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Highly Enantioselective Biohydrolysis of *sec*-Alkyl Sulfate Esters with Inversion of Configuration Catalysed by *Pseudomonas* spp.

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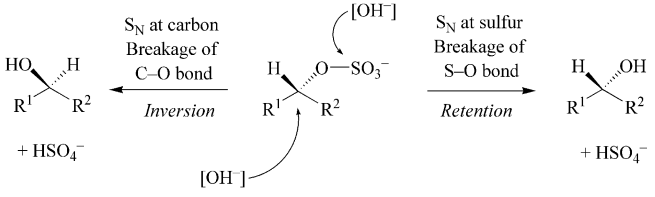
In search of highly enantioselective microbial *sec*-alkyl sulfatase activity, a broad screening among bacteria, fungi and Archaea revealed several *Ralstonia* and *Pseudomonas* spp. as valuable sources, whereas fungi were completely inactive. In particular, *Pseudomonas* sp. DSM 6611 was able to hydrolyse the (*R*) enantiomers of a broad range of *rac-sec*-alkyl sulfate esters with excellent enantioselectivities ($E > 200$) to furnish the corresponding inverted (*S*)-*sec*-alcohols in high *ee*'s. The substrate range of this organism was remarkably broad and bulky groups were also nicely tolerated.

Introduction

Sulfatases are a heterogenic group of hydrolytic enzymes that catalyse the cleavage of the sulfate ester bond to yield the corresponding alcohol and hydrogen sulfate. Depending on the mechanism of action of the enzyme, this reaction may proceed either through *retention* or *inversion* of configuration at the chiral carbon centre (Scheme 1). Whereas breakage of the S–O bond leads to retention, C–O bond cleavage results in inversion of configuration (Scheme 1).[1] The rare feature of double selectivities − *stereo*selectivity in the context of inversion/retention and *enantio*selectivity regarding the preference for a given substrate enantiomer − makes them valuable candidates for the deracemisation of *sec*-alcohols by enantioconvergent hydrolysis of their corresponding sulfate esters.[2,3]



Scheme 1. Stereochemical course of the biocatalytic hydrolysis of sulfate esters by sulfatases.

According to their preferred substrate type, sulfatases were previously classified into carbohydrate, aryl and alkyl sulfatases.[4] Carbohydrate and aryl sulfatases have been found to act predominantly through retention of configuration on sulfated carbohydrates.[5] This type of enzyme is also present in humans ("human aryl sulfatase A" [EC 3.1.6.8]) where it is involved in the degradation of glycosaminoglycans or in the synthesis of steroid hormones ("STS-steroid sulfatase" [EC 3.1.6.2]). Because of the fact that various diseases are the result of (single or multiple) sulfatase deficiency,[6] this subclass of sulfatases is extensively studied. Sequence analysis among aryl sulfatases led to the identification of the highly conserved consensus motif –C/S–X–P–X–R– among eukaryotic and prokaryotic aryl sulfatases.

A more recent classification of sulfatases is based on mechanistic aspects (rather than on substrate structure) and defines them into three classes:[7] (1) In eukaryotic aryl sulfatases possessing the above-mentioned consensus motif, a cysteine (or serine) residue within the active site is post-translationally modified into an α-formylglycine moiety, which is transformed into its corresponding aldehyde hydrate species. Being a powerful nucleophile, the latter attacks the S atom thereby cleaving the S–O bond of the sulfate ester substrate; the absolute configuration at carbon is retained.[8] (2) Class 2 sulfatases belong to the Fe^{II} α-ketoglutarate-dependent dioxygenase superfamily and are involved in the oxidative cleavage of a sulfate ester at the expense of α-ketoglutarate into the corresponding aldehyde and inorganic sulfate.[9] Because the latter occurs along with the destruction of the chiral carbon centre, they are of little use for stereoselective biotransformations. (3) Members of the third class are related to metallo-β-lactamases containing two Zn^{2+} atoms in the active site, which activate a water molecule to form a (formal) hydroxide ion. The latter performs nucleophilic attack on the substrate thereby yielding the corresponding alcohol and inorganic sulfate. As a result of the fact that only *prim*-sulfate esters were used as substrates so far, the stereochemical features of this mechanism

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with respect to retention or inversion at the chiral carbon are still unknown. In addition, the exact position of nucleophilic attack (at the sulfur or carbon atom) is still unclear.[7]

