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The Transition of Human Estrogen Sulfotransferase from Generalist to Specialist Using Directed Enzyme Evolution

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Broad specificity is believed to be a property of primordial enzymes that diverged during natural protein evolution to produce highly specific and efficient enzymes. Human estrogen sulfotransferase (SULT1E1) is a broad-specificity enzyme that detoxifies a variety of chemicals, including estrogens, by the transfer of sulfate. To study the molecular basis for the broad specificity of this enzyme and to investigate the process of SULT1E1 specialization, we have adopted a directed enzyme evolution approach. Using two iterative rounds of evolution, we generated SULT1E1 mutants with increased thermostability and narrower specificity from the broadly specific wild-type enzyme. To identify mutants with enhanced specificity, we developed an unbiased screening assay to assess sulfate transfer to three different acceptors in parallel. Such an assay enabled the isolation of SULT1E1 mutants with enhanced or wild-type activity toward an estrogen acceptor and significantly reduced activity for phenol or coumarin type of acceptors, leading to up to 3 orders of magnitude increase in specificity. We found that mutations conferring novel specificity are located in the vicinity of the active site and thus may play a direct role in reshaping the acceptor-binding site. Finally, such mutations resulted in reduced SULT1E1 thermostability, revealing a trade-off between SULT1E1 thermostability and acquisition of novel function.

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Introduction

In the complex environment of the cell, enzymes have generally evolved for high specificity and efficiency, responding to fine-tuning by metabolites. However, not all enzymes share such one enzyme–one substrate efficiency and specificity. For example, detoxification enzymes, found predominantly in the mammalian liver, exhibit exceptionally broad substrate specificity, relatively poor catalytic efficiency and, in many cases, severe inhibition at high substrate concentrations.[1] There are many families of liver broad-specificity enzymes that detoxify a diverse range of endobiotics and xenobiotics by oxidation, reduction, hydrolysis or transfer of different functional groups.[1] A prominent family of detoxification enzymes is the cytosolic sulfotransferase (SULT) family, which detoxifies a variety of substrates by sulfation of acceptor molecules bearing a hydroxyl or amine group.[2–4] SULTs catalyze

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Abbreviations used: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PAP, 3′-phosphoadenosine 5′-phosphate; WT, wild type; 3CyC, 3-cyano-7-hydroxycoumarin; pNP, *p*-nitrophenol.

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the transfer of a sulfate group from the universal 3′-phosphoadenosine 5′-phosphosulfate (PAPS) donor to a variety of acceptor substrates. Sulfation results in inactivation of the majority of acceptors, thus modulating their biological activity and rendering the product more readily excretable. However, in some cases, sulfation can result in the metabolic activation of carcinogens and mutagens. Indeed, natural polymorphism in SULT-encoding genes has been shown to be associated with increased risk of cancer.[5]

In humans, 13 different *SULT* genes that can be divided into four families have been identified: SULT1, SULT2, SULT4 and SULT6.[6,7] Of these, the SULT1 family is the most extensive and is responsible for the sulfation of phenol, thyroid and steroid hormones, as well as a variety of xenobiotics and known drugs.[8] The SULT1E1 enzyme is a prominent SULT1 family member and displays a distinct preference for estrogen acceptors.[9,10] Sulfate transfer to estrogen was shown to result in a loss of estrogen-mediated biological activity due to a lack of binding to the estrogen receptor.[11] In addition, human SULT1E1 is thought to play a prominent role in producing fluctuating levels of active estrogens during the menstrual cycle.[12] Despite differences in substrate preference between the various SULTs, considerable structural homology between the SULTs exists, highlighting the difficulties in understanding the molecular basis for their broad specificity.[8]

Currently, many enzymes have been identified as exhibiting broad specificity and promiscuous activities toward related and unrelated substrates.[13–15] These activities were found to be important "starting points" for the evolution of novel enzymatic activity.[16] However, narrow-specificity enzymes must evolve in situations in which catalyzing alternative reactions becomes disadvantageous and accurate substrate discrimination endows advantage to the fitness of the cell/organism.[17] Acquiring mutations that lead to maintenance or improvement of activity toward one class of substrate but significantly reduce activity toward other class of substrates is a possible mechanism for obtaining high specificity and selectivity in enzymes. This specialization process is extremely difficult to follow in enzyme evolution due to the low pace of natural selection and the difficulties in correlating between structure and function of homologue proteins.[18]

In this work, we consider whether the process of SULT1E1 specialization from a broad- to a narrow-specificity enzyme can be performed *in vitro* using directed evolution approaches.[19,20] We first utilized directed evolution to generate thermostable SULT1E1 mutants and further evolved these mutants for narrow substrate specificity. Using an unbiased screening assay for sulfate transfer to three different acceptors in parallel, we isolated SULT1E1 mutants exhibiting increased activity toward estrogens and dramatically decreased activity toward other chemically distinct substrates. The transition from broad to narrow specificity of the SULT1E1 mutants was accompanied by a decrease in thermostability, highlighting the trade-off between thermostability and evolution of novel protein function.

Results

The main approach for generating SULT1E1 mutants with higher thermostability and specificity is directed evolution (Fig. 1). The directed evolution approach is based on two major steps: (i) the generation of genetic diversity in the gene of interest to obtain large mutant libraries and (ii) screening of these libraries for the desired catalytic or binding activity.[20,21] As a first step toward the generation of SULT1E1 with novel specificity, we used directed evolution to improve SULT1E1 thermostability. It was previously shown that highly thermostable enzymes that readily express in *Escherichia coli* serve as an excellent starting point for further directed evolution for enhanced catalytic activity and novel specificity.[22]

Generation of SULT1E1 gene libraries

An effective approach for the generation of gene libraries that are highly enriched in thermostable mutants is by targeted mutagenesis of residues that deviate from the consensus sequence of the gene family.[23] To identify such residues, we aligned 38 mammalian SULT1 sequences, containing 8 SULT1E1 homologues. We identified 17 different positions that deviate from the SULT1E1 family consensus (Supplemental Table S1 and Supplemental Fig. S1a). Previously, several back-to-consensus mutations in β-lactamase were shown to significantly increase protein thermostability and evolvability.[24] To generate a SULT1E1 gene library containing back-to-consensus mutations, we adapted a recently developed methodology termed ISOR (*i*ncorporation of *s*ynthetic *o*ligonucleotides via gene *r*eassembly[25]) for partial mutagenesis of the targeted positions. This methodology, schematically depicted in Supplemental Fig. S1b, is an adaptation of gene shuffling and allows simultaneous diversification of specific residues by spiking with synthetic oligonucleotides containing the desired mutations during the gene assembly process.[26] Following library generation, sequencing of 10 random SULT1E1 library variants indicated an average of six back-to-consensus mutations per gene. Each library variant carried a random and different subset of mutated residues, with the entire

