Inhibition of Bovine Phenol Sulfotransferase (bSULT1A1) by CoA Thioesters

EVIDENCE FOR POSITIVE COOPERATIVITY AND INHIBITION BY INTERACTION WITH BOTH THE NUCLEOTIDE AND PHENOL BINDING SITES*

(Running title: Palmitoyl-CoA Inhibits Phenol Sulfotransferase)

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SUMMARY

Previous work with the bovine phenol sulfotransferase (bSULT1A1, EC 2.8.2.1) demonstrated inhibition by CoA that was competitive with respect to the sulfuryl donor substrate, 3’-phosphoadenosine-5’-phosphosulfate (PAPS) [Leach et al. (1999) Biochem. Biophys. Res. Commun. 261, 815-819]. Here we report that long chain acyl-CoAs are more potent inhibitors of bSULT1A1, and also of human dopamine sulfotransferase (SULT1A3), when compared to unesterified CoA and short chain length acyl-CoAs. A complex pattern of inhibition was revealed by systematic variation of palmitoyl-CoA, PAPS, and 7-hydroxycoumarin, the acceptor substrate. Convex plots of apparent $K_m/V_{max}$ vs. [palmitoyl-CoA] were adequately modeled using an ordered rapid equilibrium scheme with PAPS as the leading substrate, and by accounting for the possible binding of two equivalents of inhibitor to the dimeric enzyme. Interestingly, the first $K_i$ of 2-3 µM was followed by a second $K_i$ of only 0.01-0.05 µM, suggesting that positive subunit cooperativity enhances binding of long chain acyl-CoAs to this sulfotransferase. Simultaneous interaction of palmitoyl-CoA with both the nucleotide and phenol binding sites is suggested by two experiments. First, the acyl-CoA displaced 7-hydroxycoumarin from the highly fluorescent bSULT1A1:PAP:7-HC complex in a cooperative manner. Second, palmitoyl-CoA prevented the quenching of bSULT1A1 fluorescence observed with pentachlorophenol. Finally, titrations of bSULT1A1:pentachlorophenol complex with palmitoyl-CoA caused the return of protein fluorescence, and the binding of palmitoyl-CoA was highly cooperative (Hill constant of 1.9). Overall, these results suggest a model of sulfotransferase inhibition in which the 3’- phosphoadenosine-5’-diphosphate moiety of CoA docks to the PAPS domain, and the acyl-pantetheine group docks to the hydrophobic phenol binding domain.
INTRODUCTION

Many xenobiotics, neurotransmitters, steroids and other hormones are metabolized by sulfate conjugation, which is catalyzed by a family of cytosolic sulfotransferase enzymes (1-3). The common sulfuryl group donor substrate is 3'-phosphoadenosine-5'-phosphosulfate (PAPS), with which a sulfate ester and adenosine-3',5'-bisphosphate (PAP) are generated as products. The isolation of sulfotransferase cDNAs and genes during the previous decade, along with the expression and purification of recombinant enzymes, has expanded our knowledge of the diversity and functions of this enzyme family (4).

Important insights have been gained regarding the structures and chemical mechanisms of sulfotransferases (5-7). A highly conserved feature is a histidyl residue in the vicinity of position 108, which may serve as a general base in acceptor substrate proton abstraction. The binding site of PAPS includes conserved lysyl, arginyl, and seryl groups, and acceptor substrate specificity is determined in part by the charge in its binding domain. Many cytosolic sulfotransferases are dimeric, and the dimerization domain has been identified as a relatively simple hydrophobic zipper stabilized on the ends by salt bridges (6). The simplicity of the dimerization domain may explain the observation of heterodimeric sulfotransferases purified from rat liver (8).

The role of subunit dimerization in sulfotransferase function is currently under investigation. The tight binding of only one equivalent of PAP per dimeric rat AST-IV suggests a structural limitation to ligand binding in that enzyme (9). And PAP-dependent cooperativity has been reported for the binding of pentachlorophenol to the bovine SULT1A1 (10). Nonetheless, there is little evidence to date for a role of subunit interactions during sulfotransferase catalytic turnover.

A variety of steady state kinetic mechanisms have been reported for different sulfotransferases. Partially purified guinea pig liver phenol sulfotransferase was reported to obey a rapid equilibrium random bi bi pattern (11), whereas partially purified rat brain phenol sulfotransferase displayed a sequential ordered bisubstrate mechanism with PAPS as the leading substrate (12). A random mechanism was also reported for the more highly purified rat liver aryl
(phenol) sulfotransferase IV (13), although it was noted that an ordered Theorell-Chance mechanism was also consistent with the data. Characterization of the human phenol (SULT1A1) and dopamine (SULT1A3) sulfotransferases revealed ordered bi bi mechanisms with PAPS as the leading substrate (14,15). Some caution is warranted as we look back on work performed with partially purified non-recombinant enzymes; however, all reports provide a consistent picture that PAPS can bind to the enzyme in the absence of a phenol. This is supported by the frequent use of nucleotide agaroses for affinity purification (16,17), by the presence of PAP found tightly bound to a recombinant rat enzyme (9), and by recent crystallization of mouse estrogen sulfotransferase complexes with PAP and PAPS (18).

Much emphasis has been placed on elucidating substrate specificities for various members of the sulfotransferase family (16), which often display overlapping reactivities with many acceptor substrates. Quantitative structure activity relationships have revealed clear differences between the human phenol sulfotransferase (SULT1A1) and the dopamine sulfotransferase (SULT1A3) (7,19). Equally important, however, is the likely role that biomolecules play in regulating sulfoconjugation reactions. Exogenous inhibitors include polyphenolic compounds (20,21), aryl carboxylic acids (22), aryl aldehydes (23), and polyhalogenated phenols (13,24). Endogenous inhibitors include ATP (14,15), pyridoxal phosphate (25), and CoA (17). The latter is of interest due to its overt structural overlap with PAP, another potent sulfotransferase inhibitor (26) generated by normal enzymatic turnover. CoA was found to be a competitive inhibitor with respect to PAPS of the bovine phenol sulfotransferase (bSULT1A1) (17), an enzyme with very similar sequence and substrate specificity compared to the human SULT1A1 enzyme (27).

