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STUDIES ON THE INTERACTIONS OF CHIRAL SECONDARY ALCOHOLS WITH RAT HYDROXYSTEROID SULFOTRANSFERASE STa

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ABSTRACT:

Hydroxysteroid (alcohol) sulfotransferase STa catalyzes the 3′-phosphoadenosine 5′-phosphosulfate-dependent *O*-sulfonation of a diverse array of alcohols including neutral hydroxysteroids. Many of the secondary alcohols that interact with this sulfotransferase are the metabolic products of stereoselective oxidation or reduction reactions. The role that the stereochemistry of secondary alcohol substrates plays in the catalytic efficiency of STa was investigated with a series of chiral benzylic alcohols and the enantiomeric 3-hydroxyl-containing steroids, androsterone and epiandrosterone. In the case of (*R*)-(+)- and (*S*)-(−)-enantiomers of 2-methyl-1-phenyl-1-propanol and 1-phenyl-1-butanol, the effect of stereochemistry on the catalytic efficiency of STa was small (less than 2-fold in favor of (*R*)-(+)-enantiomers). However, as the number of carbons in the α-alkyl chain increased, the stereoselectivity for the sulfation of enantiomers increased as well. The (*R*)-(+)-enantiomers of 1-phenyl-1-pentanol, 1-phenyl-1-hexanol, and 1-phenyl-1-heptanol were preferred as substrates over the (*S*)-(−)-enantiomers with a 3-fold difference in catalytic efficiency. STa showed absolute stereospecificity in the sulfation of the enantiomers of 1-phenyl-1-cyclohexylmethanol; (*R*)-(+)-1-phenyl-1-cyclohexylmethanol was a substrate for STa, while the (*S*)-(−)-enantiomer was a competitive inhibitor of the enzyme. Although a lower degree of stereoselectivity was observed with the 3-hydroxyl-containing steroids, androsterone and epiandrosterone, results with these substrates were also consistent with the conclusion that the stereochemistry of secondary alcohols is an important factor in the catalytic efficiency of STa.

Conjugation to form sulfuric acid esters is an important reaction in the metabolism of many drugs and other xenobiotics, neurotransmitters, and hormones (1–4). These biotransformations are catalyzed by sulfotransferases in reactions that involve the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate (PAPS)[1] to acceptor substrates, thereby producing products that are usually more water soluble and often less toxic. Hydroxysteroid (alcohol) sulfotransferases catalyze the sulfation of many steroids (1, 5, 6) as well as various other alcohols (7, 8). Benzylic alcohols are among the many diverse substrates for mammalian sulfotransferases. These alcohols are often found as intermediary metabolites obtained from cytochrome P-450 monooxygenase-catalyzed reactions involving the stereoselective oxidation of benzylic carbon atoms (9). Benzylic alcohols can also be obtained through metabolic reduction of benzylic carbonyls (10–12) and through the cytochrome P450- and epoxide hydrolase-mediated metabolism of polycylic aromatic hydrocarbons (13), reactions that are also stereoselective.

Although sulfation of alcohols often leads to less toxic and more readily excreted metabolites, some sulfo-oxy metabolites of benzylic and allylic alcohols are sufficiently electrophilic that they can covalently bind to cellular macromolecules (14). Some examples of these electrophilic metabolites include the sulfuric acid esters derived from 5-hydroxymethyl-chrysene (15), safrole (16), estragole (16), 7,12-dihydroxymethylbenz[*a*]anthracene (17), and 3,4-dihydroxy-3,4-dihydrocyclopenta [cd]pyrene (18). Because the metabolic formation of benzylic alcohols is usually stereoselective, the stereochemical aspects of the subsequent sulfation of these intermediates may have important implications for the carcinogenicity of these polycyclic aromatic hydrocarbons. Furthermore, a recent report on the DNA-binding properties of the antiestrogen antitumor agent, tamoxifen, also indicates that stereochemical considerations may be important in the formation of α-hydroxytamoxifen, an allylic secondary alcohol, and its subsequent sulfation to an electrophilic sulfuric acid ester capable of forming DNA adducts (19).

As implied by the name, hydroxysteroids also represent a class of chiral secondary alcohols that serve as substrates for hydroxysteroid (alcohol) sulfotransferases. Strott and co-workers have reported on the isolation and cloning of two distinct sulfotransferases from the guinea pig adrenal (20, 21) that catalyzed the stereospecific sulfation of 3α- and 3β-hydroxysteroids. These enzymes were the first characterized sulfotransferases that demonstrated substrate specificity based on the stereochemistry of a 3-hydroxyl group on a hydroxysteroid.

We have previously observed that one of the aryl (phenol) sulfotransferases, AST IV, displayed stereoselectivity, and in some cases absolute stereospecificity, in catalyzing the sulfation of benzylic alcohol substrates (22). Moreover, this stereoselectivity increased with the size of the alkyl substituent on the benzylic carbon. Phenol sulfotransferases have also been shown to stereoselectively catalyze the *O*-sulfonation of the phenolic groups in 4-hydroxypropranolol and terbutaline, molecules where the chiral center is remote from the site of sulfation (23). Although aryl sulfotransferases have shown a pronounced stereoselectivity for some benzylic alcohols, these enzymes

[1] Abbreviations used are: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, adenosine 3′,5′-diphosphate; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetic acid; HPLC, high performance liquid chromatography.

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have displayed limitations in their ability to use large polycyclic aromatic hydrocarbons as substrates (24).

In contrast to the observations with aryl sulfotransferases, several carcinogenic polycyclic aromatic hydrocarbons that bear benzylic alcohol functional groups are good substrates for both rat and human hepatic hydroxysteroid (alcohol) sulfotransferases (25, 26). The major isoform of the enzyme in rat liver was specified as sulfotransferase a (STa) (26), and it has also been designated as rHSST2 (2) and rHSTa (27). Our previous structure-activity studies on STa have shown that the catalytic efficiency of the enzyme increases with the hydrophobicity of the benzylic alcohol substrate (8). Steric factors may also influence the specificity of STa because quantitative studies on a series of seven-carbon alcohols as substrates for STa revealed that the catalytic efficiencies with primary alcohols were from 3-fold to 8-fold higher than those with secondary alcohols, and catalytic efficiencies with tertiary alcohols were approximately 10-fold lower than those observed for secondary alcohols (8).