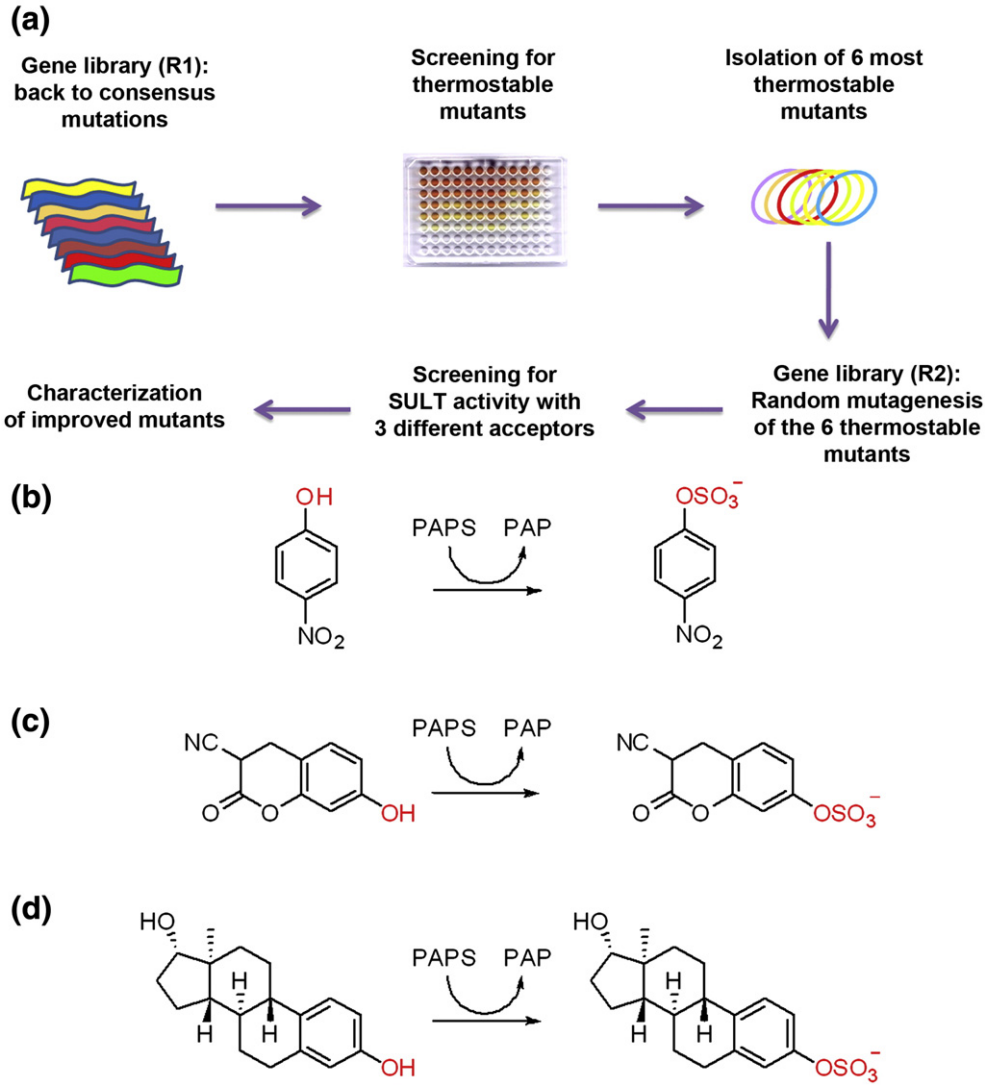

Fig. 1. Scheme describing the directed evolution process and the acceptors used for the analysis of SULT1E1 specificity. (a) The directed evolution process for the generation of SULT1E1 mutants with increased thermostability (R1) and specificity (R2). (b–d) Acceptors used in this study were pNP (b), 3CyC (c) and 17β estradiol (d).

set being represented in the complete library (data not shown).

Screening of the back-to-consensus SULT1E1 library for enhanced thermostability

To facilitate the screening of the SULT1E1 library, we have developed a simple and rapid medium-throughput screening methodology for the transfer reaction of sulfate to 3-cyano-7-hydroxycoumarin (3CyC) (Fig. 1c) based on the quenching of the 3CyC fluorescence signal upon sulfate transfer. This assay is highly sensitive and is performed in a 96-well plate format using crude cell lysates of *E. coli* expressing the mutant libraries. The low background signal from *E. coli* endogenous proteins and the automation of the process allow for the screening of hundreds, up to thousands, of mutants in a relatively short time. We, therefore, transformed the back-to-consensus SULT1E1 library into *E. coli* cells, and single colonies were grown and induced for SULT1E1 protein expression. Following cell lysis, ~600 different mutants were screened for sulfate transfer to 3CyC by following the time-dependent decrease in the 3CyC fluorescence signal. We identified a large proportion (~8%) of positive clones exhibiting wild-type (WT) levels of 3CyC transfer activity. To compare the back-to-consensus approach to the random mutagenesis approach, we also generated a random mutant library from the WT SULT1E1 gene containing an average of three mutations per gene. We analyzed 50 random

Table 1. Mutations and heat inactivation temperatures of newly evolved SULT1E1 variants

SULT1E1[a] variants	List of mutations	Heat[b] inactivation temperature, T_i	Thermal[c] denaturation, T_m
WT	—	42.0 ± 0.4	47.2 ± 0.1
rB6 (R1)	F138Y, G182S, L195M, E211G	55.8 ± 0.3	ND
bE1 (R1)	F138Y, G182S, H224K, E285G	54.6 ± 0.6	57.8 ± 0.1
bG1 (R1)	V73A, N87D, G182S, H224K, L251V, E285G	52.8 ± 0.3	56.3 ± 0.1
IE5 (R1)	E12G, N29D, V73A, N87D, F138Y, G182S, L195M, E211G, E285G	52.1 ± 0.1	ND
rG1 (R1)	E12G, N29D, F138Y, G182S, L251V	51.8 ± 0.9	ND
rG6 (R1)	N29D, L195M, H224K, L251V	48.3 ± 2.4	ND
N35 (R2)[d]	**D65N, K85E, E86K**, F138Y	43.4 ± 0.2	55.4 ± 0.1
SII9 (R2)[d]	E12G, N29D, **D65N, C83R**, N87D, **M98V**, F138Y, G182S, L195M, H224K	43.1 ± 0.5	54.6 ± 0.1
N14 (R2)[d]	**K85E**, **A135T**, F138Y, G182S, L251V	46.3 ± 0.5	ND

ND, not determined.

