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Chiral Inversion of 1-Hydroxyethylpyrene Enantiomers Mediated by Enantioselective Sulfotransferases

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The benzylic alcohol 1-hydroxyethylpyrene (1-HEP) is activated to a mutagen by sulfotransferases. The sulfuric acid ester formed is difficult to detect, as it is rapidly hydrolysed back to the alcohol. Incubation of the individual enantiomers of 1-HEP with human hydroxysteroid sulfotransferase (hHST) or estrogen sulfotransferase (hEST), expressed in bacteria, led to the formation of the other enantiomer. The rates of sulfation were determined from the initial rates of chiral inversion of the alcohol, knowing that hydrolysis follows an S_N1 mechanism and therefore produces racemic alcohol. hEST showed high enantioselectivity for *S*-1-HEP, whereas hHST strongly preferred the *R*-enantiomer. The rates of sulfation of the preferred enantiomers were high, similar to those for the prototype substrates of hEST (*β*-estradiol) and hHST (dehydroepiandrosterone). Moreover, after a 30-min incubation of *S*-1-HEP with hEST, 95% of the recovered alcohol showed the *R*-configuration, indicating that several cycles of sulfation and hydrolysis had led to the depletion of one enantiomer and to the enrichment of the other enantiomer.

Sulfotransferases transfer the sulfonyl moiety from the cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to nucleophilic groups of their substrates [1]. The cytosolic forms (EC 2.8.2) present in mammalian tissues are important in the regulation of hormone levels as well as in the elimination of xenobiotics. In general, sulfonation increases the solubility in water, decreases the penetration of membranes and, therefore, facilitates excretion. However, the sulfate group, generated by *O*-sulfonation (sulfation), is electron-withdrawing and may be cleaved off heterolytically in some molecules, for example if the resulting cation is stabilised by mesomerism [2]. This is the case with sulfuric acid esters of benzylic alcohols. Many of them are strong electrophiles, which may react with cellular nucleophiles such as DNA and water. Likewise, various benzylic sulfuric acid esters form benzylic DNA adducts and are strong mutagens [2,3]. Their reaction with water, the hydrolysis of sulfuric acid esters, leads to the corresponding alcohol [4,5], which again is a substrate for sulfotransferases and may undergo a new cycle of activation.

The secondary benzylic alcohol 1-hydroxyethylpyrene [1-(1-pyrenyl)ethanol, 1-HEP, structural formula in Fig. 1] is a potent mutagen to *Salmonella typhimurium* strains engineered for expression of human sulfotransferases, but is inactive in the sulfotransferase-deficient parental strain [6]. 1-HEP is chiral due to the presence of an asymmetric benzylic carbon center. Interestingly, the *S*-enantiomer of 1-HEP is about 150 times more potent than its optical antipode to the strain TA1538-hEST, engineered for the expression of human estrogen sulfotransferase (hEST), whereas the reverse enantioselectivity (15 : 1) was detected using strain TA1538-hHST, which expresses human hydroxysteroid sulfotransferase (hHST). It is very likely that sulfuric acid esters were formed and induced the mutations in the recombinant bacteria. However, direct demonstration of the metabolically formed sulfuric acid ester is difficult, as it is rapidly hydrolysed in aqueous media. The rate constant of the hydrolysis was determined using chemically synthesised 1-HEP sulfate [7]. At 37 °C, it amounted to $1*10^{-2}$ s^{-1}, equivalent to a half-life of 1 min. The hydrolysis of benzylic sulfuric acid esters proceeds via a carbocation (S_N1 mechanism) [5] and thus leads to a racemic product.

We therefore have hypothesised that sulfotransferases may act as invertases for the enantiomers of 1-HEP. Moreover, the rate of sulfation may be deduced from the rate of chiral inversion. These rates may be compared with the mutagenic activities that have been