As a ubiquitous biomolecule, CoA fulfills several metabolic and regulatory roles. While the mitochondrial CoA pool is probably not relevant for this investigation, cytosolic CoA is a vital component in fatty acid catabolism, lipid biosynthesis, and drug metabolism (28,29). The acylation status of this CoA pool is a dynamic parameter that can shift dramatically depending on cellular physiological conditions (28,30). This, in turn, could alter its effects on enzymes such as sulfotransferases. Therefore, we have sought to extend our prior work by comparing the
inhibition of bSULT1A1 by various acylated CoAs. The complete characterization of the kinetics of this inhibition indicates an unanticipated complex kinetic pattern that required accounting for inhibitor binding to a dimeric enzyme. These results may reveal an aspect of sulfotransferase catalysis not previously uncovered, namely positive subunit interactions.
EXPERIMENTAL PROCEDURES

Reagents

Buffers and salts were the highest purities available from either Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). 2-Naphthol and 2-naphthyl-sulfate were from Fluka (Milwaukee, WI) and Research Organics (Cleveland, OH), respectively. CoA and acyl-CoAs, PAPS, PAP, pentachlorophenol (PCP), and 7-hydroxycoumarin (7-HC) were from Sigma. CoAs, PAPS, and PAP were dissolved in water, quantified by absorbance \( A=15.4/mM \) at 260 nm, and stored frozen as 1-200 mM aliquots. PCP and 7-HC were dissolved in ethanol at 1 M and 0.1 M by mass, respectively, and kept at -20°C in the dark. These reagents were diluted in water to approximately 100 µM working stocks immediately prior to use.

For some experiments, the commercially available PAPS was purified by ion exchange FPLC. Approximately 0.2 µmol of PAPS was bound to a Resource Q column equilibrated with 50 mM triethylamine carbonate/pH 7.6, followed by a 15 ml linear gradient to 1 M buffer. The PAPS peak was collected and lyophilized to remove volatile buffer components, and then dissolved in water and quantified by absorbance. When directly comparing unpurified to purified PAPS in bSULT1A1-catalyzed reactions, however, we did not observe any significant differences in either rates or steady state kinetic parameters (not shown).

Recombinant bovine phenol sulfotransferase (bSULT1A1) was purified from \( E. \ coli \) DH5α harboring the pTrc-PST cDNA expression vector as previously described (10, 27), except that the final molecular exclusion over AcA34 was replaced by FPLC through Sephacryl S-200 (Pharmacia, Hi-Prep 26/60). Aliquots of enzyme were stored at -80°C at 1-2 mg/ml in Buffer B (20 mM Tris, 1 mM EDTA, 10% (w/v) sucrose, 5 mM 2-mercaptoethanol, pH 7.4).

Recombinant human dopamine sulfotransferase (SULT1A3) was purified from \( E. \ coli \) DE3 harboring hM-PST-pET-11a, kindly provided by Dr. Michael Coughtrie. The purification method followed was that used for the bovine SULT1A1 enzyme.
Protein concentrations were determined using the BioRad reagent and bovine serum albumin as the relative standard. bSULT1A1 molarity was calculated assuming a $M_r$ of 32 kDa (27), and hSULT1A3 concentration assumed a subunit $M_r$ of 34 kDa (31).

Methods

Measurement of bSULT1A1 activity was performed by observing the decrease in 7-HC fluorescence essentially as described by Leach et al. (17), in which 50 µl reactions initiated in timed sequence were then sequentially stopped after 5-20 min. with an equal volume of 0.5 M Tris-Cl/0.1% SDS/pH 8.5. The assay buffer was 25 mM sodium phosphate/25 mM sodium succinate/0.5 mM EDTA/pH 5.5, a previously reported optimum for this enzyme (32). If the concentrations of fluorescent 7-HC exceeded 1 µM, then sufficient Tris/SDS was added to dilute the total 7-HC to 1 µM. The fluorescence intensities of the quenched reactions were then measured using a Hoefer DyNA Quant fluorimeter and 50 µl capillary cuvettes. The instrument was frequently blanked and calibrated with a sample of 1.0 µM 7-HC, which was within the linear instrumental response. Several precautionary measurements were made to ensure assay validity: first, no substrate was ever depleted beyond 20% of its starting value; second, the incubation times and enzyme concentrations fell within linear dependence; third, the inhibition by the CoAs in the assay buffer was time-independent. Concentrations of PAPS and 7-HC were varied as indicated in the figure legends. In order to accurately measure a decrease in 7-HC fluorescence during the PAPS variations, we found it necessary to hold the acceptor substrate at 0.2 µM. This is sub-saturating and has the consequence of reduced rates in comparison to the 7-HC variation experiments (e.g. Fig. 3 vs. Fig. 4). In contrast, for assays in which 7-HC was varied, the PAPS concentration was held at 10 µM. This higher concentration drove greater reaction rates and allowed estimation of the true turnover number of the reaction.

Human SULT1A3 activity was determined fluorimetrically by the method of Arand (33), in which the formation of 2-naphthyl sulfate is measured. Incubations of 100 µl included 5 mM
sodium phosphate, pH 6.8, 5 μM PAPS, and 25 μM 2-naphthol. CoA and palmitoyl-CoA concentrations in the assays were varied as indicated (Fig. 2). Emission intensities were measured with an SLM-AMINCO 8000 spectrofluorimeter (285 nm excitation, 335 nm emission), and relative activities were calculated compared to controls in the absence of inhibitors. Care was taken to select incubation times and enzyme concentration which displayed linearity, and all measurements were conducted in triplicate.

Kinetic data (Figs. 3 and 4) were numerically analyzed as follows. Initial rates were divided by bSULT1A1 subunit concentration, thereby directly providing turnover number values. Due to the apparent complexity of the inhibition patterns, attempts were made to use a sophisticated software package (Enzfitter, Biosoft, Cambridge UK) to fit the data to several common mechanisms. However, no equation or algorithm provided a satisfactory model of the data as revealed by excessive standard errors or non-random residual distribution (not shown). The apparent cooperativity of inhibition by palmitoyl-CoA (Fig. 1) suggested that a model would need to account for subunit-subunit interactions. Therefore, additional binding schemes were considered that included mixed substrate and inhibitor binding to a dimeric enzyme. Derivation of rapid equilibrium equations was by standard approaches (34), and the simplest scheme that yielded graphical predictions consistent with the data is provided (Fig. 5).