In addition to hydrophobic and steric factors, the stereochemistry of chiral alcohols is a property that might influence the catalytic efficiency of STa. Indeed, the catalytic efficiency of STa with (*R*)-(−)-2-heptanol was 35% higher than with the (*S*)-(+)-enantiomer of 2-heptanol, and this relatively small difference was the first indication of stereoselectivity in the sulfation of alcohols catalyzed by this enzyme (8). We now report on a more extensive investigation of the stereoselectivity of STa.

Materials and Methods

Thin layer chromatography (TLC) was performed on 250 μm-thick precoated silica gel GF plates (Analtech, Newark, DE). Purification by flash chromatography was accomplished with silica gel (200–400 mesh, 60 Å) from Aldrich Chemical Co. (Milwaukee, WI). Androsterone (5α-androstan-3α-ol-17-one) and epiandrosterone (5α-androstan-3β-ol-17-one) were purchased from Sigma Chemical Co. (St. Louis, MO). (*R*)-(+)-1-phenyl-2-methyl-1-propanol, (*S*)-(−)-1-phenyl-2-methyl-1-propanol, (*R*)-(+)-1-phenyl-1-butanol, (*S*)-(−)-1-phenyl-1-butanol, (+)-*B*-chlorodiisopinocampheylborane [(+)-DIP-chloride], (−)-*B*-chlorodiisopinocampheylborane [(−)-DIP-chloride], and diethanolamine were obtained from Aldrich. Optical rotations of all benzylic alcohols were determined at 589 nm and at 25°C using a Perkin-Elmer model 141 polarimeter. The specific rotations of the commercially obtained benzylic alcohols were as follows: (*R*)-(+)-2-methyl-1-phenyl-1-propanol, +23° (*c* 0.02, C_2H_5OH); (*S*)-(−)-2-methyl-1-phenyl-1-propanol, −25° (*c* 0.02, C_2H_5OH); (*R*)-(+)-1-phenyl-1-butanol, +50.6° (*c* 0.02, $CHCl_3$); (*S*)-(−)-1-phenyl-1-butanol, −49.2° (*c* 0.02, $CHCl_3$). PAPS was prepared according to a published procedure (28), and adenosine 3′,5′-diphosphate (PAP) was obtained from Sigma. Tetrahydrofuran was freshly distilled from sodium metal using benzophenone ketyl as indicator. All other chemicals, assay reagents, and buffer components were obtained from commercial sources.

Synthesis of Racemic Benzylic Alcohols. Racemates of *sec* benzylic alcohols were synthesized by reduction of the appropriate alkyl phenyl ketones using the general procedure described by Nystrom and Brown (29). To a solution of lithium aluminum hydride (10 ml of a 1.0 M solution in diethyl ether) was added 1 g of the appropriate alkyl phenyl ketone in 5 ml of diethyl ether at a rate that produced a gentle reflux. The reaction mixture was refluxed for 1.5 hr. After cooling the reaction mixture to 0°C, water was added to decompose the excess lithium aluminum hydride. Following addition of 20 ml of 10% (v/v) sulfuric acid, the solution was extracted with diethyl ether (4 × 50 ml). The combined organic layers were evaporated to yield the crude benzylic alcohol, which was subsequently purified by flash column chromatography with hexane/ethyl acetate (8:2) as the mobile phase. Yields of 70–75% were obtained for each of the four racemic benzylic alcohols prepared by this procedure.

Synthesis of Chiral Benzylic Alcohols. (*R*)-(+)-1-Phenyl-1-pentanol, (*S*)-(−)-1-phenyl-1-pentanol, (*R*)-(+)-1-phenyl-1-hexanol, (*S*)-(−)-1-phenyl-1-hexanol, (*R*)-(+)-1-phenyl-1-heptanol, (*S*)-(−)-1-phenyl-1-heptanol, (*R*)-(+)-1-phenyl-1-cyclohexylmethanol, and (*S*)-(−)-1-phenyl-1-cyclohexylmethanol were synthesized by asymmetric reduction of the corresponding alkyl phenyl ketones according to a previously published general procedure (30). All glassware was oven-dried overnight before use. All operations were carried out under an argon atmosphere. The appropriate alkyl phenyl ketone (11.3 mmol) was added to a solution containing 3.62 g (11.3 mmol) of either (+)- or (−)-DIP-chloride in tetrahydrofuran (10 ml) at −25°C. A yellow color developed immediately, and the reaction was allowed to proceed at −25°C for 11 hr. The volatiles were removed on a rotary evaporator, and the α-pinene was removed by placing the reaction product under reduced pressure (0.1 mmHg) overnight. The residue was dissolved in diethyl ether (75 ml), and diethanolamine (2.2 equiv.) was added. The separated solid was filtered off after 2 hr and washed twice with *n*-pentane (30 ml). The combined diethylether and *n*-pentane filtrates were concentrated. The product chiral alcohol was purified by flash column chromatography with hexane/ethylacetate (9:1) as the mobile phase. Yields of 60–75% were obtained for each of the eight chiral benzylic alcohols prepared by this procedure.

Chemical structures of the product chiral benzylic alcohols were verified by proton NMR spectroscopy (Bruker WM-360), IR spectroscopy (Nicolet Model 205), and mass spectrometry (Hewlett-Packard 5989 Quadrupole GC/MS). Specific rotations were determined as described above.

Preparation of α-Methoxy-α-(trifluoromethyl)phenylacetic Acid (MTPA) Esters of Chiral Secondary Benzylic Alcohols. MTPA esters of product benzylic alcohols were prepared by mixing the appropriate alcohol (1 mmol), (*S*)-(−)-MTPA (1 mmol), dicyclohexylcarbodiimide (1 mmol), and 4-dimethylaminopyridine (0.1 mmol) in methylene chloride (10 ml) and stirring overnight at room temperature. The dicyclohexylurea that precipitated was removed by filtration. The filtrate was washed successively with 50-ml portions of 0.5 N HCl, 2 N Na_2CO_3, and brine. The crude product obtained after evaporation of the organic layer was purified by flash column chromatography with hexane/ethyl acetate (8:2) as the mobile phase. The NMR spectrum of the MTPA esters derived from each of the racemic benzylic alcohols displayed two sets of signals due to the methoxy and benzylic methine protons, whereas each enantiomerically pure benzylic alcohol showed only a single set of signals.