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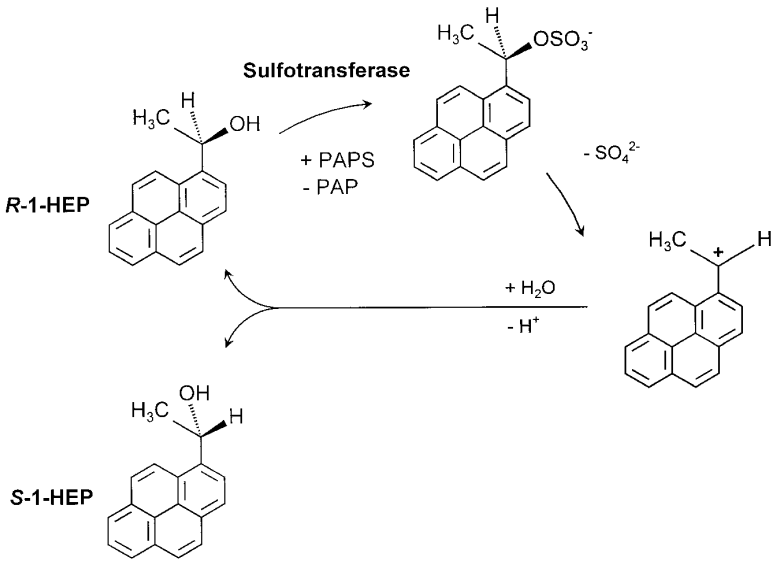

FIG. 1. Structural formulas of the investigated compounds and postulated pathway of the chiral inversion. Sulfotransferase-mediated sulfation of the enantiomers of 1-hydroxyethylpyrene should not change the configuration, as it proceeds via *O*-sulfonation. The resulting sulfuric acid ester is highly reactive and undergoes rapid hydrolysis. The hydrolysis proceeds via an achiral carbocation and therefore results in the formation of racemic 1-hydroxyethylpyrene. When an enantioselective sulfotransferase is used, such as human hydroxysteroid sulfotransferase (which selectively metabolises the *R*-enantiomer) or human estrogen sulfotransferase (which selectively metabolises the *S*-enantiomer), several passes through this cycle may lead to the depletion of one enantiomer and the accumulation of the other enantiomer.

determined in sulfotransferase-proficient *S. typhimurium* strains under incubation conditions optimised for the mutagenicity assay rather than for studying enzyme kinetics. If the high enantioselectivity of sulfotransferases indicated by the results of the mutagenicity assay is confirmed in these biochemical assays, then it may also be possible to produce a highly enriched preparation of one enantiomer by incubating the other enantiomer (or racemic 1-HEP) for an extended period with the appropriate sulfotransferase, as illustrated in Fig. 1. In the present study we have tested these hypotheses. To do this, it was necessary to devise methods for separating the enantiomers of 1-HEP in preparative and analytical scales.

MATERIALS AND METHODS

Chemicals. [^{3}H]-17β-Estradiol and [^{3}H]-dehydroepiandrosterone were purchased from NEN Life Science Products (Köln, Germany). PAPS was purchased from Dr. S. Singer (University of Dayton, Dayton, Ohio, USA). Solvents were of HPLC grade. All other chemicals were of analytical grade.

Synthesis and characterisation of 1-HEP enantiomers. Racemic 1-HEP was synthesised as described [8] and the identity of the product was confirmed by 400 MHz ^{1}H-NMR. The racemate was separated by preparative HPLC at 40 °C on a 500 × 20 mm column filled with cellulose tris(4-methylbenzoate) coated on 20 μm silicagel particles (Chiralcel OJ, Mallinckrodt-Baker, Gross-Gerau, Germany), using 15 ml/min *n*-heptane/ethanol (85:15, v/v) as eluent. The purity of (+)-*R*- and (−)-*S*-1-HEP was > 99% and their enantiomeric excess (ee) was > 99.0 and > 99.7 %, respectively. The optical rotation was $[\alpha]_D^{20}$ = +71.1° (c = 7.45 mg/ml in methanol) and −76.6° (c = 8.63mg/ml in methanol). The absolute configurations were assigned by an empirical ^{1}H-NMR-based method taking into account the differences seen in the splitting of the signals for the diastereotopic methylene protons of the (−)-menthoxyacetic acid esters [9 and references cited therein] and by comparison with the spectra of the (−)-menthoxyacetic acid esters of the known *R*-(+)- and *S*-(−)-1-phenylethanol enantiomers. The *S*-configuration was assigned to the (−)-1-HEP based on the much higher magnetic equivalence seen for the diastereotopic methylene protons in the NMR spectrum of the corresponding (−)-menthoxyacetic acid ester, whereas the *R*-configuration was assigned to the (+)-1-HEP. The (−)-menthoxyacetic acid ester of the latter showed in the NMR spectrum a well separated AB-system which was indicative for an absolute *R*-configuration.

