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Stereochemistry and Mechanism of Enzymatic and Non-Enzymatic Hydrolysis of Benzylic *sec*-Sulfate Esters

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The substrate scope of inverting alkylsulfatase Pisa1 was extended towards benzylic *sec*-sulfate esters by suppression of competing non-enzymatic autohydrolysis by addition of dimethyl sulfoxide as co-solvent. Detailed investigation of the mechanism of autohydrolysis in ^{18}O-labeled buffer by using an enantiopure *sec*-benzylic sulfate ester as substrate revealed that from the three possible pathways (i) inverting S_N2-type nucleophilic attack of [OH^-] at the benzylic carbon represents the major pathway, whereas (ii) S_N1-type formation of a planar benzylic carbenium ion leading to racemization was a minor event, and (iii) Retaining S_N2-type nucleophilic attack at sulfur took place at the limits of detection. The data obtained are interpreted by analysis of Hammett constants of *meta* substituents.

Introduction

Enantioselective hydrolysis of ester and amide bonds catalyzed by lipases, esterases, and proteases represents a landmark in biotransformations.[1] Their (industrial) application was significantly widened by introduction of dynamic resolution concepts that make use of in situ racemization[2] to overcome the 50%-yield threshold of kinetic resolution. As an alternative, simultaneous (or stepwise) transformation of a pair of substrate enantiomers through stereochemically opposite pathways leads to deracemization.[3] For the latter concepts, hydrolytic enzymes acting through retention or inversion of configuration are a crucial prerequisite.[4] In this context, we recently developed a deracemization protocol for *rac*-*sec*-alcohols through enantio-complementary hydrolysis of their corresponding sulfate monoesters by using a pair of sulfatases acting through stereo-complementary pathways.[5] The key enzymes employed were the retaining aryl sulfatase, PAS, from *Pseudomonas aeruginosa*[6] and the inverting alkyl sulfatase, Pisa1, from *Pseudomonas* sp. DSM 6611.[7] Fortunately, Pisa1 displayed a very broad substrate spectrum encompassing linear and branched *sec*-sulfate esters that bear various functional groups, such as allylic C=C and propargylic C≡C bonds, which are prone to undergo side reactions with transition metal catalysts used in dynamic resolution protocols.[8] In contrast, benzylic sulfate ester **2a** gave poor results with Pisa1, presumably owing to its hydrolytic instability at pH ≈ 8 going alongside competing spontaneous (non-enzymatic) hydrolysis, thereby eroding the *ee* of product **2b**.[8] By aiming to suppress the background hydrolysis by optimization of reaction conditions, we initiated a detailed study on the mechanism of enzymatic and non-enzymatic hydrolysis of *sec*-allylic and benzylic sulfate esters *rac*-**1a**–**8a**. Although aryl and *n*-alkyl sulfates have been thoroughly investigated regarding their stability towards hydrolysis,[9,10] no detailed studies are available on the hydrolysis of *sec*-alkyl sulfate esters. The majority of investigations deal with detergents, such as sodium dodecyl sulfate [11] or related anionic surfactants,[12] which predominantly consist of primary alkyl sulfates, in which the stereochemical consequences of hydrolysis are not an issue. Studies on highly branched neopentyl sulfate reported rearrangement issues.[13]

Results and Discussion

During our initial studies[8] we attempted to improve incomplete stereoselectivities observed with several allylic, propargylic and benzylic *sec*-sulfate esters by addition of dimethyl sulfoxide (DMSO). Although positive effects were observed, the exact molecular reason for this selectivity-enhancement – suppression of spontaneous (non-enzymatic) hydrolysis and/or alteration of the catalytic properties of the enzyme[14] – remained unknown.

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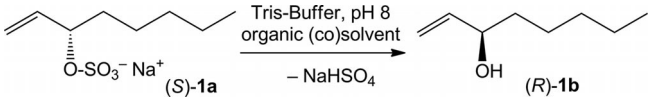
The influence of the polarity of water-miscible organic co-solvents on the *ee* of **1b** obtained from non-enzymatic hydrolysis of enantiopure (*S*)-**1a** was investigated (Table 1). Although significant racemization took place in neat buffer [*ee* of (*R*)-**1b** 34%, E_T^N of $H_2O \approx 1$], this effect gradually diminished upon decreasing the polarity (as indicated by the Dimroth–Reichardt parameter E_T^N)[15] of the organic co-solvent used [*ee* of (*R*)-**1b** 48%, E_T^N of DMSO 0.44]. Reducing the reaction temperature from 60° to 20 °C in Tris-buffer in the absence of organic co-solvent had a similar effect (ee_P 25% versus 52%, respectively). Both effects indicate the involvement of a polar (e.g. an allylic carbenium ion) species.

