

A Stereoselective Inverting *sec*-Alkylsulfatase for the Deracemization of *sec*-Alcohols

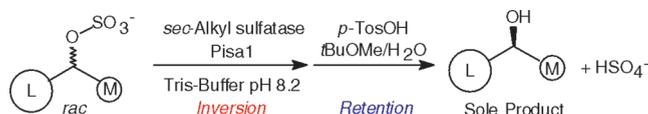
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ABSTRACT



A metallo- β -lactamase-type alkylsulfatase was found to catalyze the enantioselective hydrolysis of *sec*-alkylsulfates with strict inversion of configuration. This catalytic event, which does not have an analog in chemocatalysis, yields homochiral (*S*)-configured alcohols and nonreacted sulfate esters. The latter could be converted into (*S*)-*sec*-alcohols as the sole product in up to >99% ee via a chemoenzymatic deracemization protocol on a preparative scale.

Sulfatases are a heterogenic group of enzymes which catalyze the cleavage of the sulfate ester bond yielding the corresponding alcohol and hydrogen sulfate.^{1–4} In contrast to the majority of hydrolases,⁵ which do not alter the stereochemistry of the substrate during catalysis, the stereochemical course of sulfate ester hydrolysis may proceed via cleavage of the S–O or the C–O bond of a *sec*-alkyl sulfate going in hand with retention or inversion of the stereogenic carbon atom, respectively (Scheme 1).¹

Recently, sulfatases were reclassified based on their mode of catalysis.⁶ Aryl and carbohydrate sulfatases act on sulfated carbohydrates and steroids.⁴ These enzymes possess a highly conserved -C/S-X-P-X-A-X₄-T-G- consensus motif,² which codes for a Cys or Ser residue within the active site. The latter is posttranslationally modified into a hydrated α -formylglycine moiety, which attacks the S-atom of the sulfate ester going in hand with S–O bond cleavage while the absolute configuration at C is retained.⁷ On the other hand, sulfatases belonging to the Fe²⁺-dependent group of dioxygenases oxidatively cleave a sulfate ester at the expense of α -ketoglutarate as an electron acceptor to yield an aldehyde and inorganic sulfate, leading to destruction of the stereogenic center at C.⁸ The third class

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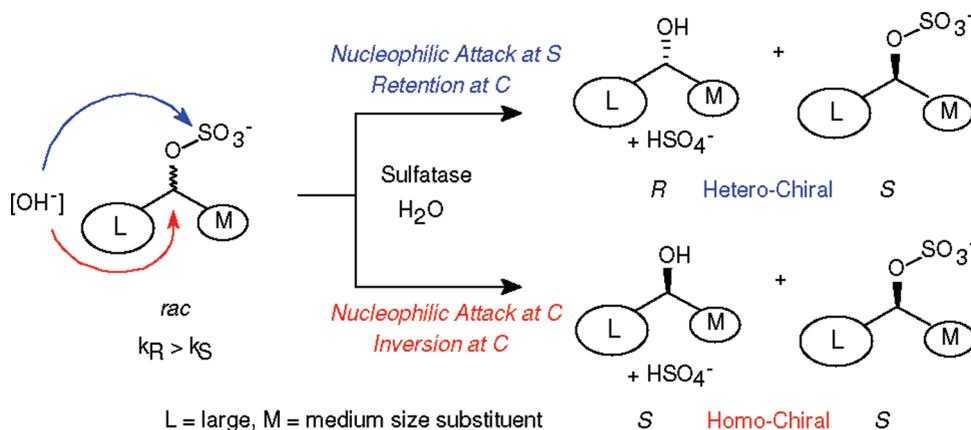
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Scheme 1. Stereochemical Course of Retaining and Inverting Alkylsulfatases



of sulfatases is related to metallo- β -lactamases and (to date) harbors only a single member: Sodium dodecyl sulfatase (SdsA1).⁶ Since SDS is an achiral *prim*-sulfate ester, the stereochemical course of SdsA1-hydrolysis is not ‘visible’. However, since the nucleophilic water molecule (W2 in the crystal structure 2cfu) strongly interacts with the sulfur atom of the substrate surrogate inhibitor 1-decanesulfonate, retention at carbon was assumed.⁶

Inverting alkylsulfatases⁹ were previously studied in *Pseudomonas* C12B,¹⁰ *Comamonas terrigena*,¹¹ and *Rhodococcus ruber* DSM 44541,¹² but due to the lack of biochemical and structural data the mechanism of inverting alkylsulfatases remained unknown. In the search for a well-characterized stable *sec*-alkylsulfatase, which would allow the design of a deracemization process for *sec*-alcohols via enantioconvergent hydrolysis of the corresponding sulfate esters, an extended whole-cell screening for *sec*-alkylsulfatase activity was conducted, which revealed *Pseudomonas* sp. DSM 6611 as the most promising candidate.¹³