Enzyme preparations. S. typhimurium TA1538-derived strains, engineered for the expression of hHST [10] and hEST [6], were grown overnight in Nutrient Broth No. 2 (Oxoid GmbH, Wesel, Germany) with ampicillin (100 μg/ml). The parental strain, TA1538, was grown in the absence of ampicillin. The overnight cultures were centrifuged for 10 min at 3000 *g*, washed twice with KCP buffer (10 mM sodium phosphate buffer, pH 7.4, in 150 mM KCl), and resuspended in a small volume of KCP buffer. The suspension was homogenised by ultrasonication. The homogenate was centrifuged for 60 min at 100,000 *g*. The supernatant was dialysed three times against KCP buffer supplemented with $MgCl_2$ and Na_2SO_4 (5 mM each). The protein content of the cytosol preparations was 7 to 10 mg/ml.

Assay of sulfotransferase activity using standard substrates. The assays were conducted as described [11] with the following specifications. The total volume amounted to 250 μl. ^{3}H-Labelled 17β-estradiol and dehydroepiandrosterone, diluted with the appropriate amount of unlabelled steroid, were dissolved in a mixture of ethanol/propylene glycol (1/99, v/v) (10 μl per incubation). The radioactivity used per incubation amounted to 0.1 μCi, the final concentrations of the substrates were in the optimal range for the corresponding enzyme, 20 nM 17β-estradiol and 30 μM dehydroepiandrosterone for hEST, 20 μM 17β-estradiol and 3 μM dehydroepiandrosterone for hHST [1]. The final concentration of PAPS was 20 μM. The protein amounts used per incubation were 1 μg and 9 μg with the cytosol

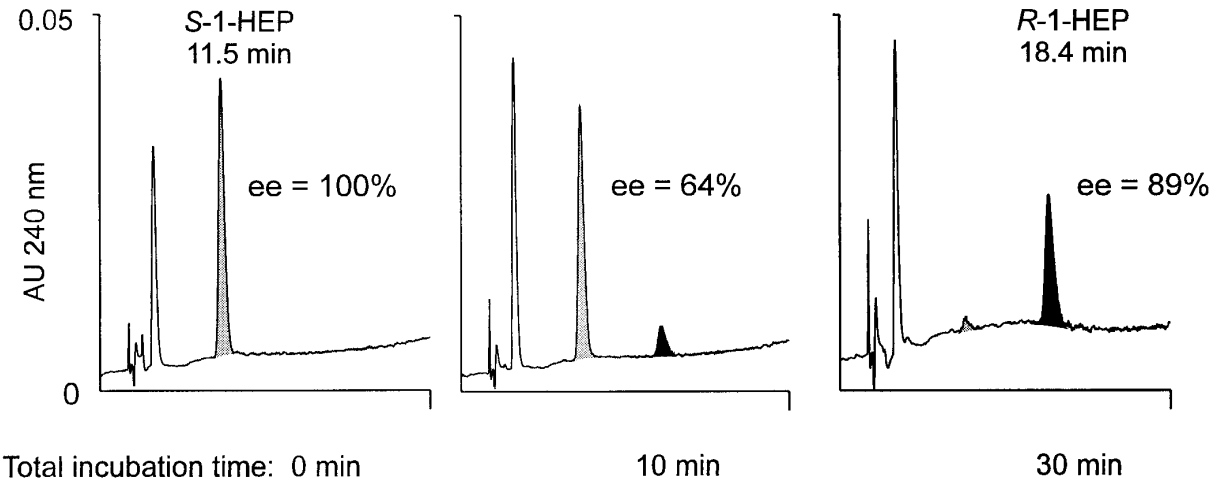

FIG. 2. Chiral inversion of *S*-1-hydroxyethylpyrene catalysed by human estrogen sulfotransferase. *S*-1-Hydroxyethylpyrene was incubated with cytosolic fraction from *S. typhimurium* strain TA1538-hEST, engineered for expression of human estrogen sulfotransferase, and cofactor (3′-phosphoadenosine-5′-phosphosulfate), for 10 min. Products were isolated, analysed, and added to a fresh incubation mixture. A total of 3 sequential 10-min incubations were carried out. The figure shows the elution profiles of the products isolated after 0, 10, and 30 min.

preparation from strains TA1538-hEST and TA1538-hHST, respectively. All incubations were adjusted to a total protein content of 10 μg by adding cytosol preparation from the control strain TA1538.