Synthesis of 1-Phenyl-1-pentanols. Chiral 1-phenyl-1-pentanols were prepared from valerophenone. IR (KBr) ν = 3370 cm^{-1} (O-H stretch), 3080–3010 cm^{-1} (C-H stretch, aromatic), 2950–2860 cm^{-1} (C-H stretch, aliphatic), 1800–1590 cm^{-1} (C=C, aromatic), 1453 cm^{-1} (O-H bending); ^{1}H-NMR ($CDCl_3$) δ 0.9 ppm (t, 3H, -CH_2CH_3), δ 1.2–1.4 ppm (m, 4H, -CH_2-CH_2-CH_3), δ 1.65–1.86 ppm [m, 2H, -CH(OH)-CH_2-], δ 1.85 ppm (s, 1H, O*H*), δ 4.69 ppm [t, 1H, -C*H*(OH)-], δ 7.2–7.38 ppm (m, 5H, C_6H_5-); GC-MS m/z 164 ($M^{+\cdot}$), 107 (M-C_4H_9), 91 ($C_7H_7^+$), 77 ($C_6H_5^+$), 51 (77-C_2H_2). For (*S*)-(−)-1-phenyl-1-pentanol: $[\alpha]^{25}_D$ = -19.2° (*c* 0.18, CH_3OH) [lit. −20°, neat, ee 100% (31)]. ee = 100% based on ^{1}H-NMR of MTPA ester. The MTPA ester of racemic 1-phenyl-1-pentanol displayed two sets of signals due to methoxy (s, δ 3.41 and 3.51 ppm) and benzylic methine protons (t, δ 5.84 and 5.91 ppm), whereas the MTPA ester of (*S*)-(−)-1-phenyl-1-pentanol showed only a single set of signals at δ 3.41 and 5.91 ppm. For (*R*)-(+)-1-phenyl-1-pentanol: $[\alpha]^{25}_D$ = +20.2° (*c* 0.19, CH_3OH) [lit. +20°, neat, ee 100% (32); +40.3°, *c* 1.0, $CHCl_3$, ee 94% (33)]. ee = 100% based on ^{1}H-NMR of MTPA ester. The MTPA ester of (*R*)-(+)-1-phenyl-1-pentanol showed only a single set of signals at δ 3.51 and 5.84 ppm.

Synthesis of 1-Phenyl-1-hexanols. Chiral 1-phenyl-1-hexanols were prepared from hexanophenone. IR (KBr) ν = 3378 cm^{-1} (O-H stretch), 3085–3020 cm^{-1} (C-H stretch, aromatic), 2951–2879 cm^{-1} (C-H stretch, aliphatic), 1950–1700 cm^{-1} (C=C, aromatic), 1454 cm^{-1} (O-H bending); ^{1}H-NMR ($CDCl_3$) δ 0.8 ppm (t, 3H, -CH_2CH_3), δ 1.22–1.49 ppm [m, 6H, -$(CH_2)_3$-CH_3], δ 1.58–1.88 ppm [m, 2H, -CH(OH)-CH_2-], δ 1.75 ppm (s, 1H, O*H*), δ 4.61 ppm [t, 1H, -C*H*(OH)-], δ 7.21–7.42 ppm (m, 5H, C_6H_5-); GC-MS m/z 178 ($M^{+\bullet}$), 107 (M-C_5H_{11}), 91 ($C_7H_7^+$), 77 ($C_6H_5^+$), 51 (77-C_2H_2). For (*S*)-(−)-1-phenyl-1-hexanol: $[\alpha]^{25}_D$ = -16.3° (*c* 0.23, CH_3OH). ee = 100% based on ^{1}H-NMR of MTPA ester. The MTPA ester of racemic 1-phenyl-1-hexanol displayed two sets of signals because of methoxy (s, δ 3.40 and 3.49 ppm) and benzylic methine protons (t, δ 5.84 and 5.91 ppm), whereas the MTPA ester of (*S*)-(−)-1-phenyl-1-hexanol showed only a single set of signals at δ 3.40 and 5.91 ppm. For (*R*)-(+)-1-phenyl-1-hexanol: $[\alpha]^{25}_D$ = +16° (*c* 0.23, CH_3OH) [lit. +34.6°, *c* 0.9, $CHCl_3$, ee 93% (33)]. ee = 100% based on ^{1}H-NMR of

MTPA ester. The MTPA ester of (R)-(+)-1-phenyl-1-hexanol showed only a single set of signals at δ 3.49 and 5.84 ppm.

Synthesis of 1-Phenyl-1-heptanol. Chiral 1-phenyl-1-heptanols were prepared from heptanophenone. IR (KBr) v = 3400 cm^{-1} (O-H stretch), 3085–3020 cm^{-1} (C-H stretch aromatic), 2950–2870 cm^{-1} (C-H stretch, aliphatic), 1900–1700 cm^{-1} (C=C, aromatic), 1450 cm^{-1} (O-H bending); ^{1}H-NMR ($CDCl_3$) δ 0.8 ppm (t, 3H, -CH_2CH_3), δ 1.22–1.49 ppm [m, 8H, -$(CH_2)_4$-CH_3], δ 1.58–1.88 ppm [m, 2H, -CH(OH)-CH_2-], δ 1.7 ppm (s, 1H, O*H*), δ 4.61 ppm [t, 1H, -C*H*(OH)-], δ 7.21–7.42 ppm (m, 5H, C_6H_5-); GC-MS m/z 192 ($M^{+\bullet}$), 107 (M-C_6H_{13}), 91 ($C_7H_7^+$), 77 ($C_6H_5^+$), 51 (77-C_2H_2). For (S)-(−)-1-phenyl-1-heptanol: $[\alpha]^{25}_D$ = -12° (c 0.058, CH_3OH) [lit. -20.5, c 0.88, C_2H_5OH, ee 99.5% (34)]. ee = 100% based on ^{1}H-NMR of MTPA ester. The MTPA ester of racemic 1-phenyl-1-heptanol displayed two sets of signals because of methoxy (s, δ 3.41 and 3.5 ppm) and benzylic methine protons (t, δ 5.84 and 5.91 ppm), whereas the MTPA ester of (S)-(−)-1-phenyl-1-heptanol showed only a single set of signals at δ 3.41 and 5.91 ppm. For (R)-(+)-1-phenyl-1-heptanol: $[\alpha]^{25}_D$ = +12.1° (c 0.05, CH_3OH) [lit. +19.7, c 1.7, C_2H_5OH, ee 99.5% (34)]. ee = 100% based on ^{1}H-NMR of MTPA ester. The MTPA ester of (R)-(+)-1-phenyl-1-heptanol showed only a single set of signals at δ 3.5 and 5.84 ppm.

