905, CZ-166 28 Prague, Czech Republic, and National Institute of Public Health, Šrobárova 48, CZ-100 42 Prague, Czech Republic

Received November 23, 2009

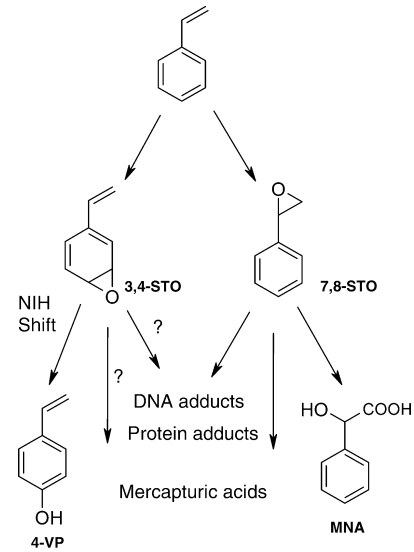
The urine from mice exposed to styrene vapors (600 and 1200 mg/m^3, 6 h) was analyzed for ring-oxidized metabolites of styrene. To facilitate the identification of metabolites in urine, the following potential metabolites were prepared: 2-, 3-, and 4-vinylphenol (2-, 3-, and 4-VP), 4-vinylpyrocatechol, and 2-, 3-, and 4-vinylphenylmercapturic acid (2-, 3-, and 4-VPMA). For the analysis of vinylphenols β-glucuronidase-treated urine was extracted and derivatized with acetanhydride/triethylamine before injection into GC/MS. Three isomers, 2-, 3-, and 4-VP, were found in the exposed urine using authentic standards. Additionally, three novel minor urinary metabolites, arylmercapturic acids 2-, 3-, and 4-VPMA, were identified by LC-ESI-MS^2 by comparison with authentic standards. Excretion of the most abundant isomer, 4-VPMA, amounted to 535 $\pm$ 47 nmol/kg and 984 $\pm$ 78 nmol/kg, representing approximately 0.047 and 0.043% of the absorbed dose for the exposure levels of 600 and 1200 mg/m^3, respectively. The ratio of 2-VPMA, 3-VPMA, and 4-VPMA was approximately 2:1:6. In model reactions of styrene 3,4-oxide (3,4-STO) with *N*-acetylcysteine in aqueous solutions and of its methyl ester in methanol, 4-vinylphenol was always the main product, while 3-vinylphenol has never been detected. No mercapturic acid was found in the reaction of 3,4-STO with *N*-acetylcysteine in aqueous solution at pH 7.4 or 9.7, but a small amount of 4-VPMA methyl ester was detected by LC-ESI-MS after the reaction of 3,4-STO with *N*-acetylcysteine methyl ester. In contrast, no mercapturic acid was found in the reaction of 3,4-STO with *N*-acetylcysteine in aqueous solution at pH 7.4 or 9.7. These findings indicate a capability of 3,4-STO to react with cellular thiol groups despite its rapid isomerization to vinylphenol in an aqueous environment. Moreover, the *in vivo* formation of 2- and 3-isomers of both VP and VPMA, neither of which was formed from 3,4-STO *in vitro*, strongly suggests that another arene oxide, styrene 2,3-oxide, might be a minor metabolic intermediate of styrene.

Introduction

Biotransformation of styrene in both humans and rodents (Scheme 1) proceeds mainly through an oxidation at the vinyl group. Styrene 7,8-oxide (7,8-STO[1]) is the key reactive metabolite, which is further biotransformed by two main metabolic routes, i.e., epoxide hydrolase-catalyzed hydrolysis and glutathione (GSH) conjugation. More than 90% of the styrene biotransformation proceeds via 7,8-STO (*1*). 4-Vinylphenol (4-VP) is the only metabolite with an intact vinyl group hitherto identified. Its detection in rat urine was first reported by Bakke and Scheline (*2*). Pantarotto et al. suggested that 4-VP is indicative of arene oxidation in the metabolism of styrene (*3*). It is most likely formed by a rearrangement of styrene 3,4-oxide (3,4-STO), known as NIH shift (*4, 5*). 4-VP was found also in the urine of workers exposed to styrene (*6–8*).

Watabe et al. studied the mutagenicity of 3,4-STO and its isomers, 1,2-STO and 7,8-STO, toward *Salmonella typhimurium*.

Scheme 1. Main Metabolic Routes of Styrene[a]



[a] More than 90% of biotransformation proceeds via 7,8-STO, while 3,4-STO is a minor metabolic intermediate.

Despite its instability in aqueous solutions (isomerization to 4-VP with a half time as short as 4 s at pH 7.4), 3,4-STO was found to be more mutagenic and more cytotoxic than its more stable isomers (*9*). It has been assumed that although 4-VP

* Corresponding author. Igor Linhart, Department of Organic Chemistry, ICT Prague, Technická 1905, CZ-166 28, Prague, Czech Republic. Tel: +420 220 444 165. E-mail: linharti@vscht.cz.

† Institute of Chemical Technology, Prague.

‡ National Institute of Public Health.

[1] Abbreviations: STO, styrene oxide; VP, vinylphenol; VPC, vinylpyrocatechol; VPMA, vinylphenylmercapturic acid; ACC, *N*-acetyl-L-cysteine; MeACC, *N*-acetyl-L-cysteine methyl ester; MAP, mercapturic acid pathway; MNA, mandelic acid; DCM, dichloromethane; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; MCPBA, *m*-chloroperoxybenzoic acid.

10.1021/tx9004192
Published on Web 12/18/2009

reflects a minor biotransformation pathway of styrene, i.e., the metabolic activation to 3,4-STO, this pathway may underlie a substantial part of the cytogenetic damage associated with styrene exposure (*8*). The role of arene oxidation in the biotransformation of styrene and its toxicological significance should be clarified. Some other phenolic metabolites, e.g., 4-hydroxymandelic acid (*3*) and 2-(4-hydroxyphenyl)ethanol (*10*) have been identified; however, no VP isomers, which would indicate a formation of arene oxides other than 3,4-STO, have been reported.