Assay of chiral inversion of 1-HEP enantiomers. For the determination of the sulfotransferase activity, the incubation mixture comprised bacterial cytosol preparation (1.25 mg/ml protein), ATP (1 mM), $MgCl_2$ (5 mM), Na_2SO_4 (5 mM) and PAPS (50 μM) in potassium phosphate buffer (50 mM, pH 7.4). The incubation volume was 4 ml. After warming for 2 min at 37 °C, the 1-HEP enantiomer (50 μM, final concentration), dissolved in dimethylsulfoxide (final concentration 0.5%), was added. The reaction was stopped after 10 min by adding 1 ml of acetone (which inhibits the sulfotransferase activity completely). The incubation was continued at 37 °C for 5 min, in order to hydrolyse the sulfuric acid ester of 1-HEP. The enantiomers of 1-HEP were analysed as described below. The sulfation rate was calculated by multiplying the figure for the initial rate of formation of the optical antipode with the factor 2.

In other incubations, it was attempted to convert an extensive proportion of the substrate into its antipode, rather than to determine the initial rate of inversion. In these experiments, the substrate concentration for the 1-HEP enantiomer was reduced to 20 μM. The products were isolated after incubation for 10 min, analysed and added to a fresh incubation mixture. A total of 3 sequential incubations were performed. This procedure was used in order to follow the time course of chiral inversion and to compensate for the inactivation of the enzyme during extended incubation with 1-HEP.

Analysis of the enantiomers. 1-HEP was extracted from the incubation mixture by liquid-liquid extraction with 25 ml *n*-heptane kept over diatomaceous earth. 25 μl dimethylsulfoxide was added and the *n*-heptane was evaporated *in vacuo*. The residue was dissolved in 1000 μl *n*-heptane/propanol (1/1, v/v). An aliquot of 20 μl of this solution was used for the analysis of the enantiomeric composition by chiral-stationary phase HPLC. *R*- and *S*-1-HEP were separated on a Chiralcel OJ (10 μm particles) column (250 × 4.6 mm) using a mixture of *n*-heptane and ethanol as eluent (initially 75/25, v/v, isocratic for 5 min, then a linear gradient to 40/60 for 25 min). The elution rate was 1 ml/min, the column temperature was 15 °C. The eluted analytes were detected by their u.v. absorption (240 nm) and fluorescence (λ_{ex} 273 nm, λ_{em} 385 nm). The identity of the isolated 1-HEP enantiomers was further confirmed by their circular dichroism spectra, recorded between 210 and 400 nm.

RESULTS

1-HEP enantiomers are stable in aqueous solution at pH 7.4. Even after incubation of *S*-1-HEP and *R*-1-HEP in 100 mM potassium phosphate buffer, at pH 7.4, for 1-week, we could not detect the formation of the other enantiomer or any other products. Taking into account the detection limit, the first-order rate constant for the racemisation was below $2*10^{-6}$ s^{-1}. However, hydronium ions effectively catalysed the racemisation. For example, in 100 mM phosphoric acid (pH < 2), the pseudo-first-order rate constant of racemisation was approximately $2*10^{-3}$ s^{-1}. Under either conditions, formation of other products, such as 1-vinylpyrene or 1-acetylpyrene (which were available as reference compounds), was not detectable, indicating that neither β-elimination nor oxidation of the benzylic alcohol occurred under these conditions.

When the individual 1-HEP enantiomers were incubated in the presence of sulfotransferase and PAPS, the formation of the other enantiomer was detected by chiral-stationary phase HPLC (Fig. 2). The reaction rate was high when *S*-1-HEP was incubated with hEST and when *R*-1-HEP was incubated with hHST. It was low or even below the detection limit when the enantiomers were incubated with the enzyme preparations in the reverse permutation. The findings suggest that 1-HEP was sulfated by the appropriate sulfotransferase, and the resulting sulfuric acid ester was hydrolysed. Since the hydrolysis proceeds via an S_N1 mechanism [5], it involves a racemisation (reaction scheme in Fig. 1). This additional information allows the calculation of the rate of sulfation from the initial rates of the formation of the optical antipode (Table 1).

With hEST, the deduced rate of sulfation of *S*-1-HEP was even higher than that of β-estradiol, which normally is used as reference substrate [1]. The K_M value, which is low for β-estradiol, was not determined for *S*-1-HEP. Sulfation of β-estradiol was studied using the optimal, very low, substrate concentration, whereas with the 1-HEP enantiomers a single, not optimised, relatively high substrate concentration was used. In contrast to *S*-1-HEP, its *R*-enantiomer was not detectably metabolised by hEST. The detection limit was about 40 times below the rate observed with the *S*-enantiomer. However, this enantiomer was effectively metabolised by the other human sulfotransferase studied. Using hHST, the rate of sulfation of *R*-1-HEP was about a quarter of that of the sulfation of its standard substrate, dehydroepiandrosterone, and about 11 times higher than that of its enantiomer, *S*-1-HEP (Table 1). hHST showed also substantial sulfation activity towards β-estradiol, which however had to be used at a much higher substrate concentration than with the actual estrogen sulfotransferase, hEST.