Synthesis of 1-Phenyl-1-cyclohexylmethanol. The chiral 1-phenyl-1-cyclohexylmethanols were prepared from cyclohexylphenyl ketone. IR (KBr) v = 3409 cm^{-1} (O-H stretch), 3100–3030 cm^{-1} (C-H stretch, aromatic), 2927–2850 cm^{-1} (C-H stretch, aliphatic), 1850–1600 cm^{-1} (C=C, aromatic), 1450 cm^{-1} (O-H bending); ^{1}H-NMR ($CDCl_3$) δ 0.8–1.92 ppm [m, 12H, cyclohexyl and -CH(O*H*)-], δ 4.31 ppm [d, 1H, -C*H*(OH)-], δ 7.21–7.39 ppm (m, 5H, C_6H_5-); GC-MS m/z 190 ($M^{+\bullet}$), 107 (M-C_6H_{11}), 91 ($C_7H_7^+$), 77 ($C_6H_5^+$), 51 (77-C_2H_2). For (S)-(−)-1-phenyl-1-cyclohexylmethanol: $[\alpha]^{25}_D$ = -18.8° (c 0.1, CH_3OH) [lit. -18.5°, c 10.3, ether (35)]. ee= 100% based on ^{1}H-NMR of MTPA ester. The MTPA ester of racemic 1-phenyl-1-cyclohexylmethanol displayed two sets of signals due to methoxy (s, δ 3.41 and 3.49 ppm) and benzylic methine protons (t, δ 5.56 and 5.65 ppm), whereas the MTPA ester of (S)-(−)-1-phenyl-1-cyclohexylmethanol showed only a single set of signals at δ 3.41 and 5.65 ppm. For (R)-(+)- 1-phenyl-1-cyclohexylmethanol: $[\alpha]^{25}_D$ = +19.2° (c 0.07, CH_3OH). ee= 96% based on ^{1}H-NMR of MTPA ester. The MTPA ester of (R)-(+)-1-phenyl-1-cyclohexylmethanol showed a major single set of signals at δ 3.49 and 5.56 ppm.

Purification of Hydroxysteroid (Alcohol) Sulfotransferase STa. STa was purified to apparent homogeneity from female Sprague Dawley rats (9–10 weeks of age), using a modification (8) of previously published procedures (26, 36). The enzyme was homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie Blue staining. Protein concentrations were determined using a modified Lowry procedure (37), with bovine serum albumin as standard.

Assay of Hydroxysteroid Sulfotransferase STa with Benzylic Alcohols. The various benzylic alcohols were evaluated as both substrates and inhibitors of purified STa using a published HPLC procedure for determination of the concentration of PAP formed in the reaction (38). Reaction mixtures of 0.03 ml total volume contained 0.25 M potassium phosphate buffer at pH 7.0, 8.3 mM 2-mercaptoethanol, 0.3 mM PAPS, and various concentrations of the alcohols in acetonitrile (final concentration of acetonitrile in the assay was no more than 5% v/v). Reactions were initiated by the addition of 1.0 μg of enzyme, incubated at 37°C for 10 min., and terminated by addition of 0.03 ml of methanol. The substrate-dependent concentration of PAP formed in the reaction was determined by HPLC. Linear standard curves relating HPLC peak areas to concentrations of PAP were determined daily. At least six different concentrations of each alcohol were assayed, and these included concentrations both greater than and less than the apparent K_m values. The substrate concentrations used to determine the kinetic constants for the enantiomeric benzylic alcohols were as follows: 1-phenyl-2-methyl-1-propanols, 0.5–7.0 mM; 1-phenyl-1-butanols, 0.4–7.0 mM; 1-phenyl-1-pentanols, 0.25–4.0 mM; 1-phenyl-1-hexanols, 0.25–2.0 mM; 1-phenyl-1-heptanols, 0.15–2.0 mM; 1-phenyl-1-cyclohexylmethanol, 0.25–3.0 mM. Apparent K_m and V_{max} values are presented as ± the standard error obtained by non-linear least squares curve fitting (39) of the velocity data to the Michaelis-Menten equation. Values for k_{cat} were calculated using the relative molecular mass for a subunit of STa, 33,124, as determined from the deduced amino acid sequence (40).

Calculation of Hydrophobicity Constants for Chiral Benzylic Alcohols. Partition coefficients for chiral benzylic alcohols were calculated using the ACD/LogP computer program from Advanced Chemistry Development Inc. (Ontario, Canada).

Results

Role of the Configuration of a Benzylic Alcohol in the Catalytic Efficiency of STa. STa was previously reported to show a small degree of stereoselectivity (35%) for (R)-(−)-2-heptanol over (S)-(+)-2-heptanol (8). Based on this observation, we have now investigated the stereochemical preferences of this enzyme with a series of chiral benzylic alcohols. The catalytic efficiency of STa with each chiral benzylic alcohol was determined by examination of the k_{cat}/K_m values. As seen in table 1, the effect of stereochemistry at the benzylic carbon on the kinetic constants for sulfation catalyzed by STa was small for (S)-(−)- and (R)-(+)-2-methyl-1-phenyl-1-propanol and (S)-(−)- and (R)-(+)-1-phenyl-1-butanol (1.3-fold and 1.6-fold, respectively). However, as the carbon number on the n-alkyl side chain that was α to the benzylic hydroxyl increased, the stereoselectivity increased to 3-fold, as seen in table 1. In the case of 1-phenyl-1-cyclohexylmethanol, STa catalyzed stereospecific sulfation of the (R)-(+)-enantiomer; the (S)-(−)-enantiomer of 1-phenyl-1-cyclohexylmethanol was not a substrate for the enzyme. Further investigation revealed that the (S)-(−)-1-phenyl-1-cyclohexylmethanol was a competitive inhibitor of the STa-catalyzed sulfation of p-butylbenzyl alcohol (fig. 1).