The aim of this study was to assess the possible formation of other metabolites derived from ring oxidation of styrene, namely, vinylphenols or vinylphenylmercapturic acids. These metabolites would indicate the ring oxidation of styrene, which has been shown to be a relevant toxicity-related event (*8, 9*).

Experimental Procedures

General. Acetonitrile for LC/MS Chromasolv was from Riedel de Haën, formic acid, puriss. p.a. from Fluka, and 3-chloroperoxybenzoic acid also purchased from Fluka, and the actual content of the peracid was determined by iodometric titration. 3- and 4-Vinylphenylboronic acids were from Aldrich, and 2-vinylphenylboronic acid was from Molekula (Germany). Other chemicals were of analytical grade and were used as received. Redistilled water was used for LC/MS and for SPE. β-Glucuronidase type H-2 from *Helix pomatia* (EC 3.2.1.31), glucuronidase activity ≥100,000 units/mL with residual sulfatase activity ≤7,500 units/mL, was from Sigma. Analytical standards of vinylphenols, 2-VP (*11*), 3-VP (*12*), 4-VP (*13*), and 4-VPC (*11*), were prepared as described in the literature and identified comparing their ^{1}H NMR spectra with those reported therein.

GC/MS analyses were carried out using a gas chromatograph−ion trap mass spectrometric system GCQ (Thermo Finnigan). LC/MS analyses were carried out on a Thermo Scientific LXQ linear trap mass spectrometer in tandem with a Janeiro LC system consisting of two Rheos 2200 pumps and a CTC PAL autosampler. NMR spectra were taken on a Varian Gemini 300 MHz spectrometer.

Vinylphenylmercapturic Acids. Three isomeric mercapturic acids, 2-, 3-, and 4-VPMA, were prepared by alkaline hydrolysis of their methyl esters whose synthesis is described elsewhere (*14*). The methyl ester of the corresponding mercapturic acid (30.6 mg, 0.110 mmol) was dissolved in a mixture of 2 mL of methanol and 4 mL of water, 120 μL of 1 M aqueous solution of NaOH was added, and the mixture was stirred at room temperature for 2 h. The solution was then neutralized with 120 μL of 1 M hydrochloric acid, diluted with 30% aqueous methanol to 58 mL, resulting in a 0.5 mg/mL solution of VPMA which was then used for calibration. Analytical samples of 3- and 4-VPMA in the form of ammonium salts were obtained as follows. Aliquots (50 mL) of VPMA solutions were partially evaporated in a vacuum to remove methanol. Resulting turbid solutions were then diluted with water to 50 mL, acidified with 1 M HCl to pH 2.5, and extracted with DCM (3 × 20 mL). Extracts were dried with anhydrous sodium sulfate and filtered, diluted ammonium hydroxide (1:5, 100 μL) was added, and the extracts were evaporated to dryness in a vacuum to afford solid residues.

2-Vinylphenylmercapturic Acid (2-VPMA). ^{1}H NMR (DMSO-d_6): δ = 1.76 (s, 3H, CH_3CO); 3.08 (dd, J = 12.9 and 7.6 Hz, 1H, CH_2S); 3.31 (dd, J = 12.9 and 5.3 Hz, 1H, CH_2S); 4.14 (m, 1H, SCH$_2$CH); 5.30 (d, J = 11.0 Hz, 1H, CH_2=CH); 5.70 (d, J = 17.6 Hz, 1H, CH_2=CH); 7.09 (dd, J = 17.7 and 11.0 Hz, 1H, CH$_2$=CH); 7.20 (m, 1H, aromatic CH), 7.40 (d, J = 6.7 Jz, 1H, aromatic CH); 7.52 (dd, J = 7.3 and 1.5 Hz, 1H, aromatic CH); 7.82 (d, J = 7.6 Hz, 1H, aromatic CH).

ESI-MS: m/z 266 (M + H)$^+$, 264 (M − H)$^-$. MS^2 at 266: m/z 248 (MH − H_2O)$^+$, 224 (MH − CH_2CO)$^+$, 130 (MH − $CH_2CHC_6H_4SH$)$^+$. MS^2 at 264: m/z 135 (M − CH_2CH(COOH)NHCOCH$_3$)$^-$.

3-Vinylphenylmercapturic Acid (3-VPMA). ^{1}H NMR (DMSO-d_6): δ = 1.75 (s, 3H, CH_3CO); 3.13 (dd, J = 13.8 and 9.1 Hz, 1H, CH_2S); 3.34 (dd, J = 13.8 and 4.6 Hz, 1H, CH_2S); 4.12 (m, 1H, SCH$_2$CH); 5.26 (d, J = 10.8 Hz, 1H, CH_2=CH); 5.87 (d, J = 17.6 Hz, 1H, CH_2=CH); 6.68 (dd, J = 17.7 and 10.9 Hz, 1H, CH$_2$=CH); 7.20 (m, 3H, aromatic CH), 7.40 (s, 1H, aromatic CH).

ESI-MS: m/z 266 (M + H)$^+$, 264 (M − H)$^-$. MS^2 at 266: m/z 248 (MH − H_2O)$^+$, 224 (MH − CH_2CO)$^+$; 130 (MH − $CH_2CHC_6H_4SH$)$^+$. MS^2 at 264: m/z 135 (M − CH_2CH(COOH)-NHCOCH$_3$)$^-$.