Experimental data were then fit to the model as follows. Triplicate rates for a set of varied substrate concentrations, for a single concentration of inhibitor, were entered into a spreadsheet (Cricket Graph) configured to perform the statistical weighting calculations of Wilkinson (35). For visualization, traditional Lineweaver-Burk plots were constructed displaying the average velocities and the weighted fits. The slopes of these fits (Km/TN) were subsequently plotted against palmitoyl-CoA concentration. Fittings of the slope replots according to the slope portions of equations 3 and 4 (Table 1) were conducted manually in an iterative process. Initial selections of the equation parameters Ks1, Ks2, and TN were made based on rate data in the absence of inhibitor. Cosubstrate concentrations were known experimental parameters. The IC50 value (0.24 μM) of palmitoyl-CoA inhibition (Fig. 1) was used as initial estimates of Ki1 and Ki2, and "α"
was initially set to 1. It was decided to restrain Ki2 from decreasing below 0.01 µM, the point at which inhibition of bSULT1A1 activity could be detected (Fig. 1). The parameters for the two sets of data (Fig. 3 and 4) were then varied independently until visual fits of the experimental slope replots were achieved. Refinements of the fits were then made while minimizing the sum of the squared residuals for each replot, and parameters between the two experiments were progressively converged in small increments. After six iterations no further minimization of summated squared residuals could be achieved, and the final fits and parameters are reported in Figs. 3 and 4 and Table 2.

Fluorescence emission spectra were measured using an SLM-AMINCO 8000 spectrofluorimeter with an excitation wavelength of 280 nm. Excited tryptophanyl emission energy is apparently transferred to bound 7-HC, which displays an enhanced emission around 400-420 nm (submitted). All spectra were recorded at ambient temperature and were corrected for the small dilutions due to titrant additions. For most experiments, 1.50 ml samples in Buffer B included a magnetic stirrer in the quartz cuvette. Fluorimeter shutters were closed unless spectra were being recorded, in order to minimize UV exposure time. Titrations with stock solutions of CoA, palmitoyl-CoA, or PCP were such that 2-5 µl additions of reagent were made, followed by at least two minutes for mixing and equilibration. Preliminary experiments demonstrated that this was sufficient time for binding equilibration to occur (not shown).

Quenching of bSULT1A1 fluorescence with PCP was performed by titrations of 2.0 µM solutions of enzyme essentially as previously described (10). Emission intensities at 338 nm were recorded using 280 nm excitation, and mock titrations were performed in order to provide empirical dilution correction factors. Inclusion of PAPS, CoA, or palmitoyl-CoA (5 µM) reduced the initial emission intensity by at most 5%, which is likely due to an excitation inner filter effect. Therefore, fluorescence values are expressed as percent relative to the sample lacking PCP. Final concentrations of PCP could not exceed about 5 µM without introducing a further inner filter artifact (10), thus precluding reliable titration data at greater PCP concentrations (Fig. 8).
It was also possible to titrate the quenched bSULT1A1:PCP complex with palmitoyl-CoA (Fig. 9). The 335 nm emission of 0.50 µM enzyme in Buffer B was recorded, followed by addition of 3 µM PCP, which reduced the fluorescence by 25-30%. Subsequent additions of palmitoyl-CoA caused the return of protein emission to near starting values. Data were corrected for dilution and inner filter effects by performing mock titrations of free enzyme with palmitoyl-CoA, or PCP-bound enzyme with 5’-AMP, which apparently did not displace PCP from the enzyme. Averaged data from three titrations that varied by less than 5% were modeled with a Hill-type equation (36) by minimizing the sum of squared residuals.

Analytical molecular exclusion column chromatography (Fig. 10) was performed with a Pharmacia (Amersham) FPLC system. The Superdex 200HR column was eluted at 1 ml/min with Buffer B (above) containing 50 mM NaCl. Size standards (BSA, chicken ovalbumin, and β-lactoglobulin) were from Sigma. Preliminary experiments indicated that 40 µM palmitoyl-CoA increased the apparent size of bSULT1A1 in a time-dependent manner over a period of several hours. Therefore, to obtain final equilibrated states, the enzyme (0.6 mg/ml) was incubated with varied concentrations of palmitoyl-CoA for 24 h at ambient temperature prior to chromatography. Fractions (1 ml) were collected and assayed for bSULT1A1 activity (see above).
RESULTS

Effect of acyl chain length on sulfotransferase inhibition

Previous work demonstrated the inhibition of bSULT1A1 by CoA, and we sought to determine if various CoA thioesters might exhibit similar or different inhibition in comparison. An overall assessment was therefore made of CoA, acetyl-CoA, malonyl-CoA, octanoyl-CoA, and palmitoyl-CoA across a broad range of concentrations (Fig. 1). The IC$_{50}$ value for CoA of 33 µM was consistent with prior work (17), and this dose-response overlapped those of acetyl-CoA (IC$_{50}$ = 39 µM) and malonyl-CoA (IC$_{50}$ = 40 µM). The longer chain-length acyl-CoAs, however, were clearly more potent bSULT1A1 inhibitors, with palmitoyl-CoA (IC$_{50}$ = 0.24 µM) being five times more effective than octanoyl-CoA (IC$_{50}$ = 1.25 µM).

To determine if the effect of acyl-chain length might also apply to other sulfotransferases, we tested the response of human dopamine sulfotransferase (hSULT1A3) to CoA and palmitoyl-CoA (Fig. 2). This enzyme is highly related to bSULT1A1, although its phenol binding site microenvironment is less hydrophobic due to the existence of Glu146 (7). Overall, hSULT1A3 is less sensitive to inhibition by CoAs compared to bSULT1A1, although there remains a shift in inhibition between CoA (IC$_{50}$ = ca. 1 mM) and palmitoyl-CoA (IC$_{50}$ = 100 µM). The assays of hSULT1A3 were conducted at a greater pH of 6.8 (see Methods) compared to that optimal for bSULT1A1 of pH = 5.5 (Fig. 1), so it was possible that the apparent reduced sensitivity of the human enzyme may have been due to assay conditions. Therefore, the inhibition of bSULT1A1 by palmitoyl-CoA was also re-tested and found to display an IC$_{50}$ of 1 µM. Thus, bSULT1A1 is 100-fold more sensitive to inhibition by palmitoyl-CoA compared to hSULT1A3 under identical conditions, which reveals that different enzymes in the sulfotransferase superfamily will likely display variable sensitivities to CoA and its thioesters.