[a] Back-to-consensus mutations, including E12G, N29D, V73A, N87D, G182S, E211G, H224K, L251V, E285G, F138Y and 195M identified in the first round (R1) of evolution mutants.

[b] Heat inactivation temperatures were measured by testing SULT1E1 residual activity following incubation at different temperatures (see Fig. 3 and Materials and Methods). The heat inactivation temperature was derived by fitting the data to a logistic model (see Materials and Methods).

[c] Thermal denaturation, as measured by CD analysis. Measurements were taken during thermal denaturation upon gradual heating of the samples from 30 °C to 80 °C at a wavelength of 222 nm. The melting temperature (T_m) was derived by fitting the normalized data to a logistic model (see Materials and Methods).

[d] Mutations inserted by random mutagenesis in the second round (R2) of evolution are marked in boldface.

mutants from this library and observed a dramatic decrease in activity of 70–100% for all mutants, relative to the WT SULT1E1. This comparison shows that the back-to-consensus library is significantly more active and enriched in mutants exhibiting WT levels of 3CyC transfer activity relative to the random mutant library.

To further screen the selected mutants for improved thermostability, we tested the top 21 SULT1E1 mutants exhibiting the highest activity for sulfate transfer activity following heat inactivation treatment. The crude cell lysates containing the WT and selected mutants were incubated at 40 °C and 50 °C for 10 min, and the residual sulfate 3CyC transfer activity was measured and compared to the transfer activity noted without heat inactivation. The heat inactivation step significantly reduced the activity of the WT protein and, thus, was optimal for the identification of SULT1E1 mutants showing increased thermostability (Supplemental Fig. S2). We found six mutants exhibiting increased thermostability, relative to the WT protein, and subjected these clones to sequence analysis. Such analysis indicated that, out of the 18 possible mutations in the back-to-consensus library, 11 were identified in the group of selected SULT1E1 mutants (Table 1 and Supplemental Table S1). This result indicates that ~60% of the back-to-consensus mutations that were identified in the group of selected R1 mutants are either beneficial or neutral, leading to enhanced thermostability. Mapping the mutations on the crystal structure of WT SULT1E1[27] revealed that most are scattered on the outer surface of the protein (Fig. 2). Due to the large number of mutations, it is difficult to dissect the contribution of each mutation to the overall thermostability of the protein. However, it is clear that enhanced thermostability is obtained by diverse contributions from several back-to-consensus mutations. Some of these mutations were characterized by substitution of hydrophobic residues to charged residues (Table 1 and Fig. 2), thus resulting in favorable interactions with the polar solvent and reduction of surface hydrophobicity. However, we found two mutations, that is, F138Y and G182S, that are present in the majority of the isolated mutants (Table 1). By the addition of a hydroxyl group to the aromatic ring of the phenylalanine, the F138Y mutation might stabilize the protein due to additional hydrogen bond formation with S48 (see Supplemental Fig. S3).

To accurately measure improvements in thermostability of the different SULT1E1 mutants, relative to the WT protein, we over-expressed the proteins in *E. coli* and purified them by affinity chromatography. The residual activity of the proteins was measured following incubation at a range of temperatures from 30 °C to 65 °C to obtain heat inactivation curves for each of the purified proteins (Fig. 3 and Table 1). We observed an up to 15° increase in the heat inactivation temperature of the SULT1E1 mutants, relative to the WT protein. To further evaluate the effects of the mutations on SULT1E1 thermostability, we utilized CD spectroscopy to monitor the thermal denaturation of the WT protein and selected SULT1E1 mutants. We examined changes in the CD signal at 222 nm, indicative of denaturation of the α-helical structure of SULT1E1 upon gradual heating of the samples from 30 °C to 80 °C. We observed an increase of 10.6 °C and 9.1 °C in the thermostability of two of the first-round mutants, that is, bE1 and bG1, respectively, relative to the WT protein (Supplemental Fig. S4 and