4-Vinylphenylmercapturic Acid (4-VPMA). ^{1}H NMR (DMSO-d_6): δ = 1.74 (s, 3H, CH_3CO); 3.11 (dd, J = 12.3 and 6.5 Hz, 1H, CH_2S); another signal of CH_2S (AB system) overlapped by HOD; 4.05 (m, 1H, SCH$_2$CH); 5.16 (d, J = 11.1 Hz, 1H, CH_2=CH); 5.76 (d, J = 17.9 Hz, 1H, CH_2=CH); 6.65 (dd, J = 17.7 and 10.8 Hz, 1H, CH$_2$=CH); 7.23 (d, J = 8.2 Hz, 2H, aromatic CH); 7.34 (d, J = 8.2 Hz, 2H, aromatic CH).

ESI-MS: m/z 266 (M + H)$^+$, 264 (M − H)$^-$. MS^2 at 266: m/z 248 (MH − H_2O)$^+$, 224 (MH − CH_2CO)$^+$; 130 (MH − CH_2-CHC_6H_4SH)$^+$. MS^2 at 264: m/z 135 (M − CH_2CH(COOH)-NHCOCH$_3$)$^-$.

Preparation of Styrene 3,4-Oxide (3,4-STO). 3,4-STO was synthesized in 4 steps from 2-(cyclohexa-1,4-dien-1-yl)ethanol. In brief, its bromination according to Watabe et al. (*15*) afforded a mixture of 2-(1,6-dibromo-cyclohex-3-en-1-yl)ethanol and 2-(4,5-dibromo-cyclohex-1-en-1-yl)ethanol. Isolation by column chromatography on silica gel afforded 30% of the former.

Bromination of 2-(1,6-Dibromo-cyclohex-3-en-1-yl)ethanol with Bromotriphenyl-phosphonium Bromide. To a suspension of freshly prepared Ph_3PBr_2 (6,03 g, 13.3 mmol) in 30 mL of DCM, a solution of 3.38 g (11.9 mmol) of 2-(1,6-dibromo-cyclohex-3-en-1-yl)ethanol in 20 mL of DCM was added dropwise under external cooling with crushed ice. The cooling bath was then removed, and the reaction mixture was stirred for 20 h at room temperature. The resulting yellowish solution was washed subsequently with saturated aqueous solutions of sodium bicarbonate and sodium chloride, and dried with potassium carbonate. DCM was removed by evaporation in a vacuum, and the solid residue was then extracted with three 40 mL portions of hexane. The resulting hexane solution was evaporated to dryness yielding 3.76 g (91%) of the crude product, which was purified by flash chromatography (10:1 hexane/DCM) to yield 1.66 g (40%) of pure 4,5-dibromo-4-(2-bromoethyl)-1-cyclohexene identified by comparing its ^{1}H NMR spectra with that reported in the literature (*15*).

Oxidation of 4,5-Dibromo-4-(2-bromoethyl)-1-cyclohexene with *m*-Chloroperoxybenzoic Acid (MCPBA). A solution of 4,5-dibromo-4-(2-bromoethyl)-1-cyclohexene (1.032 g, 2.97 mmol) and MCPBA (0.914 g, 80% pure, 4.24 mmol) in 15 mL of chloroform was stirred for 24 h at 40 °C. The reaction mixture was then washed subsequently with saturated aqueous solutions of sodium bisulphite (20 mL), sodium bicarbonate (20 mL), and sodium chloride (20 mL), and dried with magnesium sulfate. Evaporation of the solvent in a vacuum yielded 0.979 g (91%) of a crude product, which was purified by flash chromatography (5:1 hexane/ethyl acetate) to afford 0.705 g (65%) of white crystals, mp 83−85 °C, identified by comparing the ^{1}H NMR spectrum with that reported in the literature as 4,5-dibromo-4-(2-bromoethyl)cyclohexane 1,2-oxide.

Elimination with 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU). 4,5-Dibromo-4-(2-bromoethyl)cyclohexane 1,2-oxide (135 mg, 0.372 mmol) was placed in a reaction flask, which was then evacuated and rinsed with argon to remove air and moisture. Dry ether (4 mL) was added through a septum followed by 330 μL (2.21 mmol) of DBU. The reaction mixture was stirred for 20 h at room temperature under argon. A white precipitate was gradually formed while the solution became intensely yellow. The reaction flask was then evacuated to remove the ether, and the residue was extracted with three portions of pentane (3 × 9 mL). Combined pentane solutions were washed quickly with a saturated aqueous bicarbonate solution (10 mL) followed by brine (10 mL). After 1 h of drying over sodium sulfate, a clear yellow solution was evaporated in a vacuum evaporator, which was thoroughly dried and protected from atmospheric moisture by a drying tube filled with molecular sieves. Styrene 3,4-oxide (30 mg, 67%) was obtained

as a yellow liquid and identified by ^{1}H NMR and GC/MS as 3,4-STO (*15*). It contained only a trace of 4-VP.

Model Reaction of 3,4-STO with *N*-Acetylcysteine Methyl Ester (MeACC). To a solution of MeACC (26 mg, 0.148 mmol) in methanol (5 mL), sodium hydride (5.9 mg, 60% suspension in mineral oil, 0.148 mmol) was added to generate the thiolate anion. The reaction mixture was stirred under argon and cooled externally with crushed ice. Freshly prepared 3,4-STO (15 mg, 0.125 mmol) in pentane (4 mL) was added. After 2 h, the solvents were removed in a vacuum, the residue was redissolved in diethyl ether (10 mL), and the resulting turbid ether solution was washed with brine (10 mL) followed by water (10 mL) and dried with magnesium sulfate. The extract was then filtered, evaporated to dryness in a vacuum yielding 25 mg of oily product. ^{1}H NMR analysis confirmed 4-VP as the predominating product. The product was further analyzed by LC/MS using a 250 × 2 mm Phenomenex Gemini C18 column, particle size 5 μm, eluted with aqueous methanol, the concentration of which was increased linearly from 50 to 80% in 20 min and then held for another 10 min at a flow rate of 200 μL/min. ESI-MS (positive ions) was taken in full scan *m/z* 100−400. A peak corresponding to 4-VPMA methyl ester was detected showing ions at *m/z* 280 $(M + H)^+$, 302 $(M + Na)^+$, and 318 $(M + K)^+$.