Role of Hydrophobic Characteristics of Chiral Benzylic Alcohols in the Catalytic Efficiency of STa. In addition to the orientation of the benzylic hydroxyl with respect to the phenyl ring and a alkyl substituent on the benzylic carbon, the hydrophobicity of these benzylic alcohols contributed to their ability to serve as substrates for the sulfotransferase STa. Calculated values of log p for each of the benzylic alcohols are seen in table 1. Comparison of these log p values with the catalytic efficiency of STa for these substrates (k_{cat}/K_m) revealed that k_{cat}/K_m values generally increased in relation to increasing log p values. These data were consistent with a previous study on the effect of hydrophobicity of primary benzylic alcohols on their ability to serve as substrates for STa (8). The small decrease in the k_{cat}/K_m values for 1-phenyl-1-heptanol when compared with the k_{cat}/K_m values for 1-phenyl-1-hexanol was not in good agreement with its high lipophilic character. However, it suggested that the ability of chiral benzylic alcohols to serve as substrates for STa depended on both the overall hydrophobicity of the molecules and the differences between the steric interactions of each enantiomer with the active site of the enzyme.

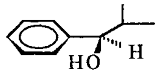
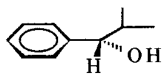
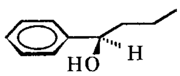
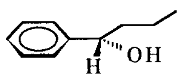
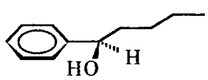
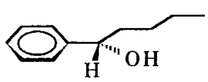
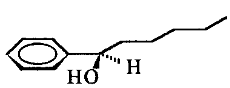
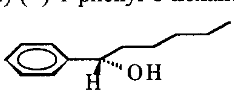
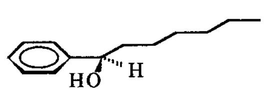
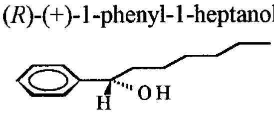
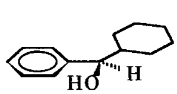
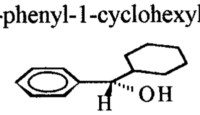
Androsterone and Epiandrosterone. Steroids with 3α and 3β-hydroxy groups represent another class of chiral secondary alcohols that are substrates for STa. Thus, we investigated the STa-catalyzed sulfation of androsterone (3α-hydroxyl) and epiandrosterone (3β-hydroxyl) as model hydroxysteroid substrates. Although both epiandrosterone and androsterone showed substrate inhibition above 60 μM and 80 μM, respectively, a higher maximum velocity was demonstrated for epiandrosterone. This result indicated a small degree of stereoselectivity with epiandrosterone as the preferred substrate for STa (fig. 2).

Discussion

Hydroxysteroid (alcohol) sulfotransferase STa catalyzes the sulfation of various benzylic alcohols and steroids (1–4). We have shown previously that the hydrophobicity of a benzylic alcohol is a major determinant of its ability to act as a substrate for the enzyme (8). In the present study, we investigated both stereochemical and steric interac-

TABLE 1

Kinetic constants for STa-catalyzed sulfation of chiral benzylic alcohols

Benzylic Alcohol	K_m (app)	V_{max}	k_{cat}/K_m	(R)/(S) Ratio[a]	log P	K_i
 (*R*)-(+)-1-phenyl-2-methyl-1-propanol	2.8 ± 0.8	27.0 ± 3.4	0.33 ± 0.1	1.3	2.26	—
 (*S*)-(-)-1-phenyl-2-methyl-1-propanol	3.0 ± 0.6	22.8 ± 2.0	0.25 ± 0.05			—
 (*R*)-(+)-1-phenyl-1-butanol	3.3 ± 0.4	85.5 ± 6.0	0.9 ± 0.1	1.8	2.44	—
 (*S*)-(-)-1-phenyl-1-butanol	3.7 ± 0.3	58.8 ± 2.0	0.5 ± 0.05			—
 (*R*)-(+)-1-phenyl-1-pentanol	1.4 ± 0.2	154.4 ± 7.8	3.6 ± 0.4	3.0	2.98	—
 (*S*)-(-)-1-phenyl-1-pentanol	1.9 ± 0.3	70.3 ± 5.8	1.2 ± 0.2			—
 (*R*)-(+)-1-phenyl-1-hexanol	0.46 ± 0.03	157.3 ± 4.0	11.3 ± 0.9	4.1	3.5	—
 (*S*)-(-)-1-phenyl-1-hexanol	0.53 ± 0.06	59.2 ± 2.4	3.7 ± 0.4			—
 (*R*)-(+)-1-phenyl-1-heptanol	0.38 ± 0.03	94.3 ± 2.0	8.1 ± 0.6	2.8	4.04	—
 (*S*)-(-)-1-phenyl-1-heptanol	0.42 ± 0.04	37.3 ± 1.3	2.9 ± 0.3			—
 (*R*)-(+)-1-phenyl-1-cyclohexylmethanol	1.0 ± 0.3	17.8 ± 2.4	0.6 ± 0.2	—	3.44	—
 (*S*)-(-)-1-phenyl-1-cyclohexylmethanol	—	—	—			0.75 ± 0.1

Values for apparent K_m, V_{max}, k_{cat}/K_m, and K_i are expressed in mM, nmol min^{-1} (mg of STa)$^{-1}$, mM^{-1} min^{-1}, and mM, respectively. Values for log P were calculated as described under *Materials and Methods*.

[a] k_{cat}/K_m for the (*R*)-(+)-enantiomer divided by k_{cat}/K_m for the (*S*)-(−)-enantiomer.