Table 1. Non-enzymatic hydrolysis of (*S*)-1-octen-3-yl sulfate (**1a**) in the presence of water-miscible organic co-solvents.[a]

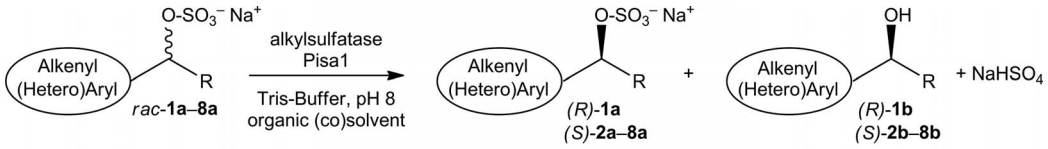


Co-Solvent	*ee* of (*R*)-**1b** [%]	Dimroth–Reichardt parameter (E_T^N)[b]
None	34	≈ 1
Methanol	44	0.76
Ethanol	45	0.65
2-Propanol	46	0.55
DMSO	48	0.44

[a] Conditions: Tris-buffer 100 mM, pH 8.0, 20% (v/v) co-solvent, 5 mg/mL (*S*)-**1a**, 90 h at 30 °C. [b] E_T^N values are given for pure solvents.

To support the hypothesis that a polar carbenium ion species causes racemization during non-enzymatic hydrolysis, a series of benzylic *sec*-sulfate esters (**2a**–**8a**) were subjected to non-enzymatic and enzymatic hydrolysis with Pisa1 (Scheme 1, Table 2). Of special interest were the *meta* substituted derivatives **2a**–**6a**, because the electronic effects of the *meta* substituents on the (de)stabilization of a benzylic carbenium ion can be easily correlated to their Hammett constants.[16] Substrate **7a** was incorporated from ref.[8] for comparison and pyridyl-analog **8a** was used as an electron-deficient heterocyclic candidate.

Substrates *rac*-**2a**–**8a** were subjected to enzymatic hydrolysis under standardized reaction conditions by using Pisa1,



	Alkenyl/(Hetero)Aryl	R
1a,b	CH_2=CH-	*n*-C_5H_{11}
2a,b	C_6H_5-	CH_3
3a,b	*m*-MeO-C_6H_4-	CH_3
4a,b	*m*-F-C_6H_4-	CH_3
5a,b	*m*-Cl-C_6H_4-	CH_3
6a,b	*m*-CF_3-C_6H_4-	CH_3
7a,b	*m,m*-$(CF_3)_2$-C_6H_3-	CH_3
8a,b	3-Pyridyl-	CH_3

Scheme 1. Stereoselective hydrolysis of allylic and benzylic *sec*-sulfate esters by using inverting alkylsulfatase Pisa1.

Table 2. Enzymatic and non-enzymatic hydrolysis of benzylic *sec*-sulfate esters *rac*-**2a**–**8a**.

Substrate	Co-solvent[a]	Conversion [%][b]	ee_P [%]	*E*[c]	Autohydrolysis [%][d]	σ_m constant[e]
rac-**2a**[f]	none	> 99	3.6	< 2	> 96	0.00
	DMSO	> 99	4.1	< 2	> 96	
rac-**3a**	none	99	40	2.6	96	0.12
	DMSO	82	82	10	34	
rac-**4a**	none	60	60	12	13	0.34
	DMSO	50	93	96	2	
rac-**5a**	none	60	61	13	10	0.37
	DMSO	50	93	84	1	
rac-**6a**	none	54	85	70	4	0.43
	DMSO	50	99	> 200	0.3	
rac-**7a**[g]	none	10	> 99	> 200	< 0.3	n.a.
	DMSO	13	> 99	> 200	< 0.3	
rac-**8a**	none	50	99	> 200	12	n.a.
	DMSO	48	> 99	> 200	6	

[a] Standard conditions: Pisa1 (0.13 mg), Tris-buffer, 100 mM, pH 8.0, substrate **2a**–**8a** (5 mg/mL), 24 h at 30 °C; [a] 20% v/v. [b] Calculated from $ee_S/(ee_S + ee_P)$. [c] Enantiomeric Ratio (*E*) calculated from ee_P and ee_S: $E = \{\ln[(1-ee_S)/(1 + ee_S/ee_P)]\}/\{\ln[(1 + ee_S)/(1 + ee_S/ee_P)]\}$;[17] for the application of *E* values to kinetic resolutions with competing autohydrolysis, see ref.[18] [d] Conversion in the absence of enzyme. [e] Hammett constant of substituent R in the *meta* position (Scheme 1). [f] For data from 6 h reaction time, see ref.[8]. [g] For data from 72 h reaction time, see ref.[8]; n.a. = not applicable.