Model Reaction of 3,4-STO with *N*-Acetylcysteine (ACC) in Aqueous Solutions. To a 0.1 M solution of ACC (2 mL), the pH of which was adjusted with ammonium hydroxide to 7.4 or 9.7, freshly prepared 3,4-STO (4 mg) in pentane (4 mL) was added in four portions of 1 mL each. After each addition, the reaction mixture was stirred vigorously for 20 min, and pentane was removed by evaporation in a vacuum. The yellow color of the pentane solution disappeared rapidly. To ensure the conversion of possible primary hydroxycyclohexadienyl adducts to vinylphenylmercapturic acids, the reaction mixtures were acidified with acetic acid to pH 2, incubated for 1 h at 37 °C, and then neutralized with aqueous ammonia. In a control experiment without ACC, 3,4-STO was added in the same way to a 20 mM ammonium formate buffer (pH 7.4 or 9.7). The resulting solutions were analyzed by LC with UV detection and by LC/MS. A peak coeluting with 4-VP was detected by UV detection as a sole major product uniformly in all samples, whereas VPMA was not detected.

Animal Treatment. Adult male NMRI mice (mean weight 29 g) were exposed for 6 h to styrene vapor in a dynamic exposure chamber with controlled concentrations of 600 and 1200 mg/m^3. Animals were divided into five groups, six animals per group. Each group was placed into a glass metabolic cage with free access to food and water. To enhance diuresis, sucrose (8 mg/mL) was added to the drinking water. Animals were exposed by inhalation, two groups at each concentration level, while one group remained unexposed. Urine was collected 24 h after the beginning of the exposure. During sample collection, we filtered the urine through a gauze filter to remove pieces of feces and crumbs of food pellets. The walls of the metabolic cages were rinsed with distilled water, and the resulting solution was added to the main portion of collected urine. Samples were stored at −20 °C until analyzed. Animal experiments were approved by the Central Committee for Animal Protection of the Czech Republic.

Analysis for the Phenolic Metabolites. To the urine samples (2 mL) adjusted with 10 M acetic acid to pH 5.0−5.5, a suspension of β-glucuronidase from *Helix pomatia* was added (10 μL). The samples were incubated at 37 °C for 1 h and then extracted with ethyl acetate (4 mL). The extract was evaporated to dryness, and the residue was acetylated with a mixture of acetonitrile/acetanhydride/triethylamine at 3:1:1 (100 μL) at room temperature for 20 min. The solvents were again evaporated to dryness, and the residue was dissolved in ethyl acetate (50 μL) and injected onto a DB-5 fused silica capillary column 30 m × 0.25 mm I.D., film thickness of 0.25 μm (J&W). The injector was set in splitless mode (1 min). Helium was used as a carrier gas at a linear velocity of 30 cm/s. Temperature of the column was held at 60 °C for 1 min, then raised at 10 °C/min to 100 °C, and thereafter at 40 °C/min to 300 °C, which was held for 4 min. Temperatures of the injector, transfer line, and ion source were 300 °C, 250 °C, and 180 °C, respectively. The detector was operated in EI mode (70 eV), and full scan data were collected in the mass range *m/z* 50−400. Analyses for vinylphenols were carried out at *m/z* 120 $(M - CH_2CO)^+$. For VPC (M = 220), the transition 178 → 136 was monitored in the SRM mode.

Analysis of Vinylphenylmercapturic Acids by LC/MS. Urine samples were filtered through 0.2 μm Nylon membrane filters, and 1 mL aliquots were diluted with 2 mL of 0.1 M HCl resulting in pH 1−2 and left standing for 1 h. Samples were then transferred onto Oasis HLB 60 mg SPE columns (Waters), which were preconditioned with 2 mL of methanol followed by 2 mL of water. Columns were washed with 2 mL of 20% aqueous methanol and then eluted with 2 mL of 80% aqueous methanol. Eluates were evaporated to dryness in a stream of nitrogen at 37 °C and redissolved in 1 mL of 20% aqueous methanol. Calibration solutions of 4-VPMA in blank urine were worked up in the same way. The calibration curve was linear in the range of concentration 0.2−5 μg/mL, $R > 0.999$. Aliquots of 10 μL were injected on a 250 × 2 mm Phenomenex Luna C18(2) column, particle size 5 μm, which was eluted isocratically with 30% acetonitrile in 0.1% aqueous formic acid at a flow rate of 200 μL/min. Full scan ESI-MS spectra were collected in the mass range of *m/z* 100−400. Collision activated daughter spectra (MS^2) were generated from the parent ions *m/z* 264 and 266 for negative and positive ion modes, respectively, using a collision energy of 20 V for both modes. Helium was used as the collision gas. Both negative and positive ions were used for the detection and identification of VPMA isomers, whereas only negative ions were used for the quantification of 4-VPMA.

Results and Discussion

Authentic Standards. To ensure unequivocal identification of the possible metabolites arising from ring oxidation of styrene, authentic samples of 2-, 3-, and 4-vinylphenol, 4-vinylpyrocatechol, as well as 2-, 3-, and 4-vinylphenylmercapturic acid were prepared and characterized by NMR and mass spectra. 4-VP was prepared by hydrolysis of commercially available 4-acetoxystyrene (*13*). 2-VP and 4-VPC were prepared by microwave induced decarboxylation of 2-hydroxycinnamic and caffeic acid, respectively (*11*). The same procedure failed to give a preparatively useful yield of 3-vinylphenol; therefore, this isomer was prepared from 3-hydroxycinnamic acid by a classical copper-catalyzed decarboxylation (*12*). Preparation of 3-VPMA and 4-VPMA methyl esters by S-arylation of MeACC with corresponding vinylphenylboronic acids is described elsewhere (*14*). Their alkaline hydrolysis afforded authentic samples of 2-, 3-, and 4-VPMA.