Chromatographic protein purification followed by tryptic digestion and peptide mass fingerprinting allowed assignment of the obtained peptide masses to the predicted open reading frames using the full genomic sequence of the strain.¹⁴ The protein band with the lowest mobility on SDS-PAGE (termed Pisa1, identified by *de novo*

sequencing and peptide matching) surprisingly turned out to be a homologue of SdsA1.¹⁵ The Pisa1 gene was amplified by PCR, cloned, and expressed in an *E. coli* BL21 strain with a C-terminal hexa-histidine tag. Pisa1 displayed the desired catalytic properties: (*R*)-2-Octyl sulfate (>99% ee) was quantitatively hydrolyzed to yield (*S*)-2-octanol (>99% ee) through strict inversion of configuration, whereas the (*S*)-enantiomer was completely unreactive. Hydrolysis of *rac*-2-octyl sulfate ceased at 50% conversion to furnish a homochiral product mixture of (*S*)-2-octanol and unreacted (*S*)-2-octyl sulfate, indicating perfect enantioselectivity ($E > 200$).

Although Pisa1 shares a 44% sequence identity with SdsA1 the substrate preference of both proteins differs significantly: Based on k_{cat} and K_M values, SdsA1 has a 150-fold affinity for the *prim*-sulfate ester **1a**, whereas Pisa1 has a pronounced (190-fold) opposite preference for the *sec*-sulfate ester analog **2a**. Hence, Pisa1 is the first inverting *sec*-alkylsulfatase that is characterized on a molecular level.^{15,16}

The stereochemical course of hydrolysis for both enzymes was investigated in detail using unlabeled 1-octyl-**1a** and *rac*-2-octyl sulfate (**2a**) in ¹⁸O-enriched buffer (label >98%, Scheme 2, part A). GC-MS analysis of the formed 1-octanol (**1b**) and (*S*)-2-octanol (**2b**, ee >99%) showed complete incorporation of the ¹⁸O-label in the product within analytical limits, proving C–O bond cleavage in both cases.

The nucleophilic attack of (formal) [OH⁻] at the chiral carbon atom of an alkyl sulfate ester as exerted by alkylsulfatases SdsA1 and Pisa1 leading to inversion of configuration at C is a remarkable catalytic event, which does not have a direct counterpart in chemical catalysis. The latter event would generate SO₄²⁻, which is a very poor leaving group.¹⁷ Hence, inverting nucleophilic hydrolysis of sulfate

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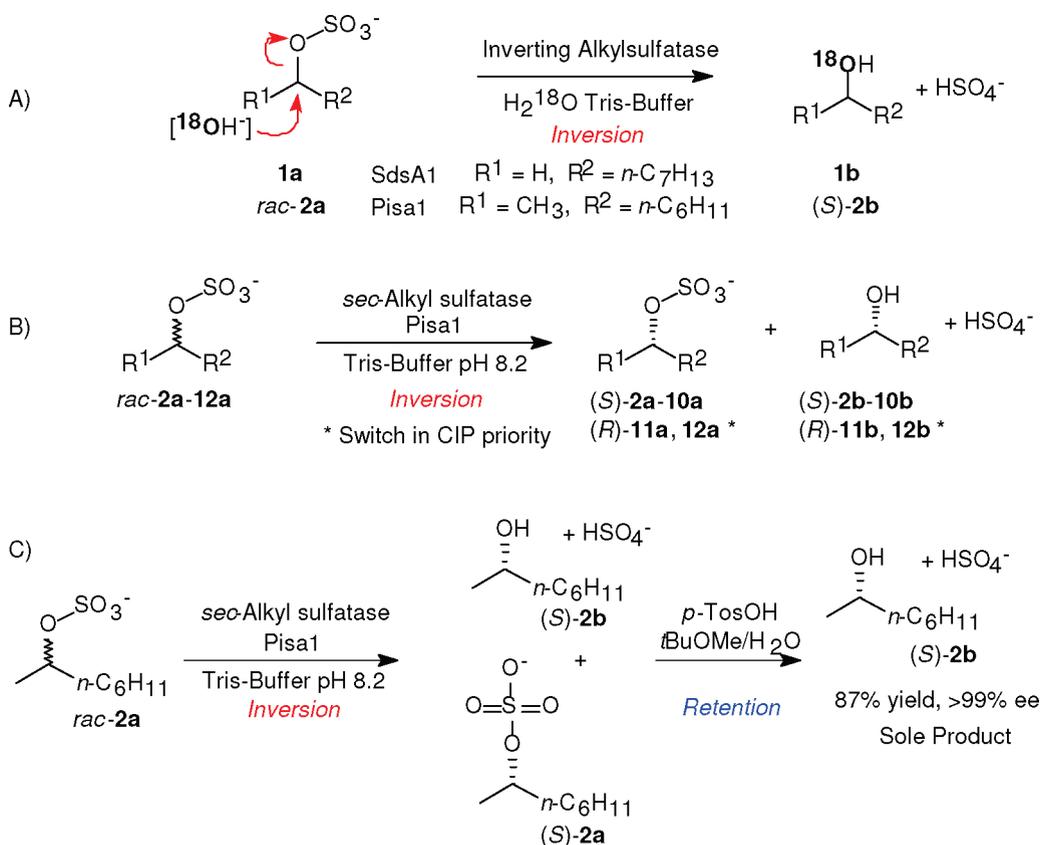
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(15) The sequence was submitted to the European Nucleotide Archive (accession #: FR850678 – confidential until 01.07.2011). No measurable sequence similarities of Pisa1 with other sulfatases, such as the α -ketoglutarate-dependent alkylsulfatase AtsK from *Pseudomonas putida*, the arylsulfatase PAS from *P. aeruginosa*, and the human steroid sulfatases HArS, N-acetylgalactosamin 4-sulfatase, or cerebroside-3-sulfate sulfatase, could be detected ($\leq 10\%$).