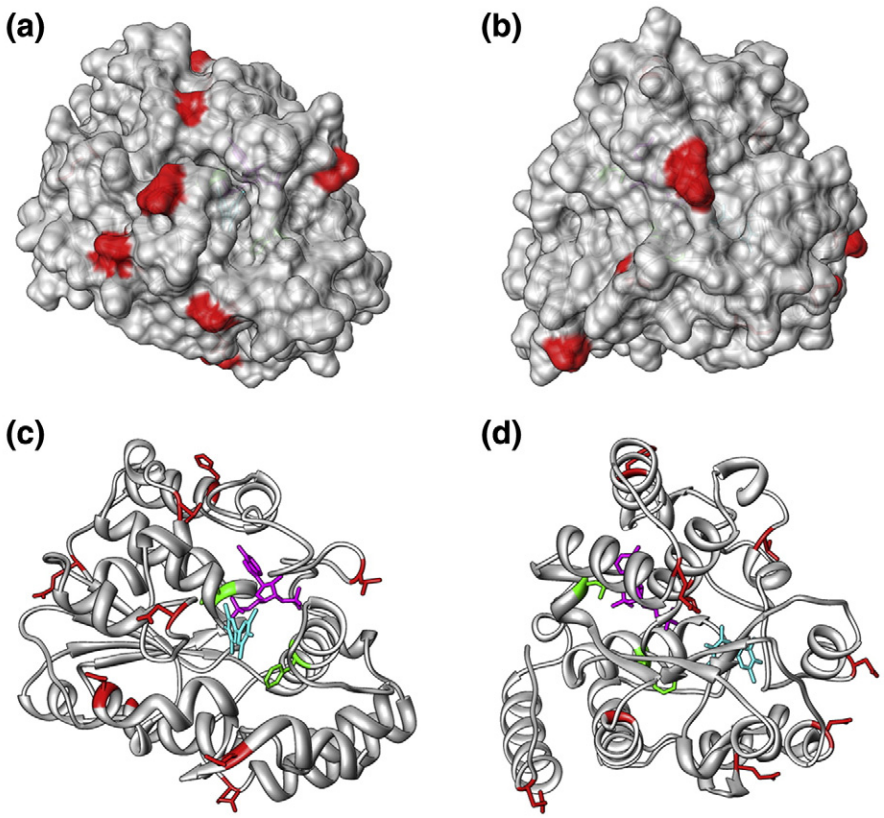

Fig. 2. Surface (a and b) and ribbon (c and d) representations of the human SULT1E1 structure complexed with PAP and hydroxylated polychlorinated biphenyl (OH-PCB; Protein Data Bank code 1G3M). The surface mutations identified in thermostable SULT1E1 mutants (Table 1) are highlighted in red (E12G, N29D, V73A, N87D, G182S, E211G, H224K, L251V and E285G). The buried mutations identified in these variants are highlighted in green (c and d) (F138Y and 195M). The OH-PCB acceptor is shown in blue, and PAP shown in purple marks the location of SULT1E1 active site. The model of the structure was generated using the UCSF Chimera program.

Table 1). The significant increase in heat inactivation temperature, as measured by residual activity, and the melting temperature, as measured by CD spectroscopy, of the first-round mutants highlights the potential of the back-to-consensus mutations in increasing the thermostability of the SULT1E1 protein. It is important to note that we found that the thermal denaturation was irreversible, probably due to aggregation of the denaturated state. Thus, our analysis does not allow for a determination of the true levels of unfolding according to the classical reversible two-state model for protein denaturation and renaturation.

Generation of second-round SULT1E1 random mutant libraries

To select for SULT1E1 mutants with novel substrate specificity, we used the top six highly thermostable SULT1E1 genes (Table 1) as templates for the generation of second-round random mutant libraries. Random mutations were generated using nucleoside wobble base analogues, inserted during the PCR amplification process.[28] Several libraries were generated at different mutation rates, and the library pools were analyzed for sulfate transfer to 3CyC. We found that an average mutation rate of three mutations per gene results in a residual activity of 30–50% in the SULT1E1 library pool (data not shown). This library was further screened for mutants exhibiting novel specificity (see below).

Screening of the second-generation SULT1E1 library for mutants with novel specificity

To identify and isolate SULT1E1 mutants with narrow specificity in an unbiased manner, we screened the SULT1E1 random mutant library with three different acceptor substrates. Around 400 SULT1E1 mutants were screened for transfer activity to 3CyC and *p*-nitrophenol (pNP) acceptors (Fig. 1b and c) in parallel in a multi-well plate format. pNP was used as a representative of phenolic acceptors, while 3CyC served as a representative of two larger fused ring acceptors.[8] In addition, we screened the mutants for transfer activity to the

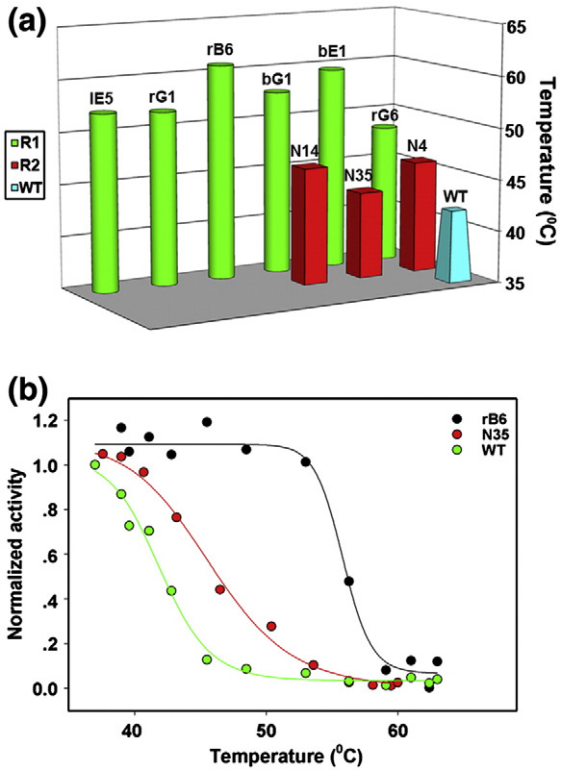

Fig. 3. Heat inactivation temperatures and curves for WT and SULT1E1 mutants isolated after the first round (R1) and the second round (R2) of evolution. (a) Bar graph of the heat inactivation temperatures for the WT (blue) and selected mutants from the first round (R1, green) and from the second round (R2, red) of evolution. (b) Heat inactivation curves for WT (green) and selected SULT1E1 mutants isolated after the first round of evolution (rB6, black) and after the second round of evolution (N35, red). The heat inactivation temperatures derived from the fits are presented in Table 1.

bulky 17β estradiol estrogen acceptor labeled with fluorescein (17FE; Supplemental Fig. S5). We synthesized the fluorescently labeled estrogen acceptor 17FE by conjugating a fluorescein moiety to position 17 of estrone via a hydrazine linker, in order to minimize any steric clash with the SULT1E1 protein (Ref. 29; Supplemental Note). The design of 17FE was based upon careful examination of the marine SULT1E1 crystal structure complexed with estrogen.[9] Screening with 17FE was performed by thin layer chromatography and visual monitoring of the sulfated product on a silica plate by exploiting the decreased mobility of sulfated 17FE, relative to the unmodified 17FE (Supplemental Fig. S6).