Phenolic Metabolites. Mice were exposed to styrene vapor at two concentration levels, 600 and 1200 mg/m^3 for 6 h. To release phenolic metabolites from their conjugates, urine was treated with β-glucuronidase/arylsulfatase, neutralized, and extracted with ethyl acetate. The extracts were then acetylated to improve the chromatographic properties of the analytes. Systematic GC/MS analysis of the neutral extracts using authentic standards indicated the presence of 3-VP, 4-VP, and probably also 2-VP isomer. The latter could not be unequivocally identified because of its insufficient separation from other styrene metabolites. In the exposed urine samples, a cluster of incompletely separated peaks appeared at *m/z* 120 near the retention time of the authentic 2-VP (Figure 1). Our attempts to improve separation by changing the column temperature gradient were unsuccessful. 4-VPC was present in both exposed and control urine at similar concentration levels so that its occurrence cannot be attributed to styrene exposure. In fact, it has been reported that in both rodents and humans this compound is formed by intestinal microflora from caffeic acid, which is present in the diet (*16*).

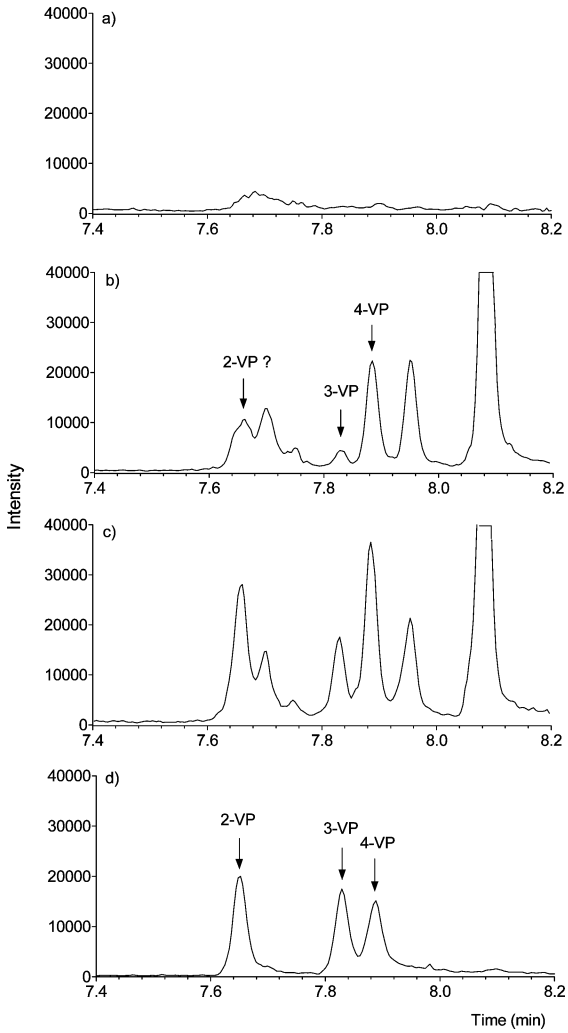


Figure 1. GC/MS chromatograms of vinylphenols (*m/z* 120) in the neutral extracts of urine: (a) control urine; (b) urine from mice exposed to styrene at the concentration of 1200 mg/m^3 for 6 h; (c) urine from exposed mice spiked with authentic 2-, 3-, and 4-VP; (d) standards of 2-, 3-, and 4-VP.

Mercapturic Acids. In our initial search for VPMAs, acidic extracts of mice urine were methylated and analyzed by GC/MS. No VPMA was detected in the full scan or SIM modes, but in SRM mode using a single transition of *m/z* 220 → 161, 3- and 4-VPMA isomers were detected in the form of their methyl esters. Ultimate identification of all three VPMA isomers was achieved by LC/MS. Urine samples were purified by SPE, and the extracts were analyzed by LC-ESI-MS^2 in both positive and negative ion modes. Peaks coeluting with the authentic standards of 2-, 3-, and 4-VPMA were detected (Figure 2). Fragmentation of the quasimolecular ion $(M + H)^+$ at *m/z* 266 in all VPMA isomers gave characteristic main fragments at *m/z* 248 $(MH - H_2O)^+$ and 224 $(MH - CH_2CO)^+$. Further fragmentation of these daughter ions revealed significant differences between 2-VPMA and the other two isomers. In Figures 3 and 4, the fragmentation patterns (MS^2 and MS^3) of 2-VPMA with those of 3- and 4-VPMA are compared. While 2-VPMA tends to stabilize the fragment ions by intramolecular interaction with the ortho vinyl group giving thiatropylium ions and/or by the stabilization of three membered thiiranium ions through interaction with the vinyl π-electron (Figure 3), these stabilizing effects are not possible with the more distant vinyl group in the 3- and 4-isomers, which did not differ significantly in their fragmentation (Figure 4). However, in the negative ion mode,