(16) The coordinates of the crystal structure of Pisa1 (2.7 Å resolution) are available from the PDB (code 2YHE); A detailed study on the structural and mechanistic aspects of Pisa1 will be published: Knaus, T.; Schober, M.; Kepplinger, B.; Gadler, P.; Macheroux, P.; Faber, K.; Wagner, U. G. Manuscript in preparation.

(17) Being the anion of the weak acid HSO₄⁻ ($pK_a = +1.9$ to $+2.7$).

Scheme 2. Proof of Inversion for Pisa1 and SdsA1 via ^{18}O -Labeling (A), Substrate Spectrum of Pisa1 (B), and Deracemization of *rac*-**2a** (C)



esters by hydroxide, acetate, or methoxide proceeds extremely slowly and is not feasible for preparative purposes.^{18,19}

In order to facilitate the departure of the sulfate moiety, it has to be converted into a good leaving group, i.e. HSO_4^- rather than SO_4^{2-} .²⁰ Consequently, acid-catalyzed *sec*-sulfate ester hydrolysis is a fast process, which proceeds through retention at C.^{21,18} Based on the crystal structure of Pisa1,¹⁶ an acid–base-type mechanism for the inverting enzymatic alkyl sulfate hydrolysis can be proposed (Figure 1).

(i) The anionic substrate is positioned by a sulfate binding site consisting of positively charged (Arg328/323) and H-bonding residues (Asn318, Thr321); (ii) a highly conserved ‘nucleophile site’ consisting of a binuclear Zn^{2+} -binding cluster (Asp310/183, His179/181, Glu291) activates a water molecule to provide the nucleophile

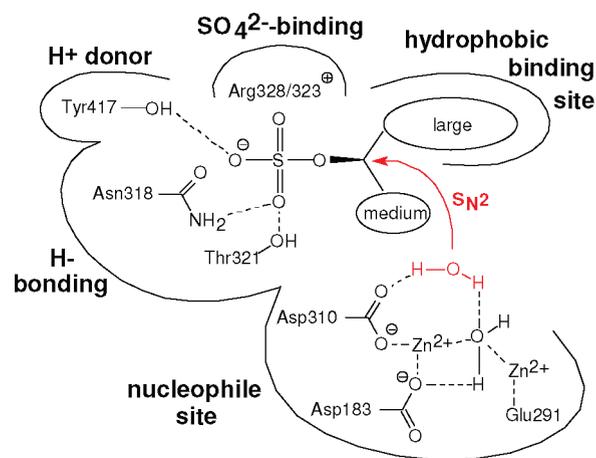


Figure 1. Schematic proposal for an acid–base mechanism for alkyl sulfate ester hydrolysis catalyzed by Pisa1 (His179/181/184/355 coordinating the Zn^{2+} ions were omitted for clarity).

$[\text{OH}^-]$, which attacks the carbon atom bearing the sulfate ester moiety. The latter is facilitated through (iii) concomitant protonation of the liberated inorganic sulfate (presumably by Tyr417), to yield HSO_4^- as a good leaving

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(20) Based on the pK_a of methyl monosulfate, which was calculated/estimated as $\text{pK}_a = -8.4$ or $\text{pK}_a = -3.4$, thus protonation of a sulfate ester requires a strong acid of pK_a ca. < 2 ; see: Klages, F.; Jung, H. A.; Hegenberg, P. *Chem. Ber* **1966**, *99*, 1704–1711. Fulford, J. E.; Dupuis, J. W.; March, R. E. *Can. J. Chem.* **1978**, *56*, 2324–2330.