These experiments had demonstrated high but opposite substrate selectivity of hHST and hEST for one enantiomer of 1-HEP. In further experiments, the sulfotransferase and its preferred enantiomer were incubated for an extended period. Under these conditions, a major portion of the enantiomer could be converted to its antipode. The results of an experiment in which *S*-1-HEP was incubated with hEST are presented in Fig. 2. After incubation for 30 min, 94.5 % of the 1-HEP (or ee = 0.89) showed the *R*-configuration. Likewise, the analogous incubation of *S*-1-HEP with hHST produced alcohol which comprised 65 % of the *R*-enantiomer (ee = 0.30).

TABLE 1

Sulfation of 1-Hydroxyethylpyrene Enantiomers and Endogenous Substrates by Human Estrogen and Hydroxysteroid Sulfotransferase[a]

Substrate	Rate of sulfation (pmol/min/mg protein)	
	hHST	hEST
β-Estradiol	240 ± 40	560 ± 140
Dehydroepiandrosterone	0 ± 5	1200 ± 90
R-1-Hydroxyethylpyrene	0 ± 10	290 ± 50
S-1-Hydroxyethylpyrene	410 ± 40	27 ± 12

[a] Cytosol preparations from *S. typhimurium* strains TA1538-hEST and TA1538-hHST were used as the enzyme source. The rate of sulfation of the enantiomers of 1-hydroxyethylpyrene was deduced from the initial rate of chiral inversion, whose value was multiplied by a factor of 2. Values are mean and SE of 4 incubations.

DISCUSSION

Change of the configuration at the benzylic carbon was used to determine the enzyme activities of hHST and hEST towards the 1-HEP enantiomers without the need to analyse low concentrations of short-lived sulfuric acid esters. hHST showed a strong selectivity for the *R*-enantiomer (preference by a factor of 11), whereas hEST preferred its optical antipode (preference by a factor of $\geq$40). This result agree with similar enantioselectivities previously observed in mutagenicity experiments (15-fold higher mutagenic activity of the *R*-enantiomer than of the *S*-enantiomer to strain TA1538-hHST, 150-fold higher mutagenic activity of the *S*-enantiomer than of the *R*-enantiomer to strain TA1538-hEST). These congruent results, using very different experimental conditions, corroborate their validity. Furthermore, the high turnover rates of the 1-HEP enantiomers by sulfotransferases, similar to those for the major endogenous substrates, suggest that the sulfation pathway may be important also *in vivo*.

Various sulfotransferase forms are expressed with high tissue and cell specificity [1,12,13]. For example, both hEST and hHST are expressed in the liver. However, in the endometrium only hEST, but not hHST, is found [14]. Likewise, high levels of hHST are expressed in the adrenal gland [15]. Thus, *R*- and *S*-1-HEP may be metabolically activated to reactive sulfuric acid esters in different tissues, and these might be the primary target tissues for their toxicological effects. However, the situation is complicated by the occurrence of a rapid enzyme-mediated chiral inversion of 1-HEP. Although the inversion of the configuration of 1-HEP enantiomers has been studied until now only under *in vitro* conditions, chiral inversion of a structurally related compound, the allylic alcohol stiripentol, has been observed in the rat [16]. The inversion was unidirectional from the *R*- to the *S*-enantiomer. Pentachlorophenol, an inhibitor of some phenol sulfotransferases, substantially inhibited the inversion. The results of the present study with 1-HEP demonstrate directly that sulfotransferases can act as invertases, supporting the notion that they may be involved in the inversion of stiripentol.

In conclusion, metabolic sulfation does not always lead to the formation of products which can be readily excreted. In some cases, the conjugates are reactive and form adducts with cellular macromolecules and then lead to mutagenic, carcinogenic and cytotoxic effects. If water is a major reactant for the sulfuric acid ester formed, sulfotransferases may also act as invertases for hydroxyl groups at chiral carbon atoms. Even if the alcohol formed by hydrolysis is racemic, multiple cycles of selective sulfation of one enantiomer may eventually lead to a strong excess of the other enantiomer.

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