the ee_P of *sec*-alcohols (*S*)-**2b**–**8b** formed was determined by GC analysis on a chiral stationary phase after extractive separation from the remaining non-hydrolyzed sulfate esters (*S*)-**2a**–**8a**. The latter were subjected to acid-catalyzed hydrolysis through strict retention of configuration[8] to yield corresponding alcohols (*S*)-**2b**–**8b** for *ee*-determination. Autohydrolysis was measured under identical conditions in the absence of enzyme. Absolute configurations were elucidated by co-injection with authentic reference materials with known absolute configuration.[8] DMSO was selected as co-solvent because it showed the strongest selectivity-enhancing effects (Table 1).

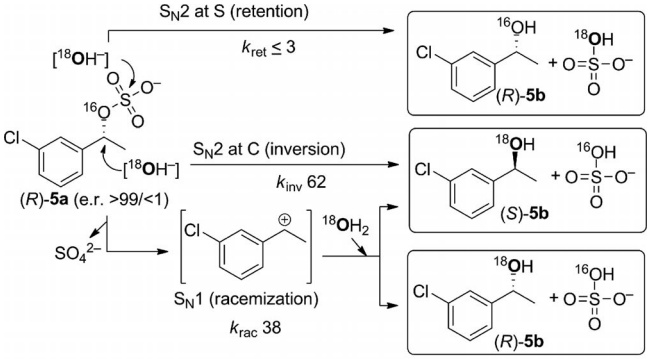
The enzymatic hydrolysis of substrate *rac*-**2a** was strongly outcompeted by non-selective autohydrolysis and consequently gave alcohol **2b** in near racemic form. Although the addition of DMSO showed a positive trend, the effects were too small to be truly beneficial.

Introduction of electron-withdrawing substituents in the *meta* position (substrates **3a**–**6a**) gave increasingly better results, i.e. the gradual suppression of autohydrolysis gave a strong improvement in the apparent enantioselectivities[18] from barely detectable (E = 2.6) to a respectable value (E = 70). In line with the suppression of autohydrolysis, the overall reaction rates slowed from **2a** to **6a**, indicated by decreasing conversion values. The addition of DMSO (20% v/v) further decreased autohydrolysis and hence gave even better overall enantioselectivities of up to $E > 200$. The correlation between the electronic properties of the *meta* substituents, as denoted by their Hammett σ_m-values, is remarkably strong: there is a drastic improvement in selectivity owing to decreased autohydrolysis going from R = H (0.00) through R = MeO (0.1) to R = Hal (> 0.3), whereas both halo-derivatives with comparable σ_m-values of 0.34 and 0.37 gave similar results. A further significant improvement was achieved with the CF_3-derivative (0.43).

The beneficial effect of electron-deficient substituents in the *meta* position is nicely underlined by doubly *meta* substituted substrate **7a**, which could be resolved with perfect enantioselectivity.[8] To test whether this electronic effect could also be extended to heteroaromatic benzylic analogs, electron-deficient 3-pyridyl derivative **8a** was investigated. In line with the above trends, it could be resolved with excellent results ($E > 200$). Unfortunately, attempts to synthetize electron-rich derivatives, such as 1-(furan-2yl)ethyl sulfate, 1-(thiophen-2-yl)ethyl sulfate, 1-(1*H*-pyrrol-2-yl)ethyl sulfate or imidazole analogs, which could serve as counterproof, were unsuccessful owing to the instability of the corresponding *sec*-alcohols.

The hydrolysis of *sec*-alkyl monosulfate esters is a complex process: Acid catalysis proceeds by protonation of the negatively charged sulfate ester moiety[19] at the C–O–S bridge atom, which allows nucleophilic attack of H_2O at sulfur, along with release of the alcohol and HSO_4^- as a good leaving group.[20] Consequently, it is a fast process and proceeds with retention of configuration at the chiral C-atom bearing the sulfate ester moiety. However, nucleophilic attack of [OH^-] at C under basic conditions would proceed through inversion at C, but it is hardly possible, because the approach of [OH^-] onto the negatively charged substrate is disfavored and the process would generate SO_4^{2-} as a poor leaving group; hence, it is an exceedingly slow process.[21,22] In contrast, the enzymatic hydrolysis as exemplified by inverting alkylsulfatase Pisa1 is a masterpiece of cooperative acid-base catalysis:[7] Nucleophilic attack of [OH^-] onto C (derived from H_2O by a binuclear Zn^{2+} cluster in the active site of the enzyme) is complemented by simultaneous protonation of the sulfate ester moiety through histidine 317 to generate HSO_4^-. All of these processes basically proceed through S_N2 at C, because the generation of an aliphatic carbenium ion would be energetically too costly.