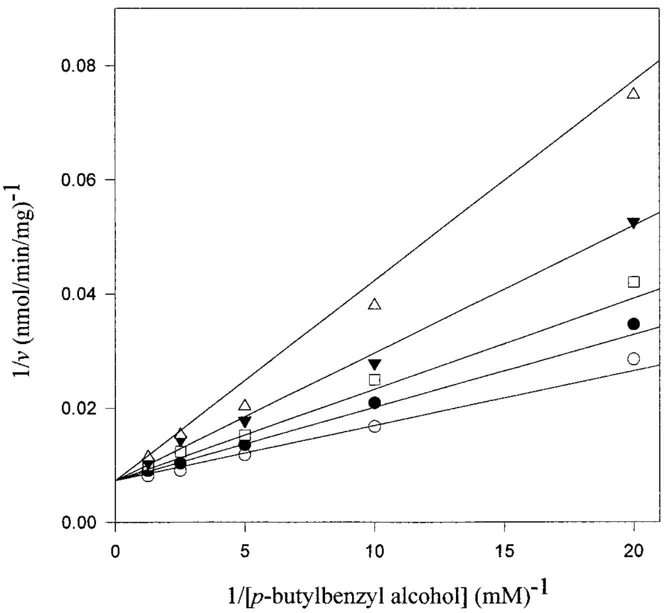


FIG. 1. *Inhibition of STa by (S)-(−)-1-phenyl-1-cyclohexylmethanol.*

Data points are observed values, with the *lines* representing the theoretical values for competitive inhibition of the sulfation of *p*-butylbenzyl alcohol as calculated by non-linear least squares analysis (39). Concentrations of the inhibitor are represented as follows: ○, no inhibitor; ●, 0.25 mM; □, 0.5 mM; ▲, 1 mM; △, 2 mM.

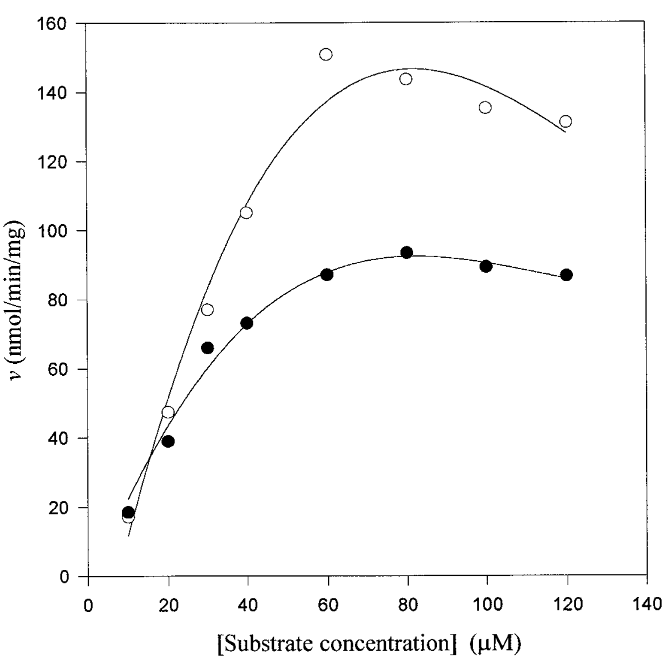


FIG. 2. *Initial velocities for the sulfation of epiandrosterone and androsterone catalyzed by STa.*

Assays were conducted as described under *Materials and Methods*. Data are represented as follows: ●, androsterone; ○, epiandrosterone. Each *point* represents the average of duplicate determinations.

tions at the active site of STa through the use of α-alkyl-substituted chiral benzylic alcohols as model substrates. Although the difference in the configuration of the benzylic carbon will not affect the overall hydrophobic character of the two enantiomers, steric interactions with the active site of the enzyme may be affected. Thus, the enantioselectivity of STa may be one indication of the steric constraints that define the binding of substrates and inhibitors at the active site of the enzyme.

The results of our studies indicated that as the length of the α-alkyl chain on a chiral benzylic alcohol was increased, the extent of stereoselectivity in the reaction catalyzed by STa also increased. Moreover, the enzyme showed absolute stereospecificity for the enantiomers of 1-phenyl-1-cyclohexylmethanol, wherein the α-alkyl substituent was the sterically bulky cyclohexyl group. The (*R*)-(+)-enantiomer of 1-phenyl-1-cyclohexylmethanol was a substrate for STa, while the (*S*)-(−)-enantiomer was a competitive inhibitor of STa-catalyzed sulfation of *p*-butylbenzyl alcohol. In addition, the apparent K_m value for (*R*)-(+)-1-phenyl-1-cyclohexylmethanol was higher than would be expected on the basis of a comparison with either (*R*)-(+)-1-phenyl-1-hexanol or (*R*)-(+)-1-phenyl-1-heptanol. The V_{max} value for (*R*)-(+)-1-phenyl-1-cyclohexylmethanol was also significantly different from that observed with (*R*)-(+)-1-phenyl-1-hexanol and (*R*)-(+)-1-phenyl-1-heptanol. Neither this 10-fold decrease in V_{max} nor the observed increase in the apparent K_m value was consistent with the hydrophobic characteristics of these molecules. Thus, the presence of an α-cyclohexyl substituent on the benzylic carbon caused steric interactions to be much more important in the reaction. Our results support the conclusion that a specific orientation of the benzylic hydroxyl on the substrate must be achieved at the active site of STa before catalysis occurs, and that steric factors may combine with the stereochemical configuration of the substrate or inhibitor to determine the catalytic efficiency of the enzyme. The stereoselectivity observed with benzylic alcohols as substrates for STa was also seen with the hydroxysteroids androsterone and epiandrosterone. The structure of androsterone differs from epiandrosterone only in the configuration of the 3-hydroxyl group, that is, an α-orientation for androsterone and a β-orientation for epiandrosterone. Although these hydroxysteroids exhibit a stereoselectivity that is similar to some of the enantiomeric benzylic alcohols, they show a substrate inhibition at higher concentrations that has not yet been observed with the α-substituted benzylic alcohols. This substrate inhibition resembles that previously seen with dehydroepiandrosterone as substrate for STa (8). In the case of α-alkyl-substituted benzylic alcohols, concentrations approaching the limit of solubility for each substrate did not yield any substrate inhibition.