Kinetic characterization of bSULT1A1 inhibition by palmitoyl-CoA

Previous work indicated simple competitive inhibition of bSULT1A1 by CoA with respect to PAPS (17). Due to the dramatic increase in inhibition connected with long acyl chain length (Fig.
1), and to determine a true $K_i$ value, a detailed investigation into the steady state kinetics with palmitoyl-CoA was undertaken. Initial work employed systematic variation of PAPS and inhibitor, with constant concentration of phenolic substrate (7-HC). Lineweaver-Burk plots of the measured rates are provided for illustration (Fig. 3), which indicated a complex or mixed pattern of inhibition. Further measurements with systematic variation of 7-HC acceptor substrate, at a fixed concentration of PAPS, also revealed complex inhibition (Fig. 4). Attempts to numerically fit the data using standard equations, such as for competitive, non-competitive, etc., were not satisfactory (not shown). We observed non-linear slope replots (Figs. 3 and 4) as part of the graphical analyses, and this behavior suggested a quadratic dependence of inhibition on palmitoyl-CoA concentration.

Therefore, possible binding schemes were considered that could model the non-linear slope replots. Previous investigations of the highly similar human SULT1A1 and SULT1A3 steady state kinetic mechanisms suggested ordered bi bi patterns with PAPS as the leading substrate (14,15). Importantly, no investigations of sulfotransferase kinetics have, to our knowledge, taken into account the dimeric quaternary structure that is quite common for this enzyme family. It seems plausible that usual catalytic turnover may, under conditions of subsaturating substrate concentrations, proceed mechanistically via one subunit before the adjoining subunit engages in catalysis. In the presence of an inhibitor, however, we reasoned that a complete binding model should account for both subunits. We also noted, as have others, the relatively slow catalysis by this enzyme family, so rapid equilibrium assumptions in the derivation of mathematical models seemed appropriate.

The simplest model of palmitoyl-CoA binding to bSULT1A1 was to allow sequential reversible binding to the two subunits of the enzyme as an initial dead-end branch from an ordered bi bi scheme. The derived rate equations predicted a constant double reciprocal plot y-intercept (not shown), a pattern inconsistent with the experimental results. In contrast, by allowing the binding of inhibitor to an idle subunit during catalysis, both a variable y-intercept and non-linear slope replots were predicted. This scheme is depicted in Figure 5, with the binding parameters and
equations given in Table 1. The results in Figures 3 and 4 were adequately fit (Methods) using this model, and the derived values of the dissociation constants are summarized in Table 2. There is reasonably good agreement in the values, thus providing some assurance of the adequacy of the binding scheme. It is important to note that binding of the first equivalent of palmitoyl-CoA has an approximately 100-fold greater dissociation constant compared to the second inhibitor binding step. This result suggests that subunit interactions exist within the dimeric bSULT1A1 that enhance binding of palmitoyl-CoA to the second subunit.

An additional result of the slope replot analysis that suggests subunit interactions is the magnitude of "alpha". This term reflects the interaction between the PAPS and palmitoyl-CoA binding equilibria involving the adjoining bSULT1A1 subunits (Fig. 5). A numerical value of one would indicate no subunit interactions; however, the fitted value of 0.65 (Table 2) indicates that binding of PAPS reduces the dissociation constant of palmitoyl-CoA on the neighboring subunit. This effect is consistent with the sequential binding of two palmitoyl-CoAs, in which binding of the first augments binding of the second (see above). Despite the observation of "alpha" being optimized to 0.65, it should be pointed out that the significance of this numerically derived parameter should be cautiously viewed, as variations in "alpha" had relatively little impact on the least squares fitting when compared to other parameters.

Disruption of a bSULT1A1:PAP:7-HC complex by CoA and acyl-CoAs

The binding of 7-HC to bSULT1A1 is accompanied by PAP-dependent enhancement of 7-HC emission at approximately 400-420 nm, and our previous titrations suggested a binding stoichiometry of 0.5 mole PAP and 7-HC per mole of enzyme subunits (submitted). We were interested in how CoA and acyl-CoAs might participate in either the formation or disruption of such a complex. Preliminary experiments indicated that CoA or palmitoyl-CoA could not substitute for PAP in regard to enhancing the binding of 7-HC, but rather impaired the formation of the bSULT1A1:PAP:7-HC ternary complex (not shown). Therefore, preformed complex was titrated with palmitoyl-CoA (Fig. 6), and the emission of the bound 7-HC at 420 nm was analyzed.
in detail. With increasing palmitoyl-CoA, the composite emission spectrum displayed a decrease in 420 nm intensity and the overall appearance approached that of bSULT1A1 and 7-HC in the absence of PAP (Fig. 6, scan 5 vs. scan 1). This is consistent with an equilibrium binding model (Fig. 5), in which association of palmitoyl-CoA with a subunit of bSULT1A1 is mutually exclusive of PAP and 7-HC. The ability of palmitoyl-CoA to disrupt the bSULT1A1:PAP:7-HC complex suggests the reversibility of the initial binding steps.

Based on the inhibition experiments (Fig. 1), it was postulated that palmitoyl-CoA would disrupt the bSULT1A1:PAP:7-HC complex at lower concentrations than unesterified CoA. We also sought to compare disruption by CoA nucleotides to that previously observed by pentachlorophenol (PCP), a very tightly binding phenol sulfotransferase inhibitor. Therefore, spectrofluorimetric titrations of bSULT1A1:PAP:7-HC with CoA and PCP were also performed, and the results are summarized in Fig. 7. PCP was the most effective titrant in this experiment, and was completely disruptive at sub-stoichiometric concentrations. A titration endpoint of 1 µM PCP was obtained (not shown), which is consistent with our previous results that suggest the formation of a bSULT1A1:PAP:7-HC ternary complex (submitted). Palmitoyl-CoA clearly disrupted the complex at lower concentrations in comparison to CoA, which is consistent with the augmented inhibition observed with this long chain acyl-CoA (Fig. 1). As a control for possible confounding denaturation effects, aliquots of disrupted complexes were appropriately diluted in Buffer B (Methods) containing 100 µM PAPS, followed by assays of bSULT1A1 activity. Nearly complete enzyme activity was observed (not shown). The titrations data were modeled using a Hill equation (36), and both CoA and palmitoyl-CoA titrations were best fit with slope factors of 1.3. This equilibrium result provides moderate evidence for the cooperative binding of these nucleotides to bSULT1A1; however, it should be noted that the enzyme is pre-bound with the PAP nucleotide, which may affect the observed CoA binding model.
Palmitoyl-CoA inhibits PCP-induced quenching of bSULT1A1 fluorescence