With benzylic substrates, such as **5a**, a resonance-stabilized carbenium ion has to be taken into account, because clear correlation of the decrease of autohydrolysis with the electron-withdrawing effects of *meta* substituents (as indicated by their Hammett constants) strongly suggests that autohydrolysis (at least in part) occurs through an S_N1-mechanism via an intermediate benzylic carbenium ion. Our investigations on the mechanism of autohydrolysis was led by the following considerations: (i) analysis of the *ee* of formed alcohol **5b** (and its potential erosion) derived from enantiopure substrate (*R*)-**5a** would prove the existence of a transient benzylic carbenium ion responsible for racemization; (ii) use of ^{18}O-labelled water would allow determination of the site of nucleophilic attack (S versus C) through incorporation of [OH^-] either into the formed alcohol (attack at C) or into inorganic sulfate (attack at S) to prove inversion or retention of configuration, respectively (Scheme 2).



Enzyme	e.r. of (*S*)-**5b** [%]	$^{18}O/^{16}O$-ratio of **5b** (*S*)	(*R*)
Pisa1	>99:<1	79:21	—
none	81:19	83:17	79:21

Scheme 2. Elucidation of retaining (S_N2 at S), inverting (S_N2 at C) and racemizing (S_N1) pathways of non-enzymatic and enzymatic hydrolysis of (*R*)-**5a** through ^{18}O-labeling (*k* values are stated as first order relative rate constants).

To check the validity of the method, enzymatic hydrolysis of (*R*)-**5a** [enantiomer ratio (e.r.) >99:<1] by using inverting Pisa1 in $^{18}OH_2$ was performed as a control experiment.[7] For handling purposes, the medium was composed of ^{16}O-Tris-buffer (0.1 mL, 1 M, pH 8.0) diluted at a ratio of 1:10 with ^{18}O-labelled H_2O (label 97:3). Addition of Pisa1

(2.6 mg) from 4.6 μL of $^{16}OH_2$ stock solution led to a (calculated) $^{18/16}$O-ratio in the reaction medium of 84:16. After 24 h of reaction time, analysis of alcohol **5b** by GC–MS with a chiral stationary phase revealed an e.r. of >99:<1 for the (*S*)-enantiomer with an $^{18/16}$O-label of 79:21. These data confirm that Pisa1 hydrolyzed (*R*)-**5a** with complete inversion with concomitant incorporation of ^{18}O at C within the limits of accuracy (calculated 84:16, measured 79:21).

The pathways of autohydrolysis were investigated by an analogous experiment in the absence of enzyme by using an $^{18/16}$O-label of 83:17 at a fivefold-extended reaction time. The following facts were deduced:

(i) Non-enzymatic hydrolysis of enantiopure (*R*)-**5a** (e.r. >99/<1) gave (*S*)-**5b** with an e.r. of 81:19, indicating that inversion through S_N2 at C is a dominant pathway.

(ii) The (*R*)-enantiomer of alcohol **5b** derived from (*R*)-**5a** can either be formed through retention or racemization, but ^{18}O-labeling of (*R*)-**5b** can only take place through racemization, because retention retains the ^{16}O-label. Because the ratio of $^{18/16}$O-label in (*R*)-**5b** (79:21) corresponds to that of the aqueous medium (83:17) within the accuracy of measurement, it can be concluded that retention at C through S_N2 at S can be neglected and racemization through S_N1 through a benzylic carbenium ion strongly prevails.

(iii) Consequently, inversion (S_N2 at C) and racemization (S_N1) are the major pathways. Their relative proportion can be estimated by taking the erosion of e.r. from (*R*)-**5a** to (*S*)-**5b** (e.r. from >99*R*:<1*S* to 81*S*:19*R*) into account: Because racemization produces equal amounts of (*R*)- and (*S*)-**5b** (19 parts each, i.e. 38 in total), the remainder of 62 parts counts for inversion (considering retention below the limits of detectability ≤ 3). Consequently, the ratio of relative rates of k_{inv} (S_N2 at C) versus k_{rac} (S_N1) are about 1.6:1.