Characterization of SULT1E1 mutants with novel specificity

The screening of the SULT1E1 library for transfer to multiple acceptors enabled the identification and isolation of three mutants, N14, N35 and SII9, with novel specificity (Table 2). Such mutants exhibited a WT level of transfer activity toward 17FE and decreased levels of activity toward the 3CyC and pNP acceptors. To quantitatively characterize the activity of the mutants, relative to the WT protein, we over-expressed these mutants in *E. coli* and purified them by affinity chromatography. The catalytic activities of the different mutants were measured using steady-state kinetics with the pNP and 3CyC acceptors. Unfortunately, due to the high K_m of SULT1E1 toward the promiscuous pNP and 3CyC acceptors, the limited solubility and the high extinction coefficient of these acceptors, we could not accurately determine the K_m of the different SULT1E1 variants for these acceptors. Thus, the data were analyzed using the Michaelis–Menten equation at low acceptor concentrations to obtain k_{cat}/K_m values for each mutant (Fig. 4 and Table 2). In addition, we established an HPLC-based assay to monitor the activity of WT and mutant SULT1E1 with the unmodified 17β estradiol estrogen acceptor (Fig. 1d). This assay is based on monitoring the time-dependent increase in 3′-phosphoadenosine 5′-phosphate (PAP) levels generated from the PAPS donor during the sulfate transfer reaction (Fig. 4c). Due to the relatively low sensitivity of the HPLC detection and the low K_m of estrogen,[27] we could determine the activity of the SULT1E1 variants only at the maximal velocity (V_{max}). We examined the activity of SULT1E1 at three different estrogen

Table 2. Kinetic parameters of the newly evolved SULT1E1 variants

SULT variants	Sulfate transfer to pNP[a]	Sulfate transfer to 3CyC[a]	Sulfate transfer to 17β estradiol[b]
	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	V_{max} (nmol min^{-1} mg^{-1})
WT	151	694	10
N35	59 (0.39)	179 (0.26)	16 (1.6)
N14	10 (0.07)	65 (0.09)	27 (2.7)
SII9	0.9 (0.006)	0.11 (0.0002)	56 (5.6)

Kinetic analysis was performed for SULT1E1 mutants with altered specificity isolated after the second round of evolution.

[a] Sulfate transfer to pNP or 3CyC was measured as the time-dependent decrease in pNP or 3CyC absorbance following sulfate transfer (see Materials and Methods). The k_{cat}/K_m parameters were calculated from a linear fit to MM plot at low acceptor concentrations (see Fig. 4).

[b] Sulfate transfer to 17β estradiol was monitored using an HPLC-based assay by following the increase in PAP generation following sulfate transfer (for details, see Materials and Methods).

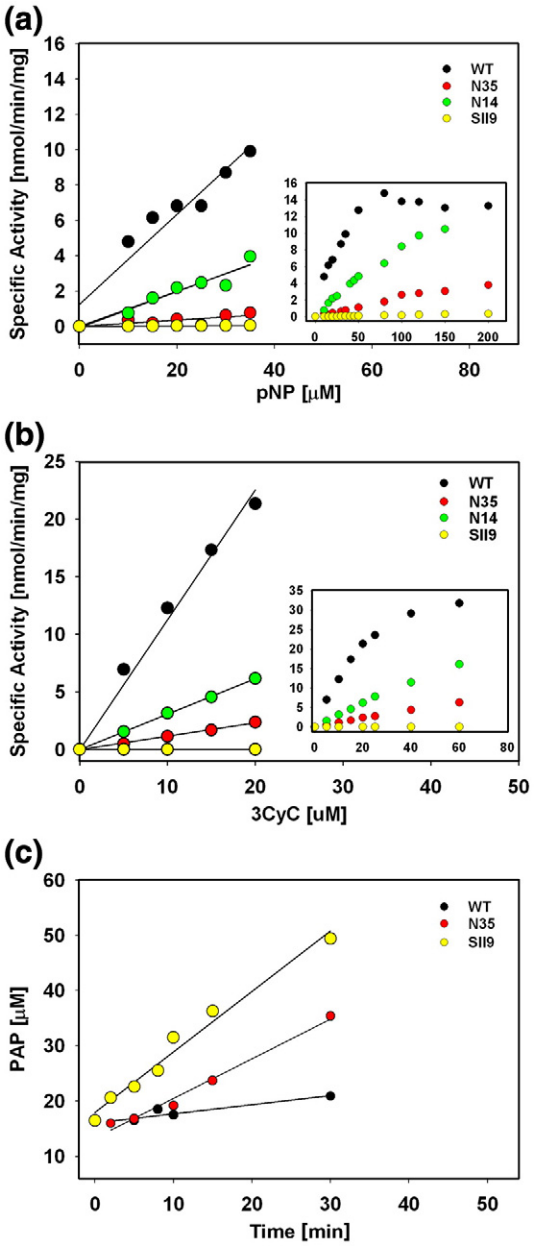

Fig. 4. Michaelis–Menten plots for the sulfate transfer activity of the WT protein and the N35, SII9 and N14 SULT1E1 mutants to pNP (a) and 3CyC (b). The k_{cat}/K_m parameters, derived from the linear fit to the data at low acceptor concentrations, are presented in Table 2. (c) Sulfate transfer activity of the WT, N35 and SII9 mutant proteins to 17β estradiol, as analyzed by the time-dependent increase in PAP concentration. PAP concentrations were assessed using HPLC (see Materials and Methods for details) at a 17β estradiol concentration of 45 μM. Similar rates were observed at 15 μM or 100 μM acceptor concentrations (data not shown).

concentrations (15 μM, 45 μM and 100 μM) and observed similar initial sulfate transfer rates for WT SULT1E1 and for the N35 and the SII9 mutants at these concentrations (Fig. 4). These results indicate that, at the estrogen concentrations tested, we measured true V_{max} rates for the different variants, in good correlation with the low K_m value reported for WT SULT1E1. Such kinetic analysis indicated that the different SULT1E1 mutants exhibit minor increases in transfer activity to the estrogen acceptor (up to a 5-fold increase in V_{max}) and a dramatic decrease in transfer activity to the 3CyC and pNP acceptors (e.g., an up to 6300-fold decrease in k_{cat}/K_m for 3CyC was noted; Table 2). We also qualitatively analyzed the sulfate transfer activity of the WT and mutant enzymes to 17FE, observing similar transfer activity in all samples (Supplemental Fig. S6). Overall, we estimate an increase of up to 3 orders of magnitude in the specificity of the mutants, relative to the WT enzyme, mainly due to a decrease in transfer efficiency to the promiscuous pNP and 3CyC acceptors (Table 2).