(21) Depending on the conditions, acid-catalyzed sulfate ester hydrolysis may be plagued by racemization,¹⁷ which can be suppressed in the presence of 1,4-dioxane as mediator; see: Batts, B. D. *J. Chem. Soc. (B)* **1966**, 547–551.

group. The hydrophobic binding sites of Pisa1 and SdsA1 differ significantly with respect to the relative size of residues, which most likely accounts for the opposite substrate specificity concerning *prim*- versus *sec*-alkyl sulfate esters.

Table 1. Substrate Spectrum of Pisa1 Compared to Results with Whole Cells of *Pseudomonas* sp. DSM 6611

substrate	R ¹	R ²	Ps. sp. DSM 6611 ^a		Pisa1	
			c [%]	E ^b	c [%]	E ^b
<i>rac</i> - 2a	CH ₃	<i>n</i> -C ₆ H ₁₃	21	>200	50	>200
<i>rac</i> - 3a	CH ₃	<i>n</i> -C ₅ H ₁₁	17	>200	50	>200
<i>rac</i> - 4a	CH ₃	<i>n</i> -C ₇ H ₁₅	7	>200	50	>200
<i>rac</i> - 5a	C ₂ H ₅	<i>n</i> -C ₅ H ₁₁	18	>200	50	>200
<i>rac</i> - 6a	<i>n</i> -C ₃ H ₇	<i>n</i> -C ₄ H ₉	20	6	57	10
<i>rac</i> - 7a	CH ₃	(CH ₂) ₂ CH=CMe ₂	9	>200	50	>200
<i>rac</i> - 8a	CH ₃	CH ₂ Ph	<1	n.d.	30	>200
<i>rac</i> - 9a	CH ₃	(CH ₂) ₂ Ph	15	>200	50	>200
<i>rac</i> - 10a	CH ₃	<i>c</i> -C ₆ H ₁₁	5	>200	10	>200
<i>rac</i> - 11a	HC≡C	<i>n</i> -C ₄ H ₉	n.i.	–	50	>200
<i>rac</i> - 12a	HC≡C	<i>n</i> -C ₅ H ₁₁	n.i.	–	50	>200

^a Whole resting cells of *Pseudomonas* sp. DSM 6611, data from ref 13.

^b Enantioselectivity expressed as Enantiomeric Ratio (*E*-value); n.d. = not determined due to exceedingly low conversion; n.i. = not investigated.

The substrate spectrum of Pisa1 proved to encompass a range of linear, branched, or cyclic ω -1 to ω -3 *sec*-alkyl sulfates (Table 1, Scheme 2, part B). Straight-chain (ω -1)- and (ω -2)-sulfate esters (**2a–5a**) were resolved with perfect enantioselectivity; the (ω -3)-analog **6a** bearing two C₃/C₄-chains of similar size gave a respectable *E*-value of *E* = 10. Substrates bearing branched (**7a**), aromatic (**8a**, **9a**), or cyclic side chains (**10a**) gave excellent results. In contrast to

the previously employed resting whole cell preparation of *Pseudomonas* sp. DSM 6611¹³ the reaction rates were far superior and conversion values usually reached the theoretical limit of 50%, which is required for a deracemization process. The differences in rates and selectivities are presumably due to competing sulfatases in whole cells. Most remarkably, substrates *rac*-**11a** and *rac*-**12a**, derived from propargylic alcohols bearing a synthetically useful terminal acetylene unit, were transformed with the same perfect stereoselectivity.

With the highly active and stereoselective inverting *sec*-alkylsulfatase Pisa1 in hand, the feasibility of a deracemization protocol for *sec*-alcohols could be demonstrated as follows (Scheme 2, part C): Treatment of *rac*-2-octyl sulfate (**2a**, 1 g) with Pisa1 in aqueous buffer gave equimolar amounts of (*S*)-**2b** and (*S*)-**2a**. The latter was hydrolyzed under acidic conditions (*p*-TosOH in *t*BuOMe/H₂O/1,4-dioxane)¹⁹ to yield enantiopure (*S*)-**2b** (>99% ee) in 87% isolated yield from the racemate (0.49 g).

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Supporting Information Available. Synthesis of sulfate esters, NMR-spectra, cloning, expression and purification of Pisa1 and SdsA1, H₂¹⁸O labeling experiments, determination of enantiomeric excess and absolute configuration, and preparative scale deracemization. This material is available free of charge via the Internet at <http://pubs.acs.org>.