Conclusions

The enantioselectivity of the enzymatic hydrolysis of benzylic *sec*-sulfate esters by using inverting alkylsulfatase Pisa1 could be significantly improved by suppressing the autohydrolysis of substrates by addition of DMSO as co-solvent. $H_2{}^{18}O$-Labeling studies revealed that the major pathway of autohydrolysis proceeded through S_N2-type inversion at carbon. In contrast, nucleophilic attack at sulfur and the S_N1-type pathway through a benzylic carbenium ion took place at the limits of detection. The data obtained are interpreted by analysis of Hammett constants of *meta* substituents. These results contribute to the understanding of the bioactivity of sulfated steroids possessing carcinogenic[23] or anabolic properties[24] and the stereo-complementary nucleophilic substitution of sulfur-based leaving groups.[25]

Experimental Section

Enzymatic Hydrolysis of Sulfate Esters 3a–6a and 8a: The corresponding sulfate ester **3a**–**6a** and **8a** (5 mg) was dissolved in Tris/HCl buffer (1 mL, 100 mM, pH 8.0), Pisa1 was added (0.13 mg) and the reaction was shaken with 120 rpm for 24 h at 30 °C. Afterwards, ethyl acetate (1 mL) was added and the mixture was centrifuged for 3 min at 13.000 rpm. The organic phase was separated and dried with Na_2SO_4 and alcohols **3b**–**6b** and **8b** were derivatized to the corresponding acetates with DMAP (1 mg) and acetic anhydride (100 μL) overnight. The reaction was quenched by addition of H_2O (300 μL) with stirring for 3 h. After centrifugation for 3 min at 13.000 rpm, the organic phase was dried with Na_2SO_4 and directly measured with GC-FID. The enzymatic hydrolysis of substrates **1a**, **2a** and **7a** is described elsewhere.[8]

Quantification of Autohydrolysis: The respective sulfate ester **3a**–**6a** and **8a** was dissolved in Tris/HCl-buffer (1 mL, 100 mM, pH 8.0) and were shaken at 120 °C and 30 rpm for 24 h. The reaction was quenched by freezing in liquid N_2 and was thawed individually prior to measurement. Quantification of autohydrolysis was done from calibration curves with the corresponding alcohol and sulfate ester.

All measurements were carried out with a Shimadzu HPLC system (CBM-20A, LC-20AD, DGU-20A5, SIL-20AC, CTO-20AC, SPD-M20A, CBM-20A) by using a ZORBAX 300-SCX (4.6 × 250 mm) IEX column and UV-detection [diode array detector set at 271 nm (**3a**), 261 nm (**4a**), 266 nm (**5a**), 262 nm (**6a**) and 259 nm (**8a**)]. The conversion was determined by using sodium formate buffer (200 mM pH 2.8) at a flow rate of 0.5 mL/min and a run time of 20 min (for retention times see Supporting Information, Table S1).

^{18}O-Labeling Experiments: ^{18}O-Enriched water (90 μL, ^{18}O content 97%) was added to a buffer solution ($^{16}OH_2$ 10 μL, 1 M Tris/HCl pH 8.0) to reach a final buffer concentration of 100 mM ($^{18/16}$O-label 83:17). Substrate (*R*)-**5a** (1 mg) was added to the solution and was shaken for 24 h at 30 °C and 120 rpm. Afterwards, alcohol **5b** was extracted with ethyl acetate (0.1 mL), the organic phase was dried with Na_2SO_4 and directly measured with GC–MS. GC–MS measurements were carried out with an Agilent 5975C MS connected to an Agilent 7890A GC fitted with a CTC Analytics PAL Autosampler by using a Chirasil Dex CB column (25 m × 0.32 mm × 0.25 μm film) and He as a carrier gas (0.69 bar). Injection temperature 250 °C, flow 0.5 mL/min, temperature program: 80° hold 1 min, 15 °C/min to 141 °C, 0.5 °C/min to 143 °C, 17 °C/min to 180 °C. Retention times: (*R*)-**5b** 7.4 min, (*S*)-**5b** 7.7 min.

Enzymatic reactions were performed analogously to the control reaction with addition of Pisa1 (26 μg, 353 pmol, 4.6 μL of stock solution in $^{16}OH_2$).

Supporting Information (see footnote on the first page of this article): Expression of PISA1, synthesis of substrates and reference compounds, analytical methods, NMR and MS spectra, and optical rotation values are presented.

Acknowledgments

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