Following library screening, we could not identify any SULT1E1 mutants that exhibited reverse specificity, namely, increased transfer activity to the pNP or 3CyC acceptors and reduced activity to the estrogen acceptor. The minor effect of the SULT1E1 mutations on transfer activity to the estrogen acceptor demonstrates the robustness of the SULT1E1 physiological activity to mutations that significantly affect transfer activity to the promiscuous pNP and 3CyC acceptors.[16] In addition, the generation of SULT1E1 mutants with novel specificities indicates structural differences in the binding modes of the SULT1E1 active site toward the different acceptors.[27,30] To further examine whether the mutations leading to novel substrate specificity affect SULT1E1 thermostability, we characterized the thermostability of the different mutants using heat inactivation analysis (Fig. 3). Interestingly, these mutants displayed lower thermostability, as reflected in differences in heat inactivation temperature, relative to first-generation highly stable precursor mutants. Similar overall results were obtained following analysis of the relative thermostability of the SII9 and N35 mutants using CD spectroscopy (Supplemental Fig. S4 and Table 1). We found that the melting temperatures of the second-round mutants, as measured by CD, are higher than the heat inactivation temperatures measured using the activity assay, indicative of the high sensitivity of the active site to elevated temperatures, relative to the rest of the protein (Table 1). Thus, the C83R, K85E and E86K mutations located in the vicinity of the active site can affect activity following heat inactivation (T_i; Table 1) but have less effect on the overall stability of the protein, as measured by CD (T_m; Table 1). Overall, these results indicate a trade-off between thermostability and acquisition of a novel protein function (Table 1 and Fig. 3). To further examine the trade-off between novel specificity and thermostability observed in the

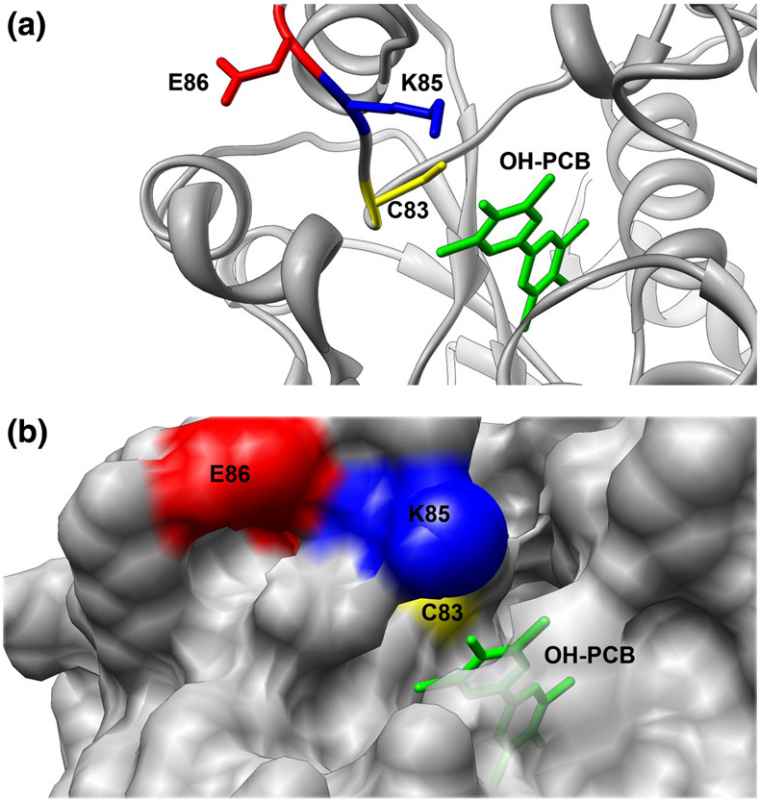

Fig. 5. Mapping mutations identified in SULT1E1 mutants with increased specificity isolated after the second round of evolution (for list of mutations, see Table 1) onto the model of the crystal structure of human SULT1E1 crystallized with PAP (green) and OH-PCB (green; Protein Data Bank code 1G3M). The C83, K85 and E86 positions are represented in yellow, blue and red, respectively. The model of the structure was generated using the UCSF Chimera program and is presented as a secondary structure (a) or as a surface view (b).

selected R2 mutants, we examined the thermostability of 16 random mutants from the R2 library that exhibited 3CyC activity similar or higher than that of the WT. We expect that if the second round of mutagenesis leads to invariable decrease in thermostability, then the vast majority of R2 mutants will exhibit reduced thermostability, since the 16 mutants were selected from the R2 library based solely on 3CyC transfer activity. To examine the relative thermostability of these mutants, we analyzed the sulfate transfer activity of all proteins without heat inactivation and following 10 min of incubation at 45 °C, 50 °C and 60 °C (Supplemental Fig. S7). We calculated the ratio of activities following heat inactivation at 50 °C and in the absence of heat inactivation to estimate the relative thermostability of these mutants (Supplemental Fig. S7b). We found that 9 out of the 16 proteins (56%) exhibit equal or increased thermostability, relative to the R1 mixture, following incubation at 50 °C (Supplemental Fig. S7b). The observation that the majority of mutants exhibit equal or higher thermostability, relative to the R1 variants, supports the trade-off between novel specificity and thermostability in the evolved R2 SULT1E1 mutants.

Sequencing of the narrow-specificity SULT1E1 mutants revealed that some of the mutations affecting substrate specificity are located in the vicinity of the active site. We found that the C83R, K85E and E86K mutations (Table 1) are located in a loop region covering the acceptor-binding site (Fig. 5). These mutations can significantly reshape the SULT1E1 active site to allow binding and turnover of the estrogen acceptor while abolishing the productive binding of the chemically distinct pNP and 3CyC acceptors. Previously, the K85 residue was identified in the vicinity of the 17 β estradiol acceptor, with its mutation to alanine resulting in a change in specificity.[27,31] We found that C83, K85 and E86 are not conserved in any other SULT isoform (data not shown), and thus, the identified mutations in SULT1E1 cannot be mapped to other SULT isoforms and explain their altered specificity. This result highlights the difference between SULT1E1 and other SULT isoforms.