In contrast to sulfatases described above, much less is known about the mechanism of inverting *sec*-alkyl sulfatases. The latter were first described during the 1970s in detergent-degrading bacteria (*Pseudomonas* and *Comamonas* spp.), which were isolated from sewage sludge.[10] Subsequent studies on these organisms revealed the presence of three *sec*-alkyl sulfatases in *Pseudomonas* C12B NCIMB 11753, two of which (termed S1 and S2) were acting through inversion of configuration on *rac*-2-octyl sulfate albeit with opposite enantiopreference,[11] whereas the third one (termed S3) was only active towards symmetrical long-chain alkyl sulfates.[12] *Comamonas terrigena* NCIMB 8193 was shown to possess two inverting *sec*-alkyl sulfatases similar to those of *Pseudomonas* C12B.[13] Interest in the enantioselectivities of these enzymes arose rather slowly.[12] First hints on a certain enantiopreference arouse from the chance observation that several *rac*-alkyl sulfates were rapidly hydrolysed until 50% conversion, whereas further reaction took place at a much slower rate.[14] However, detailed studies were undertaken only two decades later: in search for alkyl sulfatase activities for the stereoselective hydrolysis of *sec*-alkyl sulfate esters, *Rhodococcus ruber* DSM 44541[15] was identified as the first promising candidate. Whole (resting) cells of this strain were found to hydrolyse (*R*)-2-octyl sulfate into (*S*)-2-octanol. Although the stereoselectivity of sulfatase RS2 isolated from this strain[16] was excellent with regard to the strict inversion of configuration, the enantioselectivity for (ω-1)-*sec*-alkyl sulfate esters ranged from poor to a modest *E* value of 21 for *rac*-2-octyl sulfate. Although these values could be enhanced to $E > 200$ in the presence of Fe^{3+}, the gain in selectivity was paid for by a loss in activity, which rendered this "enantioselective-inhibition" method[17] unsuitable for preparative-scale experiments.[18] In addition, the substrate tolerance of RS2 proved to be limited to linear *sec*-alkyl sulfate esters and its biochemical characterisation was impeded by limited protein stability.[16]

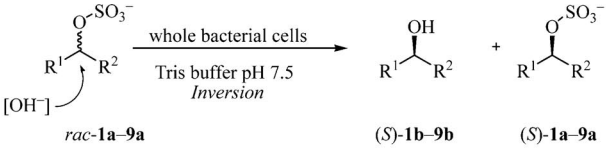
Following the assumption that microorganisms possessing strong inorganic sulfur metabolism with virtually all sulfur oxidation states from –2 to +6 might also be able to metabolise sulfated organic species, a screening among Archaea was carried out. Among anaerobic *Sulfolobus* spp., *Sulfolobus acidocaldarius* DSM 639[19] was identified to possess the desired inverting *sec*-alkyl sulfatase activity. The substrate tolerance was significantly wider and *E* values were excellent ($E > 200$). Protein purification was not attempted owing to difficult (extremophilic) cultivation conditions (85–90 °C, pH 1–2) and insufficient yields in biomass. Because of the total lack of sequence information on inverting alkyl sulfatases, genetic methods cannot be employed.

Aiming at a mesophilic microbial source for inverting *sec*-alkyl sulfatases, which would yield sufficient biomass under standard conditions within short growth periods, we turned our attention to *Pseudomonas* spp. and closely related strains.

Results and Conclusions

The screening for alkyl sulfatase activity among *Pseudomonas* spp. and related bacteria was guided by initial reports on the presence of alkyl sulfatases in *Pseudomonas* C12B NCIMB 11753 and *Comamonas terrigena* NCIMB 8193.[10] As a result of the occurrence of multiple alkyl sulfatases,[11–14] whole (resting) cells were not assumed to show reasonable enantioselectivities, but served as proof-of-principle. Phylogenetic selection of bacteria known to show a broad secondary metabolism within the *Pseudomonads*,[20] such as *Comamonas*, *Xanthomonas*, *Xanthobacter* and *Ralstonia* spp. as well as *Rhodococcus*-related actinomycetes, led to a set of 88 bacterial strains, which were tested for *sec*-alkyl sulfatase activity by using *rac*-2-octyl sulfate as a test substrate.[21] This selection was complemented by 59 Archaea and fungi, which are known to possess rich inorganic[22] and organic sulfur metabolism,[23] respectively (Scheme 2, Table 1).