These observations on substrate inhibition, as well as the relatively low degree of stereoselectivity for androsterone and epiandrosterone with rat hepatic STa, contrast with reports on two other 3-hydroxysteroid sulfotransferases that have been characterized in the guinea pig (20, 21). Although a significant sequence identity of approximately 65% was reported between the enzymes from guinea pig and the rat hydroxysteroid sulfotransferase (20, 21), it is apparent that the differences in protein sequence are sufficient to affect the kinetics and specificity of the enzymes from the two species. Although substrate inhibition was seen for 3-hydroxysteroid substrates of STa, no such interactions were reported for either of the 3-hydroxysteroid sulfotransferases from guinea pig (20, 21). Moreover, results obtained by cloning and expressing the guinea pig 3α-hydroxysteroid sulfotransferase in CHO-K1 cells indicated that this enzyme did not act on 3β-hydroxyl groups (21). Thus, there are distinct differences in specificity and kinetics between the hydroxysteroid sulfotransferases present in guinea pig and rat.

Additional documentation of species differences in the degree of stereoselectivity of hydroxysteroid sulfotransferases has been recently provided by Glatt *et al.*, who have reported that 1-(1-pyrenyl)ethanol was activated to a mutagen with higher enantioselectivity by *Salmo-*

nella strains that contained human hydroxysteroid sulfotransferase than those that contained rat STa (27). In both strains, the (*R*)-(+)-enantiomer was more mutagenic than the (*S*)-(−)-enantiomer, and this difference in mutagenicity was attributed to higher rates of sulfation (27). Thus, although there is substantial homology between the amino acid sequences (*i.e.* 76% overall similarity between human hydroxysteroid sulfotransferase and rat STa), there are differences in the magnitude of stereoselectivity displayed by the hydroxysteroid sulfotransferases from these two species. Such differences are likely to be based on relatively subtle differences in steric interactions between enzyme and substrate at the active site that give rise to selectivity for the binding of one enantiomer in the proper orientation for sulfuryl transfer. Our findings with (*S*)-(−)-1-phenyl-1-cyclohexylmethanol indicate that an enantiomer that is not properly oriented for sulfuryl transfer in the active site is nevertheless bound due to its hydrophobic characteristics and can thus serve as a competitive inhibitor. A more complete understanding of the reasons why interactions with specific amino acids at the active site would give rise to the observed stereospecificity will await futher elucidation of the structures of the active sites of these sulfotransferases.

In summary, our results clearly indicate that the stereochemistry of a chiral benzylic alcohol is an important factor in its interaction with STa. In molecules with a large bulky substituent on the chiral benzylic carbon, STa shows a marked preference for one enantiomer as substrate. Therefore, both the effect of hydrophobicity of the molecule and the steric effects that are related to the substituents on the chiral benzylic carbon atom are important determinants of the type and efficiency of interactions that occur between STa and chiral benzylic alcohols. Such relationships between chemical structure and activity as substrates and inhibitors for STa may prove useful in explaining and predicting the interactions of this sulfotransferase with other xenobiotics, as well as with molecules of endogenous origin.