Discussion

In this work, we have used directed evolution methodologies to generate SULT1E1 mutants with increased thermostability and narrow specificity. We first evolved SULT1E1 for increased thermostability and performed a subsequent round of directed evolution for the generation of mutants with narrow specificity. We chose to generate thermostable SULT1E1 mutants prior to the evolution of mutants with increased specificity to buffer the probable destabilizing mutations that can lead to novel SULT1E1 function. Indeed, we found that second-

generation (R2) mutations conferring novel specificity resulted in a decrease in thermostability (Table 1 and Fig. 3). Taking into account the relatively low thermostability of WT SULT1E1 (T_i of 42 °C; Table 1), a prior stabilization step of the enzyme could be essential in obtaining folded and stable mutants with novel specificity. In addition, we used an unbiased screening assay for sulfate transfer to three different acceptors to identify mutants with altered specificity. Thus, each mutant was screened with pNP, 3CyC and 17FE to allow for identification of mutants exhibiting low activity with pNP and 3CyC but increased activity for the estrogen acceptor (Fig. 4). Previous works have shown that the evolution of new function and protein stability trade-off with each other.[22] Notable examples are the TEM-1 β-lactamase mutations that confer resistance for third-generation antibiotics; these are destabilizing and are compensated for by a single M182T mutation.[32] In addition, stabilized P450 and TEM-1 mutants showed higher evolvability by accepting larger varieties of mutations, leading to novel function, without substantial disruption of the native folding of the protein.[24,33]

One efficient way to increase protein stability is to insert back-to-consensus mutations into the protein sequence. Such an approach specifically targets residues that deviate from the family consensus to abolish any possible deleterious effect of these residues on protein function.[24] Indeed, focused SULT1E1 libraries containing different combinations of back-to-consensus mutations were highly enriched in variants exhibiting increased thermostability (Table 1). We found that increased SULT1E1 thermostability was probably a result of several stabilizing mutations rather than one global suppressor, as in the case with the M182T mutation in TEM-1.[24,32] Previously, consensus and ancestral mutations were systematically studied and found to significantly elevate the thermostability of target proteins.[34] In addition, in neutral drift experiments addressing TEM-1 in which the protein was repeatedly mutated followed by selection for the native function, back-to-consensus mutations were identified as global suppressors of destabilizing mutations.[24]

The accepted protein evolution theory, first formulated by Jensen, claims that the broad specificity and promiscuous activities of enzymes provide many starting points for the evolution of new protein functions.[13] Following gene duplication, the enzyme specialization process requires the elimination of certain activities while strengthening others in order to generate a panel of highly specialized modern enzymes.[17,35] The SULT enzyme family is a classic example of such an ensemble of enzymes, each presenting relatively high activity for their preferred substrates and low activities for overlapping promiscuous substrates. In this respect, SULT family members may represent intermediates during the specialization process, with their ancestor having been a complete generalist with no preference for any SULT acceptor. The laboratory evolution of SULT1E1 enabled further specialization of the enzyme to increase specificity and selectivity. In recent years, promiscuous enzyme activities were shown to serve as a "springboard" for the evolution of new activities and to promote protein evolvability.[14–16,36] Such an increase in promiscuous activity could be beneficial in cases where dramatic changes under environmental or internal conditions take place, thereby allowing the organism to adapt to the novel conditions. Several directed enzyme evolution experiments enabled the increasing of low promiscuous activities by orders of magnitude by both *in vitro* and *in vivo* selection.[16,37] These improvements were achieved, in the majority of cases, by direct screening/selection for activity toward the promiscuous substrate.

In *Nature*, however, increases in promiscuous side activities can be deleterious due to the generation of toxic substances in organisms containing thousands of chemically distinct metabolites. Thus, elimination of promiscuous side activities during the enzyme specialization process is probably a common step during enzyme evolution following gene duplication.[17] Here, using an unbiased screening assay for multiple SULT1E1 acceptors, we could examine the most probable changes in SULT1E1 specificity following the introduction of random mutations into the SULT1E1 gene. Our directed evolution study of SULT1E1 indicates that specialization by elimination of promiscuous activities is much more common than are improvements in promiscuous SULT1E1 side activities. In fact, we could not identify any mutant with significant improvement in promiscuous activities in the screened library members. This phenomenon of specialization may be common to other broad-specificity enzymes exhibiting main and promiscuous side activities.[1]

Materials and Methods

Plasmids and bacterial strains

The *E. coli* DH5α and Clooni (Lucigen) strains were used for cloning. The *E. coli* BL21 (DE3) strain was used for protein expression and purification. Human liver cDNA was used as a template for the amplification of the human *SULT1E1* gene. The amplified gene was cloned into the bacterial vector pET32tr [a version of pET32 (Novagen) with truncated thioredoxin], using the NdeI and XhoI sites.

Synthetic shuffling

SULT1E1 amino acid sequences from eight mammalian species (see Supplemental Fig. S1) were aligned using the

MUSCLE software†. Oligonucleotides for synthetic shuffling were 31–33 bases in length and contained the back-to-consensus single mutation (sequence was chosen according to the *E. coli* codon usage table) flanked by 15 bases at both ends, complementary to the *SULT1E1* gene. The *SULT1E1* gene was PCR amplified, and 6–10 μg of the PCR product was digested by DNase I. Fragments of the approximate size of 80–120 bp were extracted and purified using a QIAEX II Gel Extraction Kit (Qiagen) according to manufacturer's instructions. To incorporate the oligonucleotides, we mixed the purified fragments with 5–10 nM oligonucleotides and reassembled them to form the mutated SULT1E1 variants by assembly PCR. The assembly PCR mix contained the fragmented DNA, the different oligonucleotides and Taq polymerase mix (DyNAzyme; Finnzyme). The product of the assembly PCR was directly used as a template for the amplification of the full-length SULT1E1 library containing the oligonucleotides, digested with NdeI and XhoI and cloned into the pET32tr vector, as described above.

Random mutagenesis

Target sequences were randomly mutagenized by PCR performed with the mutagenic dNTP analogues: 8-oxo-dGTP (8-oxo-2′-deoxyguanosine-5′-triphosphate) and dPTP {6-(2-deoxy-*d*-ribofuranosyl)-3,4-dihydro-8*H*-pyrimidino-[4,5-*c*][1,2]oxazin-7-one-triphosphate}. Mutagenesis stringency was controlled by the number of PCR cycles performed and dNTP analogue concentration. The reaction mix consisted of the following: 2.5 U Biotaq DNA polymerase (Bioline), 1× NH_4 buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 10 ng of plasmid containing the target sequence and 50 nM dNTP analogues (Jena Bioscience). Following PCR, the reaction mix was directly incubated for 2 h with 0.7 μl DpnI at 37 °C to digest the non-mutated template gene. The reaction mix was then purified using a PCR purification kit (Qiagen), and 1 μl of the purified mixture was taken for a second PCR amplification, using nested primers in a standard PCR amplification. Amplification products were analyzed using 1% agarose gel electrophoresis. PCR products were purified and cloned as described above.