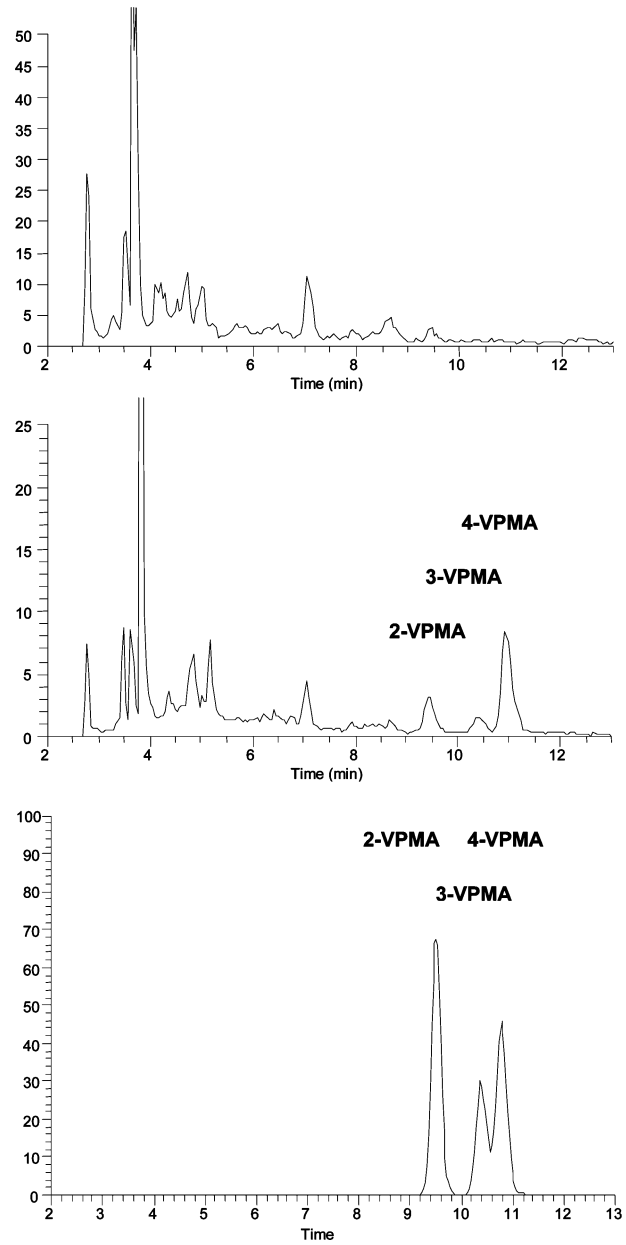


Figure 2. LC/MS chromatograms, MS^2 at *m/z* 264 for $(M - H)^-$ of VPMA. Blank urine (upper trace), urine from mice exposed to styrene, 1200 mg/m^3 for 6 h (middle trace), and authentic samples of 2-, 3-, and 4-VPMA (lower trace).

the quasimolecular ion $(M - H)^-$ at *m/z* 264 gave uniformly a single fragment at *m/z* 135 ($CH_2{=}CHC_6H_4S^-$). Negative ion MS^2 spectra, which gave a better signal-to-noise ratio in urine samples, were used for quantitative determination of the major isomer, 4-VPMA. Its concentrations found in diluted urine samples were 0.75 ± 0.10 mg/L and 1.09 ± 0.07 mg/L corresponding to the excretion of 535 ± 47 nmol/kg and 984 ± 78 nmol/kg for the lower and higher exposures, respectively.

As the formation of VPMAs proceeds through dehydration of the corresponding premercapturic acids (Scheme 2), a portion of the latter may have been still present in the native urine. To ensure the full conversion of the premercapturic acids to VPMAs, urine samples were acidified to pH 1−2 and allowed to stand in these acidic solutions for 1 h. Then, the total 4-VPMA should correspond to the sum of 4-VPMA and its premercapturic acid, i.e., *N*-acetyl-*S*-(4-vinyl-6-hydroxycyclohexa-2,4-dien-1-yl)cysteine, which may have also been present in the urine.

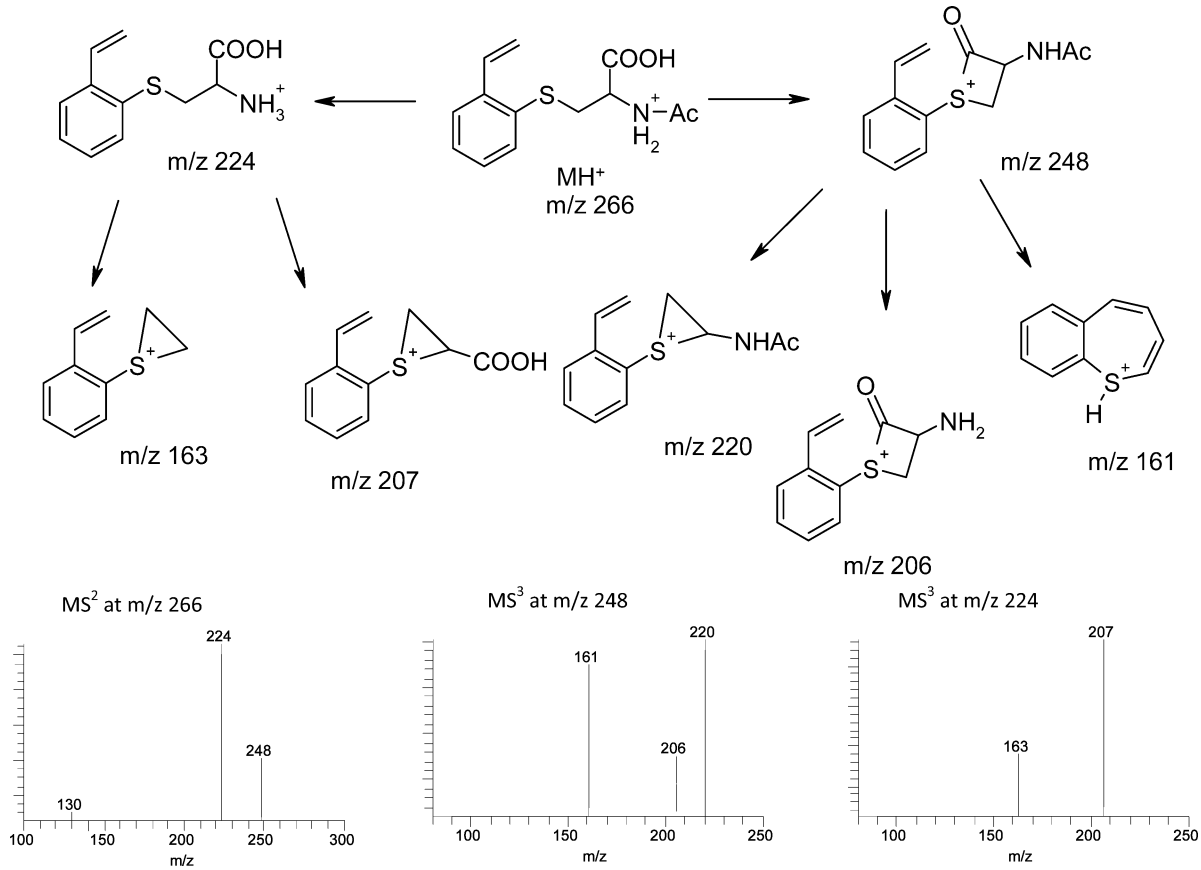


Figure 3. Mass fragmentation pattern and MS^2 and MS^3 spectra of 2-VPMA.