The kinetic data and rapid equilibrium scheme that modeled those data suggested that binding of palmitoyl-CoA excludes ligand binding to the phenolic site. This notion is supported by the disruption of the bSULT1A1:PAP:7-HC complex by palmitoyl-CoA, and by the inability of the CoA nucleotide to replace PAP in inducing the binding of 7-HC by the enzyme. To further test this model, we titrated bSULT1A1 with PCP, which decreases tryptophanyl emission by approximately 40% upon binding to the enzyme. As shown in Fig. 8, and as previously reported (10), PAPS (5 µM) enhanced the affinity of bSULT1A1 for PCP. CoA (5 µM) did not have an effect on the PCP titration, suggesting that this inhibitor did not substitute for PAPS in enhancing PCP binding. The concentration of CoA, however, may not have been sufficient to adequately saturate the enzyme based on the dose-response for bSULT1A1 inhibition (Fig. 1).

In contrast to CoA, palmitoyl-CoA (5 µM) effectively blocked the PCP-induced quenching of bSULT1A1 (Fig. 8, closed triangles). Based on the previous kinetics data, this concentration of inhibitor would be sufficient to occupy the majority of available binding sites. Some quenching (ca. 5%) was observed at the greatest PCP concentration, suggesting that the presence of palmitoyl-CoA could be overcome. But it was not possible to use greater PCP concentrations than what is reported, due to artifactual inner filter effects. These results are consistent with a model in which the binding of palmitoyl-CoA to bSULT1A1 prevents binding of a phenolic ligand.

The ability of palmitoyl-CoA to prevent PCP-induced quenching of bSULT1A1 fluorescence suggested the converse experiment of titrating PCP-quenched enzyme with the CoA thioester. This experiment (Fig. 9) revealed the predicted return of protein fluorescence intensity as palmitoyl-CoA displaced PCP from the enzyme. The dose-responses of these titrations required modeling with a Hill equation and a slope factor (Hill coefficient) of 1.9. This behavior is consistent with kinetic data that also indicated positive bSULT1A1 subunit interactions during the binding of palmitoyl-CoA. The fitted inflection point ($ED_{50}$) of 0.23 µM is sensible because the titrations were conducted with 0.50 µM bSULT1A1 subunits.
Palmitoyl-CoA affects the hydrodynamic properties of bSULT1A1

A potential confounding property of long chain acyl-CoA thioesters is detergent activity. Therefore, the possible impact of palmitoyl-CoA on the quaternary structure of bSULT1A1 was investigated by molecular exclusion chromatography (Fig. 10). Preliminary experiments indicated the elution pattern to be time-dependent (not shown), so samples of enzyme with varied palmitoyl-CoA concentrations were incubated for 24 h prior to chromatography, a time vastly exceeding the conditions of all other experiments in this report. The enzyme eluted predominately as the homodimer at palmitoyl-CoA concentrations up to 24 µM (Fig. 10, peak “c”), at which point a greater Mₐ peak appeared at a predicted tetrameric size (Fig. 10, peak “b”). Increased palmitoyl-CoA concentrations enhanced the formation of this apparent tetramer, as well as higher relative mass aggregates (Fig. 10, region “a”). Excess palmitoyl-CoA eluted much later than the protein peaks (Fig. 10, peak “d”), suggestive of a non-micellar state. Assays of bSULT1A1 demonstrated activity in the dimer (“c”) and tetramer (“b”) peaks, whereas the higher Mₐ eluate was inactive (not shown). Electrophoresis in the presence of dodecyl sulfate demonstrated monomeric 32 kDa protein after incubation with excess palmitoyl-CoA (not shown), suggesting no chemical modification of the protein. These results indicate that although palmitoyl-CoA can affect the apparent quaternary structure of bSULT1A1, the concentrations required to do so are considerably greater than what are required for inhibition (Fig. 1-4), for disruption of the ternary complex with PAP and 7-HC (Fig. 6 and 7), or for disruption of the enzyme complex with PCP (Fig. 9).
DISCUSSION

The previously published observation of bSULT1A1 inhibition by CoA (17) was anticipated based on the structural similarity to PAPS. What was not predictable was the enhanced inhibition correlated to the presence of a long chain acyl group (Fig. 1). It is very difficult to adequately propose a docked model of the bSULT1A1:palmitoyl-CoA complex, because hydrophobic interactions pose a calculational hurdle, and the acyl and pantetheine groups display considerable structural variation when complexed with other unrelated enzymes (e.g. acyl-CoA dehydrogenases, citrate synthase, etc.). At this time, we suspect that the hydrophobic acyl chain may fold into the phenol binding site of bSULT1A1. This model is supported by the rapid equilibrium kinetics scheme that describes the inhibition data, in which binding of palmitoyl-CoA precludes binding of 7-HC phenolic substrate (Fig. 5). Furthermore, neither palmitoyl-CoA nor CoA could substitute for PAP in the formation of an enzyme complex with 7-HC, but rather disrupted a preformed bSULT1A1:PAP:7-HC complex (Figs. 6 and 7). Additionally, palmitoyl-CoA impaired the ability of PCP to induce the quenching of tryptophanyl fluorescence (Figs. 8 and 9), which could be due to competition of the acyl group with PCP for binding to the phenol binding site of the enzyme.