References

1. C. A. Strott: Steroid sulfotransferases. *Endocr. Rev.* **17,** 670–697 (1996).
2. R. Weinshilboum and D. Otterness: Sulfotransferase Enzymes. In “Handbook of Experimental Pharmacology” (F. C. Kauffman, ed.), vol. 112, pp. 45–78, Springer-Verlag, Berlin, 1994.
3. G. J. Mulder and W. B. Jakoby: Sulfation. In “Conjugation Reactions in Drug Metabolism” (G. J. Mulder, ed.), pp. 107–161, Taylor and Francis, New York, 1990.
4. J. A. Miller and Y.-J. Surh: Sulfonation in chemical carcinogenesis. In “Handbook of Experimental Pharmacology” (F. C. Kauffman, ed.), vol. 112, pp. 429–458, Springer-Verlag, Berlin, 1994.
5. R. A. Ryan and J. Carroll: Studies on a 3β-hydroxysteroid sulfotransferase from rat liver. *Biochim. Biophys. Acta.* **429,** 391–401 (1976).
6. V. Luu-The, F. Bernier, and I. Dufort: Steroid sulfotransferases. *J. Endocrinol.* **150,** S87–S97 (1996).
7. E. S. Lyon, C. J. Marcus, J.-L. Wang, and W. B. Jakoby: Hydroxysteroid sulfotransferases. *Methods Enzymol.* **77,** 206–213 (1981).
8. G. Chen, E. Banoglu, and M. W. Duffel: Influence of substrate structure on the catalytic efficiency of hydroxysteroid sulfotransferase STa in the sulfation of alcohols. *Chem. Res. Toxicol.* **9,** 67–74 (1996).
9. W. F. Trager: Stereochemistry of cytochrome P-450 reactions. *Drug Metab. Rev.* **20,** 489–496 (1989).
10. B. Wermuth: Aldo-keto reductases. In “Enzymology of Carbonyl Metabolism 2” (T. G. Flynn and H. Weimer, eds.), pp. 209–230, Alan R. Liss, New York, 1985.
11. J. M. Y. Wong, W. A. Mahon, W. Kalow, and T. Inaba: Carbonyl (phenone) reductase in human liver: structure-activity relationship among substrates. *Drug Metab. Dispos.* **20,** 465–466 (1992).
12. O. A. Barski, K. H. Gabbay, C. E. Grimshaw, and K. M. Bohren: Mechanism of human aldehyde reductase: characterization of the active site pocket. *Biochemistry* **34,** 11264–11275 (1995).
13. S. K. Yang: Stereoselectivity of cytochrome P-450 isozymes and epoxide hydrolase in the metabolism of polycyclic aromatic hydrocarbons. *Biochem. Pharmacol.* **37,** 61–70 (1988).
14. J. A. Miller: Sulfonation in chemical carcinogenesis: history and present status. *Chem. Biol. Interact.* **92,** 329–341 (1994).
15. H. Okuda, N. Nojima, N. Watanabe, and T. Watabe: Sulphotransferase-mediated activation of the carcinogen 5-hydroxymethylchrysene: species and sex differences in tissue distribution of the enzyme activity and a possible participation of hydroxysteroid sulfotransferases. *Biochem. Pharmacol.* **38,** 3003–3009 (1989).
16. E. C. Miller, A. B. Swanson, D. H. Philips, T. L. Fletcher, A. Liem, and J. A. Miller: 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 (1983).
17. T. Watabe, A. Hiratsuka, and K. Ogura: Sulfotransferase mediated covalent binding of the carcinogen 7,12-dihydroxymethylbenz[α]anthracene to calf thymus DNA and its inhibition by glutathione transferase. *Carcinogenesis* **8,** 445–453 (1987).
18. Y.-J. Surh, H. Kwon, and S. R. Tannenbaum: Sulfotransferase-mediated activation of 4-hydroxy- and 3,4-dihydroxy-3,4-dihydrocyclopenta [c,d]pyrene, major metabolites of cyclopenta[c,d]pyrene. *Cancer Res.* **53,** 1017–1022 (1993).
19. L. Dasaradhi and S. Shibutani: Identification of tamoxifen-DNA adducts formed by α-sulfate tamoxifen and α-acetoxytamoxifen. *Chem. Res. Toxicol.* **10,** 189–196 (1997).
20. W. J. Driscoll, B. M. Martin, H.-C. Chen, and C. A. Strott: Isolation of two distinct 3-hydroxysteroid sulfotransferases from the guinea pig adrenal. *J. Biol. Chem.* **268,** 23496–23503 (1993).
21. Y. C. Lee, C.-S. Park, and C. A. Strott: Molecular cloning of a chiral-specific 3α-hydroxysteroid sulfotransferase. *J. Biol. Chem.* **269,** 15838–15845 (1994).
22. S. I. Rao and M. W. Duffel: Benzylic alcohols as stereospecific substrates and inhibitors for aryl sulfotransferase. *Chirality* **3,** 104–111 (1991).
23. T. Walle, U. K. Walle, J. A. Shwed, K. R. Thornburg, C. H. Mathis, and G. R. Pesola: Human phenolsulfotransferase: chiral substrates and expression in Hep G2 cells. *Chem. Biol. Interact.* **92,** 47–55 (1994).
24. S. I. Rao and M. W. Duffel: Inhibition of rat hepatic aryl sulphotransferase IV by dihydrodiol derivatives of benzo[a]pyrene and naphthalene. *Xenobiotica* **22,** 247–255 (1992).
25. H. R. Glatt, A. Seidel, R. G. Harvey, and M. W. H. Coughtrie: Activation of benzylic alcohols to mutagens by human hepatic sulfotransferases. *Mutagenesis* **9,** 553–557 (1994).
26. K. Ogura, T. Sohtome, A. Sugiyama, H. Okuda, A. Hiratsuka, and T. Watabe: Rat liver cytosolic hydroxysteroid sulfotransferase (sulfotransferase a) catalyzing the formation of reactive sulfate esters from carcinogenic polycyclic hydroxymethylarenes. *Mol. Pharmacol.* **37,** 848–854 (1990).
27. H. Glatt: Bioactivation of mutagens via sulfation. *FASEB J.* **11,** 314–321 (1997).
28. R. D. Sekura: Adenosine 3′-phosphate 5′-phosphosulfate. *Methods Enzymol.* **77,** 413–415 (1981).
29. R. F. Nystrom and W. G. Brown: Reduction of organic compounds by lithium aluminum hydride: II. Carboxylic acids. *J. Am. Chem. Soc.* **69,** 2548–2549 (1947).
30. J. Chandrasekharan, P. V. Ramachandran, and H. C. Brown: Diisopinocampheylchloroborane, a remarkably efficient chiral reducing agent for aromatic prochiral ketones. *J. Org. Chem.* **50,** 5446–5448 (1985).
31. R. Noyori, I. Tomino, Y. Tanimoto, and M. Nishizawa: Rational designing of efficient chiral reducing agents. Highly enantioselective reduction of aromatic ketones by binaphthol-modified lithium aluminum hydride reagents. *J. Am. Chem. Soc.* **106,** 6709–6716 (1984).
32. S. Itsuno, M. Nakano, K. Miyazaki, H. Masuda, K. Ito, A. Hirao, and S. Nakahama: Asymmetric synthesis using chirally modified borohydrides: Part 3. Enantioselective reduction of ketones and oxime ethers with reagents prepared from borane and chiral amino alcohols. *J. Chem. Soc. (Perkin I)* 2039–2044 (1985).

33. A. J. M. Janssen, A. J. H. Klunder, and B. Zwanenburg: Resolution of secondary alcohols by enzyme catalyzed transesterification in alkyl carboxylates as the solvent. *Tetrahedron* **47,** 7645–7662 (1991).
34. K. Mori and R. Bernotas: Enzymatic preparation of the enantiomers of some 1-phenyl-1-alkanols. *Tetrahedron* **1,** 87–96 (1990).
35. R. MacLeod, F. J. Welch, and H. S. Mosher: Asymmetric reductions: VII. The action of the Grignard reagent from (+)-1-chloro-2-methylbutane on a series of alkyl phenyl ketones. *J. Am. Chem. Soc.* **82,** 876–880 (1960).
36. T. Watabe, K. Ogura, M. Satsukawa, H. Okuda, and A. Hiratsuka: Molecular cloning and functions of rat liver hydroxysteroid sulfotransferases catalysing covalent binding of carcinogenic polycyclic arylmethanols to DNA. *Chem. Biol. Interact.* **92,** 87–105 (1994).
37. A. Bensadoun and D. Weinstein: Assay of proteins in the presence of interfering materials. *Anal. Biochem.* **70,** 241–250 (1976).
38. M. W. Duffel, T. P. Binder, and S. I. Rao: Assay of purified aryl sulfotransferase suitable for reactions yielding unstable sulfuric acid esters. *Anal. Biochem.* **183,** 320–324 (1989).
39. F. W. Perrella: EZ-FIT: a practical curve-fitting microcomputer program for the analysis of enzyme kinetic data on IBM-PC compatible computers. *Anal. Biochem.* **174,** 437–447 (1988).
40. K. Ogura, J. Kajita, H. Narihata, T. Watabe, S. Ozawa, K. Nagata, Y. Yamazoe, and R. Kato: cDNA cloning of the hydroxysteroid sulfotransferase STa sharing a strong homology in amino acid sequence with the senescence marker protein SMP-2 in rat livers. *Biochem. Biophys. Res. Commun.* **166,** 1494–1500 (1990).