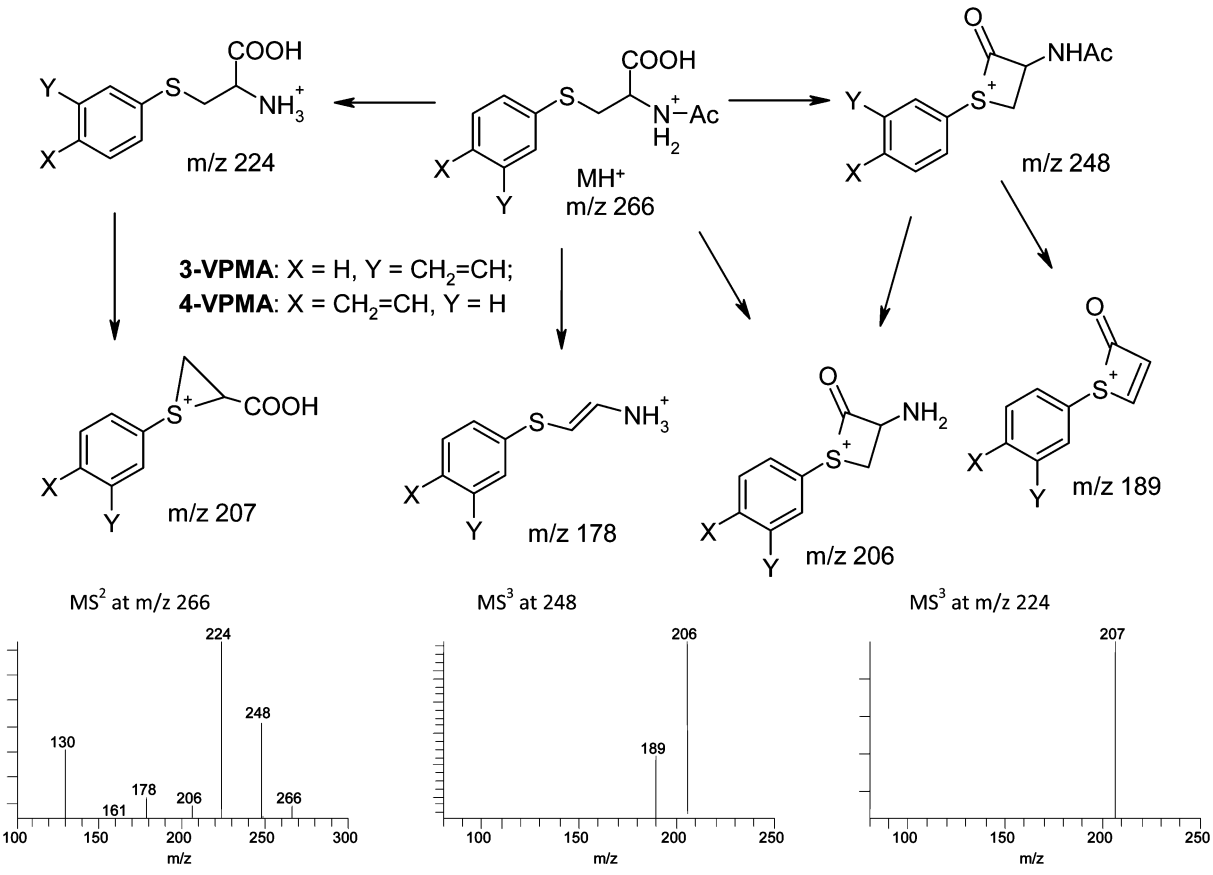
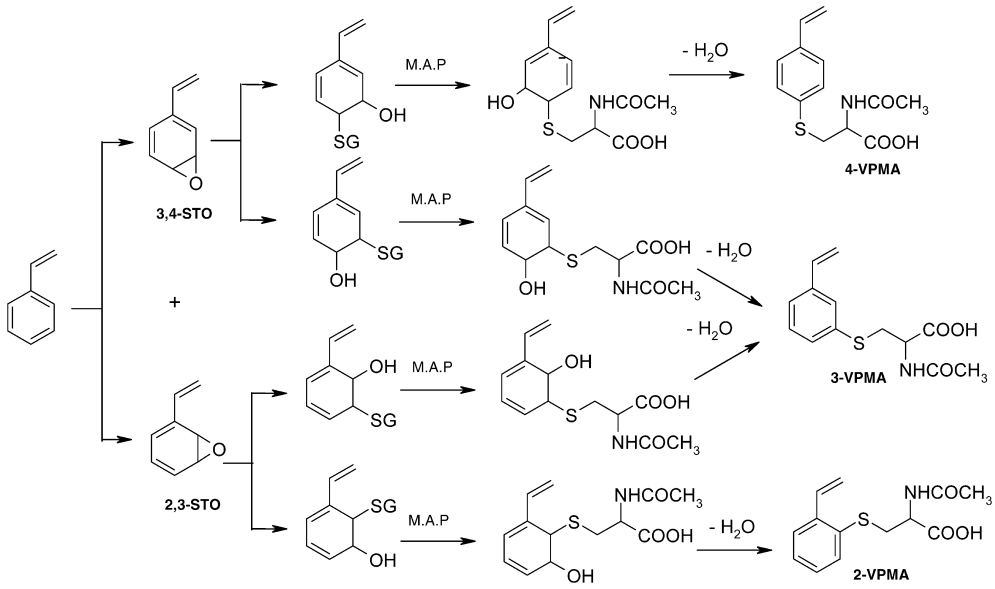


Figure 4. Mass fragmentation pattern and MS^2 and MS^3 spectra of 3-VPMA and 4-VPMA. As these isomers showed the same mass fragmentation, MS^2 and MS^3 spectra of 3-VPMA only are shown.