Will all sulfotransferases display CoA or acyl-CoA sensitivity? All members of this superfamily utilize PAPS as the sulfuryl donor, which includes the PAP moiety in common with CoA. It therefore seems quite likely that interaction with CoA will be a common feature, although we anticipate variations in the dissociation constants. The human SULT1A3 displayed decreased sensitivities to both CoA and palmitoyl-CoA in comparison to bSULT1A1 (Fig. 2). The precise basis for this differential sensitivity is not clear in the absence of structural data. It may be that the greater hydrophobicity of the SULT1A1 phenol binding site provides a stronger interaction with the acyl chain of palmitoyl-CoA. The SULT1A3 enzyme phenol binding site includes a charged residue, Glu146, important in catecholamine binding (7). This group may destabilize docking of the uncharged acyl chain, resulting in an increased dissociation constant.
A surprising feature of this investigation was the complexity of the kinetics data (Figs. 3-5). The convex slope replots suggested a quadratic dependence of inhibition on palmitoyl-CoA concentration, and this was adequately modeled by a rapid equilibrium scheme in which binding of the inhibitor to both subunits of the dimeric enzyme was taken into consideration (Fig. 5, Tables 1 and 2). Simulation of the results could only be approached as the dissociation constants for palmitoyl-CoA were allowed to diverge so that binding of the first equivalent was less favorable than the second equivalent. Refinements to enhance the numerical fit also suggested that binding of PAPS to one subunit decreases the dissociation constant for palmitoyl-CoA to the adjoining subunit. An alternative model would envision two palmitoyl-CoA binding sites per subunit and thus not require cooperativity to explain the experimental results. However, there is no evidence for two PAP binding domains on any sulfotransferase, so this model would be overly speculative. Such positive cooperativity during catalysis has not been, to our knowledge, reported for any member of this enzyme family.

The kinetic behavior observed in this investigation, however, may not be limited to the bovine SULT1A1. In their investigations of the human phenol and dopamine sulfotransferases, Whittemore et al. utilized ATP as an inhibitor to delineate kinetic mechanisms (14,15). Although double-reciprocal plots were shown by the authors, slope replots were not reported for this inhibitor. We have scrutinized those results and have observed convex slope replots (not shown). Therefore, it seems likely that other members of this family may exhibit subunit interactions that affect function.

We previously reported that unesterified CoA was a simple competitive inhibitor of bSULT1A1 with respect to PAPS (17). We now believe this to be an oversimplification, as the slope replot in that report may actually be convex. The curvature was not as great as currently observed with palmitoyl-CoA and therefore was overlooked. This comparison, in conjunction with the increased sensitivity of bSULT1A1 to palmitoyl-CoA (Fig. 1), suggests that occupation of both the nucleotide and phenol sites may be necessary to fully drive a conformational change that converts the second subunit to a higher affinity state.
The kinetic evidence for cooperative binding of palmitoyl-CoA is supported by additional equilibrium approaches. Disruption of the bSULT1A1:PAP:7-HC complex with palmitoyl-CoA (Fig. 7) was only moderately cooperative (Hill coefficient of 1.3). It should be noted, however, that the enzyme in that experiment was pre-bound with the PAP nucleotide. Titration of the ternary complex with palmitoyl-CoA probably does not begin with a protein conformation identical to the unbound state. In contrast, titration of bSULT1A1:PCP with palmitoyl-CoA (Fig. 9) revealed a high degree of cooperativity (Hill coefficient of 1.9). In this case, the enzyme is nucleotide-free at the beginning of the titration, so it seems reasonable that subunit interactions that facilitate a nucleotide-induced conformational change would be more adequately probed. The tightness of the transition (Fig. 9) is consistent with the kinetic data that indicated a 100-fold difference in the two binding constants for palmitoyl-CoA. It will be interesting to determine if this can be detected by other biophysical approaches in future experiments.

An important aspect of long chain acyl CoAs is amphilicity and detergent properties, and this can confound investigations if not adequately controlled for. If palmitoyl-CoA acted to denature bSULT1A1, several observations would be anticipated. First, the inhibition would have been dependent on the time of incubation with enzyme. We conducted experiments with varied pre-incubation of enzyme and palmitoyl-CoA, and no time-dependence of inhibition was found (not shown). Second, detergents are typically most effective in the vicinity of their critical micelle concentration (CMC), which is 30-60 µM for palmitoyl-CoA (37,38). The inhibition of bSULT1A1 by palmitoyl-CoA occurred well below this CMC value. Third, samples were tested at the end of titrations with palmitoyl-CoA (Fig. 7), and full catalytic activity was observed after dilution into assay buffer containing excess PAPS (not shown). Fourth, we have observed that the tryptophanyl emission of bSULT1A1 decreases by 20% upon denaturation with dodecyl sulfate (not shown). Very little, if any, change in emission is effected by the inclusion of 5 µM palmitoyl-CoA with 2 µM enzyme (Fig. 8). Finally, we have used FPLC gel filtration to examine the quaternary structure of bSULT1A1 after incubation with varied concentrations of palmitoyl-CoA (Fig. 10). Simple dimeric size elution was observed up to about 20 µM. Approximately 5%
tetrameric size was noted at 24.4 μM palmitoyl-CoA, and roughly 30% conversion to active
tetramer and 30% higher Mr inactive protein was observed with 50 μM palmitoyl-CoA.
Interestingly, there is evidence that palmitoyl-CoA may exist as dimers at concentrations below its
CMC (37), so the formation of tetrameric bSULT1A1 (Fig. 9, peak b) may be the result of two
protein dimers simultaneously bound to a palmitoyl-CoA dimer. The formation of higher Mr
aggregates is consistent with palmitoyl-CoA detergent properties, but these concentrations greatly
exceed the Kᵢ values determined by the kinetic analyses and titration experiments. We conclude
that the observations in this report are not due to non-specific detergent activity of the long chain
acyl-CoA.

A complete understanding of enzyme function must take into account substrate kinetics and
availability as well as possible inhibitors. In the case of the sulfotransferases, CoA is an important
biomolecule that has received little attention as a possible regulatory factor. Although this acyl
carrier is perhaps most frequently considered in mitochondrial metabolism, cytosolic CoA is also a
vital player in both catabolism and biosynthesis. Structurally, CoA includes a PAP moiety,
extended by a phosphopantetheine chain. Therefore, there is direct structural overlap between CoA
and its thioesters and PAPS, the universal sulfuryl group donor for all sulfotransferases. Given
the availability and dynamic composition of the CoA pool, it seems prudent to examine additional
members of the sulfotransferase family for sensitivity to CoA and its thioesters. It may be that
dietary and other physiological conditions regulate one or more sulfation pathways due to changes
in the acylation profile of cytosolic CoA.
REFERENCES


FOOTNOTES

1 Abbreviations used are: PAPS, 3'-phosphoadenosine-5'-phosphosulfate; PAP, adenosine-3',5'-bisphosphate; SULT, sulfotransferase; PCP, pentachlorophenol; 7-HC, 7-hydroxycoumarin.
FIGURE LEGENDS

Figure 1. Effect of CoA and acyl-CoAs on bSULT1A1 activity. Each assay in triplicate included 1 µM PAPS, 0.5 µM 7-HC, and 0.057 µM enzyme in 50 µl. Inhibitor concentrations were as indicated and standard error bars (always less than 15%) are omitted for sake of clarity. Symbols are: palmitoyl-CoA (closed circles); octanoyl-CoA (open circles); acetyl-CoA (closed triangles); malonyl-CoA (open triangles); CoA (open squares). Curves are fits according to the method of Rodbard (36).