A range of bacteroides exhibited low-to-excellent activities, but poor enantioselectivities, which typically indicates the presence of multiple sulfatase enzymes competing for the substrate (Table 1, Entries 1–6). Only slightly better selectivities were observed with a range of *Xanthobacter* spp. (Table 1, Entries 7–10, $E_{max} = 7.6$), and with *Comamonas* sp. DSM 15091 the enantioselectivity approached a synthetically useful value of $E = 16$ (Table 1, Entry 11). From two highly active *Ralstonia* spp., DSM 6428 showed an *E* value of 21 (Table 1, Entry 13). The clear hits of this screening process were found among the *Pseudomonas* spp. (Table 1, Entries 14–16). Whereas NCIMB 11753, which is known to express alkyl sulfatases S1 and S2 possessing opposite enantiopreference,[11] exhibited only modest enantio-



rac-**1a**–**9a** (*S*)-**1b**–**9b** (*S*)-**1a**–**9a**

	1	2	3	4	5	6	7	8	9
R^1	*n*-C_5H_{11}	*n*-C_6H_{13}	*n*-C_7H_{15}	*n*-C_5H_{11}	*n*-C_4H_9	$(CH_2)_2CH{=}CMe_2$	CH_2Ph	$(CH_2)_2Ph$	*c*-C_6H_{11}
R^2	CH_3	CH_3	CH_3	C_2H_5	*n*-C_3H_7	CH_3	CH_3	CH_3	CH_3

Scheme 2. Enantioselective inverting biohydrolysis of *sec*-alkyl sulfate esters *rac*-**1a**–**9a**.

Table 1. Biohydrolysis of 2-octyl sulfate (*rac*-**2a**) by using (resting) whole bacterial cells.

Entry	Strain	Activity[a]	Enantioselectivity *E*
1	*Bacillus sphaericus* FCC 098	±	1.1
2	*Achromobacter* sp. FCC 175	±	2.0
3	*Nocardia nova* DSM 43843	++	2.0
4	*Rhizobiaceae* sp. FCC 099	++	2.0
5	*Cupriavidus necator* DSM 5536	±	4.6
6	*Gulosibacter molinativorax* DSM 13485	++	5.3
7	*Xanthobacter autotrophicus* DSM 431	++	2.0
8	*Xanthobacter* sp. DSM 6696	±	2.3
9	*Xanthobacter flavus* DSM 338	±	2.7
10	*Xanthobacter autotrophicus* DSM 3874	±	7.6
11	*Comamonas* sp. DSM 15091	±	16
12	*Ralstonia eutropha* SPT0001 FCC120	++	1.0
13	*Ralstonia* sp. DSM 6428	++	21
14	*Pseudomonas* sp. NCIMB 11753	±	13
15	*Pseudomonas* sp. DSM 6978	±	>200
16	*Pseudomonas* sp. DSM 6611	++	>200

[a] Activity range denoted as: ± = low (conversion <5%), + = moderate (conversion 5–10%), ++ = high (conversion > 10%) within 24 h under standard conditions.

selectivity (E = 13), DSM 6978 and DSM 6611 hydrolysed the (*R*) enantiomer from *rac*-**2** with excellent enantioselectivity and with inversion of configuration to furnish inverted *sec*-alcohol (*S*)-**2b** ($E > 200$). The latter was proven by the fact that the biohydrolysis of enantiomerically pure (*R*)-2-octyl sulfate solely gave (*S*)-2-octanol, whereas (*S*)-**2a** was not converted at all.

These encouraging results prompted us to investigate the substrate-selectivity pattern of *Pseudomonas* spp. in more detail (Table 2).