Scheme 2. Proposed Biotransformation Routes Leading to Three Isomeric Vinylphenylmercapturic Acids from Styrene[a]



[a] GS = S-glutathionyl; M.A.P. = mercapturic acid pathway.

Assuming that the retention of styrene in lungs was 0.55 and the minute ventilation volume of mice was 1 L/min per kg body weight (*17*), the absorbed dose of styrene during the exposure can be estimated to 1.14 and 2.28 mmol/kg at 600 and 1200 mg/m^3, respectively. Hence, metabolic conversion to 4-VPMA amounted to approximately 0.05 and 0.04% of absorbed dose for the lower and the higher exposure levels, respectively. The ratio of 4-VPMA/3-VPMA/2-VPMA was approximately 6:1:2 as determined from the corresponding peak areas.

Model Reactions of Styrene 3,4-Oxide. The chemical reactivity of 3,4-STO was tested in model reactions with ACC in aqueous solutions at pH 7.4 and 9.7 and with MeACC in methanol. 3,4-Styrene oxide was prepared by a substantially modified procedure of Watabe et al. (*15*) immediately before addition to the model reaction mixture. Freshly prepared samples contained only traces of 4-VP but isomerized to 4-VP within 24 h in deuterochloroform (in the NMR cuvette). The yellow color, which indicated the presence of oxepin (a tautomeric form of 3,4-STO), disappeared immediately after dissolution in water, methanol, or in the model reaction mixture giving 4-VP as the only product, which could be isolated and identified by ^{1}H NMR. 4-VP has always been the predominating product, whereas only a trace of 4-VPMA methyl ester was detected by LC-ESI-MS when the reaction was performed in methanol. Collisionally activated MS^2 spectrum of the quasimolecular ion MH$^+$ at *m/z* 280 gave fragments at *m/z* 248 (MH $-$ $CH_3OH)^+$, 238 (MH $-$ $CH_2CO)^+$, 221 (M $-$ $CH_3CONH_2)^+$, 206 (MH $-$ CH_3OH $-$ $CH_2CO)^+$, and 144 (MH $-$ $CH_2CHC_6H_4SH)^+$. In contrast, in the model reactions performed in aqueous solutions no VPMA was detected. Unlike in mice urine, no isomers other than 4-VP or 4-VPMA could be detected in the model reactions.

Mechanistic Considerations. Formation of VPMA isomers *in vivo* represents an additional strong evidence of metabolic ring oxidation of styrene. Theoretically, three isomeric arene oxides, 1,2-, 2,3-, and 3,4-STO can be formed by this metabolic process. However, formation of 1,2-STO is rather unlikely because 1-substituted arene oxides cannot be easily formed by arene oxidases because of steric hindrance (*18*).

In accordance with theoretical predictions, which were also confirmed experimentally by model reactions of toluene 3,4-oxide (*18*) as well as of 3,4-STO, NIH shift of 3,4-STO should lead exclusively to 4-VP. Formation of the 3-VP isomer *in vivo* can be therefore explained by an enzymatic process with a different mechanism than NIH shift, namely, an epoxide hydrase catalyzed hydration−dehydration sequence, starting either from 3,4- or 2,3-STO. Ring oxidation to 2,3-STO could also explain the formation of 2-VP as a product of NIH shift as well as both 2- and 3-VPMA isomers.

2,3-STO and 3,4-STO may react with cellular GSH yielding two isomeric primary conjugates each as shown in Scheme 2. Glutathione conjugation, which is probably catalyzed by glutathione-*S*-transferases, gives rise ultimately to three mercapturic acids, 2-, 3-, and 4-VPMA. A similar biotransformation pathway consisting of dehydration of the primary conjugates and subsequent cleavage of γ-glutamyl and glycyl moieties followed by N-acetylation has been proposed for naphthalene (*19*) and benzene (*20*). Spontaneous dehydration may occur at all stages of the metabolic sequence leading from glutathione conjugates to corresponding arylmercapturic acids, which are the metabolic end-products excreted in urine, as well as during acidic sample treatment. After complete conversion of premercapturic acids to the ultimate arylmercapturic acids, the latter urinary metabolites reflect the extent of arene oxidation. For example, phenylmercapturic acid is a useful urinary biomarker of occupational and environmental exposure to benzene and its metabolic activation to benzene oxide (*20, 21*).

Conclusions. New ring oxidized metabolites of styrene, 2- and 3-VP and 2-, 3-, and 4-VPMA bring new evidence for the formation of electrophilic arene oxides in the course of styrene metabolism. In addition to styrene 3,4-oxide, which has been assumed to be a precursor to 4-VP as the only previously identified VP isomer, another arene oxide, most likely styrene 2,3-oxide, is needed to plausibly explain our findings of new metabolites, namely, 2- and 3-VPMA, 2-VP, and possibly also 3-VP.

Acknowledgment. Financial support of this work by grant numbers 2B08051 and MSM 604 613 73 01 from the Ministry of Education, Youth and Sports of the Czech Republic is gratefully acknowledged. We thank Mrs. Nad′a Kellerová and Dr. Emil Frantík for their assistance with animal exposure and handling.

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TX9004192