Library expression and screening

Single *E. coli* BL21 (DE3) colonies transformed with library plasmids or control colonies were picked to inoculate 600 μl LB media containing 100 μg/ml of ampicillin in 96-deep-well plates (Nunc). The plates were incubated with shaking at 37 °C overnight using a plate-shaking incubator (Inkubator 1000; Heidolph). The cells were diluted 1:50 in fresh selective LB media to a final volume of 600 μl in 96-well plates. The plates were incubated with shaking at 37 °C until an OD_{600} of 0.4–0.6 was reached and were induced with 0.1 mM IPTG (Calbiochem). Plates were incubated with shaking at 30 °C for an additional 5 h and centrifuged at 4000 rpm for 15 min (Centrifuge 5810R; Eppendorf). Media were discarded, and the cells were resuspended in 150 μl lysis buffer [lysis buffer: 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2% Triton X-100, 2 U/μl DNase I (NEB), 1 mg/ml lysozyme and 20 mM Hepes (pH 7.5)]. The plates were incubated with shaking at 37 °C for 30 min, followed by 15 min of centrifugation at 4000 rpm. The supernatant (120 μl) was transferred to 96-well V-shaped plates (Nunc) and stored at 4 °C for analysis (see sulfotransferase activity assay, below).

Protein expression and purification

Single colonies were picked to inoculate 5 ml of LB media containing ampicillin (100 μl/ml) and were grown for 16 h at 37 °C with shaking at 250 rpm. Culture was then diluted 1:100 into fresh selective LB media and incubated with shaking at 37 °C until an OD_{600} of 0.4–0.6 was reached and induced with 0.1 mM IPTG (Calbiochem) for an additional 18 h at 20 °C. Cells were harvested and centrifuged for 10 min at 6000*g* , resuspended in 30 ml of 20 mM Hepes, pH 7.5, and recentrifuged for 20 min to remove any access LB media. Following centrifugation, the cells were resuspended in binding buffer: 1 M NaCl, 40 mM imidazole, 1 mg/ml lysozyme and 20 mM Hepes (pH 7.4). The buffer was added at a ratio of 10 ml/g of cells, and the solution was incubated with stirring at room temperature until homogeneity was reached. Mechanical lysis was performed by sonication using an 8-min program (20 s on-cycle and 40 s off-cycle) and centrifuged, and the cleared supernatant was loaded onto a pre-equilibrated column containing 2 ml Ni-NTA resin (Qiagen). The columns containing the lysates were gently shaken by inversion for 30 min at 25 °C. The resin was then washed, and SULT1E1 was eluted in 1-ml fractions upon addition of elution buffer. Fractions containing SULT1E1 were analyzed by SDS-PAGE, pooled and dialyzed for 16 h against storage buffer [7 mM $MgCl_2$, 1.5 mM DTT, 20% glycerol and 40 mM Hepes (pH 7.5)] at 4 °C. Protein concentration was determined with a BCA protein assay kit (Pierce). The protein solutions were stored in 200-μl aliquots of 20–40 mg/ml at −20 °C. Wash and elution buffers were based on 500 mM NaCl and 20 mM Hepes, pH 7.4, and supplemented with imidazole, according to the manufacturer's recommendations.

Sulfotransferase activity assays

Clear lysates or purified proteins were added to a reaction mix consisting of the pNP or 3CyC acceptors at different concentrations [1 mM PAPS, 7 mM $MgCl_2$, 1.5 mM DTT and 20 mM Hepes (pH 7.5)] to a final volume of 200 μl. The decrease in absorbance (pNP and 3CyC, 405 nm and 408 nm, respectively) or fluorescence (3CyC, 408-nm excitation and 450-nm emission) was monitored every 15–40 s, for at least 20 min, using an ELISA plate reader (Infinite-200; Tecan). Analysis was performed using the SigmaPlot software. For heat inactivation analysis, the clear lysates or the purified protein was incubated for 15 min at the desired temperature using a DNA Engine Peltier Thermal Cycler (Bio-Rad) and then assayed as described above.

Thermal denaturation analysis by CD spectroscopy

SULT1E1 variants at a concentration of 2.8 μM in phosphate-buffered saline were denatured by raising the

† http://www.drive5.com/muscle/

temperature from 30 °C to 80 °C at a heating rate of 0.25 °C/min, a pitch of 1.0 °C and a delay time of 180 s using a JASCO Peltier PTC 423S apparatus. The CD signal at 222 nm was measured during the denaturation process using a JASCO J815 CD spectrometer. Full spectrum (190 –260 nm) measurements were taken at 20 °C and 80 °C to determine the level of SULT1E1 secondary structure. The melting temperature (T_m) was derived by fitting the normalized data to a logistic model using the SigmaPlot software.

Thin layer chromatography

Crude lysate or purified protein was added to a reaction mixture containing 1 mM PAPS and 0.5 mM 17FE to a final volume of 10 μl, and the mixture was incubated at 25 °C. Aliquots of 1 μl were removed at different time points of the reaction and spotted onto silica gel plates (Merck), dried and transferred to the mobile phase (7:3, chloroform:methanol). The plates were removed when the mobile phase was observed to reach ~80% of the plate length. 17FE was visualized using ImageMaster (VDS).

HPLC assay

Pure protein was added to a reaction mix containing 15–100 μM 17β estradiol, 100 μM PAPS, 7 mM $MgCl_2$, 1.5 mM DTT and 20 mM Hepes (pH 7.5) in a 2-ml volume. At different time points, 60 μl of sample was removed, and the reaction was terminated by freezing the samples in liquid nitrogen. For HPLC separation, 25 μl of each sample was injected into a Surveyor Plus HPLC System (Thermo Scientific), equipped with a Jupiter C18 5-μm (250 mm × 4.6 mm) column at a flow rate of 1.4 ml/min, using a mobile-phase linear gradient of 10 mM potassium phosphate, pH 3.5 (solvent A), and CH_3CN containing 0.1% trifluoroacetic acid (solvent B). Analysis was performed using the ChroQuest 4.2 software.

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Supplementary Data

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