The lead organism *Pseudomonas* NCIMB 11753 (previously denoted C12B) was able to hydrolyse linear (ω-1)-sulfate esters *rac*-**1a**–**3a** at low rates and with modest enantioselectivities (E_{max} = 13 for *rac*-**2a**). Moving the reactive group towards the centre of the molecule (*rac*-**4a**, *rac*-**5a**) led to a gradual decrease in selectivities due to the increasing spatial similarity of side chains. Bulky substituents (*rac*-**6a**–**9a**) were not tolerated at all. In contrast, *Pseudomonas* sp. DSM 6978 showed excellent enantioselectivities for long-chain linear substrates *rac*-**2a**–**4a**, albeit at moderate rates; again, steric hindrance prevented the hydrolysis of bulky substrates *rac*-**6a**–**9a**. The clear hit from this study was *Pseudomonas* sp. DSM 6611: with two exceptions, it converted all of the substrates at modest-to-good rates with almost absolute enantioselectivity ($E > 200$). Because sterically demanding compounds bearing phenyl or cyclohexyl groups were nicely accepted, steric hindrance did not seem to play a measurable role. Overall, all strains showed a more or less pronounced enantiopreference for the (*R*) enantiomer by acting with strict inversion of configuration.

Detailed biochemical characterisation of the *sec*-alkyl sulfatase from *Pseudomonas* sp. DSM 6611 is currently ongoing with the aim to elucidate its unique catalytic mechanism and its application in the deracemisation of *sec*-alcohols by enantioconvergent hydrolysis of their corresponding racemic sulfate esters.

Experimental Section

Synthesis of Alkyl Sulfate Esters: Sulfate esters *rac*-**1a**–**9a** and (*R*)- and (*S*)-**2a** were prepared from the corresponding alcohols by using $NEt_3{\cdot}SO_3$ according to a known procedure.[24] Sulfate esters were obtained as lyophilised powders, and their NMR spectra were in agreement with the reported values.[24]

Screening for Sulfatase Activity: Lyophilised whole cells (30–50 mg) were rehydrated in Tris·HCl buffer (500–600 μL, pH 7.5, 100 mM) for 1 h at 30 °C whilst shaking at 120 rpm. Then, an aliquot (200 μL) from a substrate stock solution (20 mg mL^{-1} *rac*-**2a**) was added as a test substrate. Other substrates were used in the concentration of 50 mg mL^{-1}. The reaction mixture was incubated at 30 °C whilst shaking at 120 rpm for 24 h. The samples were extracted with ethyl acetate (600 μL) and centrifuged at 13.000 rpm for 2 min to separate the organic layer from the cell/buffer suspension. The organic layer was dried with Na_2SO_4 and 100 μL of an internal standard (10 mg mL^{-1} of *rac*-2-dodecanol) was added. Conversions were measured with an achiral GC column and values were calculated from calibration curves. Afterwards, positive hits were derivatised overnight with acetic anhydride (60 μL) and catalytic DMAP for chiral analysis. The reaction was quenched with tap water (300 μL), centrifuged for 2 min at 13.000 rpm and the organic layer was treated as described above. For details on the GC analyses, see the Supporting Information.

Table 2. Substrate tolerance and enantioselectivities of *Pseudomonas* spp. NCIMB 11753, DSM 6978 and DSM 6611.

Substrate	*Pseudomonas* sp. NCIMB 11753		*Pseudomonas* sp. DSM 6978		*Pseudomonas* sp. DSM 6611	
	Conversion [%]	Enantioselectivity *E*	Conversion [%]	Enantioselectivity *E*	Conversion [%]	Enantioselectivity *E*
rac-**1a**	8	4	1	n.d.[a]	17	>200
rac-**2a**	4	13	7	>200	21	>200
rac-**3a**	2	2	4	>200	7	>200
rac-**4a**	2	8	2	>200	18	>200
rac-**5a**	10	5	2	4	20	6
rac-**6a**	8	1.2	n.c.	n.d.[a]	9	>200
rac-**7a**	n.c.	n.d.[a]	n.c.	n.d.[a]	n.c.	n.d.[a]
rac-**8a**	n.c.	n.d.[a]	n.c.	n.d.[a]	15	>200
rac-**9a**	n.c.	n.d.[a]	n.c.	n.d.[a]	5	>200

[a] n.d. = Not determined due to low (or no) conversion.

Determination of Stereochemical Pathway and Absolute Configuration: Stereopreferences were determined by using enantiopure (*R*)- and (*S*)-2-octyl sulfate (**2a**) as the substrate and prepared as described above; absolute configurations of alcohols **1b**–**9b** were elucidated by chiral GC by coinjection with commercially available or independently synthesised reference materials.

Supporting Information (see footnote on the first page of this article): Source of organisms, culture conditions and GC analyses of *sec*-alcohols **1b**–**9b**.

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