Figure 2. Inhibition of human SULT1A3 by CoA and palmitoyl-CoA. Activities were measured (Methods) in triplicate and are expressed as the average relative to controls in the absence of inhibitor. Open circles, unesterified CoA; solid circles, palmitoyl-CoA; triangles, bovine SULT1A1 with palmitoyl-CoA using the same assay for direct comparison.

Figure 3. Effect of varied PAPS concentration on the inhibition of bovine SULT1A1 by palmitoyl-CoA. Rates of 7-HC sulfation (Methods) are expressed as nmoles product/min/nmole enzyme subunits (v/E). Each point in the upper Lineweaver-Burk plot is the average of triplicate measurements that typically varied by less than 10% (error bars not shown for sake of clarity). Palmitoyl-CoA concentrations were 0 (open circles), 0.2 µM (closed circles), 0.4 µM (open triangles), 0.6 µM (closed triangles), and 0.8 µM (open squares). The lines are statistically weighted fits (Methods) from which slopes were determined, and these values are shown in the lower panel. The curve was drawn using the slope portion of equation 3 of Table 1 and the parameters summarized in Table 2.
Figure 4. Effect of varied 7-HC concentration on the inhibition of bovine SULT1A1 by palmitoyl-CoA. Rates of 7-HC sulfation (Methods) are expressed as nmoles product/min/nmole enzyme subunits (v/E). Each point in the upper Lineweaver-Burk plot is the average of triplicate measurements that typically varied by less than 10% (error bars not shown for sake of clarity). Palmitoyl-CoA concentrations were 0 (open circles), 0.2 µM (closed circles), 0.4 µM (open triangles), 0.6 µM (closed triangles), and 0.8 µM (open squares). The statistically weighted linear fits (Methods) provided the slopes that are replotted in the lower panel. The curve was drawn using the slope portion of equation 4 of Table 1 and the parameters summarized in Table 2.

Figure 5. Proposed rapid equilibrium kinetic model describing the catalysis by bSULT1A1 and its inhibition by palmitoyl-CoA. The enzyme is symbolized as “EE” to reflect its homodimeric structure. Substrates “S1” and “S2” are PAPS and 7-HC, respectively. Products “P1” and “P2” are 7-HC sulfate and PAP, respectively, although their order of dissociation is not consequential. “I” represents palmitoyl-CoA. The dissociation constants are defined in Table 1.

Figure 6. Spectrofluorimetric titration of bSULT1A1:PAP:7-HC with palmitoyl-CoA. The sample initially contained 2.0 µM bSULT1A1 (subunits) plus 2.0 µM 7-HC, and the emission wavelength was scanned as shown using 280 nm excitation (scan 1). Stock PAP was added to 2.0 µM, the sample equilibrated for 5 minutes, and then rescanned (trace 2). Subsequent additions of palmitoyl-CoA were made in 0.4 µM increments, and scans 3-5 were recorded at 0.8 µM, 2.0 µM, and 4.0 µM, respectively. Spectra from other palmitoyl-CoA concentrations are not shown for sake of clarity. All spectra are corrected for a small dilution effect of less than 2.5%.
Figure 7. Comparison of palmitoyl-CoA, CoA, and PCP toward disrupting the bSULT1A1:PAP:7-HC complex (see Fig. 6). Corrected emission intensities at 400 nm were used to calculate the relative changes in fluorescence (Methods). Each point is the average of triplicate titrations that varied by less than 5%. Open circles are CoA, closed circles are palmitoyl-CoA, and open triangles are PCP. The palmitoyl-CoA and CoA titration curves are fits (36) using slope factors (Hill coefficients) of 1.3.

Figure 8. Quenching of bSULT1A1 tryptophanyl emission by PCP. Solutions of 2.0 µM enzyme subunits at ambient temperature were titrated with PCP and the emission intensities at 338 nm recorded and corrected for dilution (Methods). The individual titrations included: enzyme only (open circles); enzyme plus 5 µM PAPS (closed circles); enzyme plus 5 µM CoA (open triangles); enzyme plus 5 µM palmitoyl-CoA (closed triangles).

Figure 9. Palmitoyl-CoA cooperatively displaces PCP from bSULT1A1. Solutions of 0.50 µM enzyme subunits containing 3.0 µM PCP were titrated with palmitoyl-CoA and the emission intensities at 338 nm recorded and corrected for dilution (Methods). The curve is a least squares fit of triplicate data using a slope factor (Hill coefficient) of 1.88 and an ED$_{50}$ value of 0.226 µM (36).

Figure 10. Effects of palmitoyl-CoA on the size exclusion of bSULT1A1. The FPLC Superdex 200HR column was loaded with 200 µl samples containing 0.6 mg/ml enzyme subunits plus varied palmitoyl-CoA (from top to bottom, 0, 24.4, 48.8, and 97.5 µM). Peak elutions of size standards are shown by the triangles (1, BSA trimer at 201,000; 2, BSA dimer at 134,000; 3, BSA monomer at 67,000; 4, ovalbumin at 45,000; 5, lactoglobulin at 35,000). Fractions from regions “a-c” were assayed for bSULT1A1 activity (see Results), and region “d” is free palmitoyl-CoA.
Table 1: Definitions of dissociation constants and rapid equilibrium-derived kinetics equations

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_s_1 = \frac{[E \cdot E][S_1]}{[E \cdot ES_1]}$</td>
<td>Dissociation constant for $S_1$</td>
</tr>
<tr>
<td>$K_s_2 = \frac{[E \cdot ES_1][S_2]}{[E \cdot ES_2]}$</td>
<td>Dissociation constant for $S_2$</td>
</tr>
<tr>
<td>$K_i_1 = \frac{[E \cdot ES_1][I]}{[E \cdot ES]_1}$</td>
<td>Dissociation constant for $S_1$ with $I$</td>
</tr>
<tr>
<td>$K_i_2 = \frac{[E \cdot ES_2][I]}{[E \cdot ES]_2}$</td>
<td>Dissociation constant for $S_2$ with $I$</td>
</tr>
<tr>
<td>$\alpha K_s_1 = \frac{[EI \cdot E][S_1]}{[EI \cdot ES_1]}$</td>
<td>Dissociation constant for $S_1$ with $EI$</td>
</tr>
<tr>
<td>$\beta K_s_2 = \frac{[EI \cdot ES_2][S_2]}{[EI \cdot ES_1]}$</td>
<td>Dissociation constant for $S_2$ with $EI$</td>
</tr>
<tr>
<td>$\alpha K_i_1 = \frac{[E \cdot E][I]}{[EI \cdot ES]_1}$</td>
<td>Dissociation constant for $S_1$ with $I$ and $EI$</td>
</tr>
<tr>
<td>$\alpha \beta K_i_2 = \frac{[E \cdot E][I]}{[EI \cdot ES]_2}$</td>
<td>Dissociation constant for $S_2$ with $I$ and $EI$</td>
</tr>
</tbody>
</table>

\[ TN = \frac{k_{cat}[E \cdot ES_1]}{[E \cdot E]} = \frac{V_{max}}{[E \cdot E]} \]

\[ \frac{v}{[E \cdot E]} = \frac{k_{cat}[E \cdot ES]}{[E \cdot E]+[E \cdot ES]+[E \cdot ES_1]+[E \cdot E]+[EI \cdot ES]+[EI \cdot ES_1]+[EI \cdot ES_2]} \]

Grouping by $S_1$:

\[ E = \frac{K_s_1 \cdot K_s_2}{TN \cdot [S_1]} \left( 1 + \frac{[I]}{K_i_1} + \frac{[I]^2}{K_i_1 \cdot K_i_2} \right) \frac{1}{[S_1]} + \frac{1}{TN} \left( 1 + \frac{K_s_2}{[S_2]} + \frac{K_s_1 \cdot [I]}{\alpha [S_1] K_i_1} + \frac{K_s_2 \cdot [I]}{\alpha \beta K_i_1} \right) \]

Grouping by $S_2$:

\[ E = \frac{K_s_2}{TN} \left( 1 + \frac{K_s_1}{[S_1]} + \frac{K_s_1 [I]}{[S_1] K_i_1} + \frac{K_s_2 [I]}{[S_2] K_i_2} \right) \frac{1}{[S_2]} + \frac{1}{TN} \left( 1 + \frac{[I]}{\alpha K_i_1} \right) \]
Table 2: Summary of Kinetic Constants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Varied PAPS (S1)</th>
<th>Varied 7-HC (S2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[PAPS], μM</td>
<td>----</td>
<td>10</td>
</tr>
<tr>
<td>[7-HC], μM</td>
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<td>----</td>
</tr>
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<td>$K_{s1}$, μM</td>
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<tr>
<td>$K_{s2}$, μM</td>
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</tr>
<tr>
<td>TN (min$^{-1}$)</td>
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</tr>
<tr>
<td>$K_{i1}$, μM</td>
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</tr>
<tr>
<td>$K_{i2}$, μM</td>
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</tr>
<tr>
<td>$\alpha$</td>
<td>----</td>
<td>0.65</td>
</tr>
</tbody>
</table>

See Fig. 5 and Table 1 for the binding scheme and parameter definitions.
Figure 1

bSULT1A1 Activity (%) vs log [CoA], M
Figure 2

- Activity (%) vs. log[CoA], M

- bSULT1A1, P-CoA
- hSULT1A3, P-CoA
- hSULT1A3, CoA

Graph showing activity percentage on the y-axis and log of CoA concentration on the x-axis.
Figure 3

![Graph showing enzyme activity (E/v) and slope vs. [P-CoA] and 1/[PAPS] concentrations.](http://www.jbc.org/)

- **X-axis:** 1/[PAPS], µM
- **Y-axis:** E/v (min.)
- **Bottom graph:** Slope vs. [P-CoA], µM
- **Data points and trend lines** indicate the relationship between enzyme activity and substrate concentrations.
Figure 5

\[ E \cdot E + S_1 \rightleftharpoons E \cdot ES_1 + S_2 \rightleftharpoons E \cdot ES_1 S_2 \overset{k_{m1}}{\longrightarrow} E \cdot EP_2 \rightarrow P_1 + E \cdot EP_2 \rightarrow P_2 + E \cdot E \]

\[ + \quad \downarrow \quad \downarrow \quad \downarrow \]

\[ l \quad I \quad I \]

\[ \downarrow K_i \quad \downarrow \alpha K_i \quad \downarrow \alpha \beta K_i \]

\[ EI \cdot E + S_1 \rightleftharpoons EI \cdot ES_1 + S_2 \rightleftharpoons EI \cdot ES_1 S_2 \]

\[ + \quad l \]

\[ \downarrow K_i \]

\[ EI \cdot EI \]

\[ E \cdot E = \text{dimeric } bSULT1A1, \text{ without ligands} \]

\[ E \cdot ES_i = bSULT1A1 \text{ with one bound PAPS, etc.} \]

\[ S_1 = \text{PAPS} \quad S_2 = \text{phenol} \quad P_1 = \text{phenylsulfate} \quad P_2 = \text{PAP} \quad I = \text{palmitoyl} - \text{CoA} \]
Figure 6
Figure 7
Figure 8
Figure 9

Fluorescence (%) vs. log [Palmitoyl-CoA], µM
Figure 10

Absorbance (280 nm) vs. time (min.)

Data FPLCSCAN

marker dot A6+.04
A5+.06
A4+.08
A1+.1

a b c d
Inhibition of bovine phenol sulfotransferase (bSULT1A1) by CoA thioesters
Gregg R. Tulik, Sundari Chodavarapu, Rick Edgar, Lenore Giannunzio, Amie Langland,
Billie Schultz and Joe D. Beckmann

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