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 18O-labeled buffer by using an enantiopure sec-benzylic sulfate ester as substrate revealed that from the three possible pathways (i) inverting Sec2-type nucleophilic attack of [OH−] at the benzylic carbon represents the major pathway, whereas (ii) Sec1-type formation of a planar benzylic carbenium ion leading to racemization was a minor event, and (iii) Retaining Sec2-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. Their (industrial) application was significantly widened by introduction of dynamic resolution concepts that make use of in situ racemization 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. For the latter concepts, hydrolytic enzymes acting through retention or inversion of configuration are a crucial prerequisite. 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. The key enzymes employed were the retaining aryl sulfatase, PAS, from Pseudomonas aeruginosa and the inverting alkyl sulfatase, Pisa1, from Pseudomonas sp. DSM 6611. 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. In contrast, benzylic sulfate ester 1a 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 2a. 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, 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 or related anionic surfactants, 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.

Results and Discussion

During our initial studies 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 – remained unknown.
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\(_2\)O \(\approx\) 1], this effect gradually diminished upon decreasing the polarity (as indicated by the Dimroth-Reichardt parameter \(E_T^N\)) 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.

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. 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,
the *ep* 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[9] 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*-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*-values of 0.34 and 0.37 gave similar results. A further significant improvement was achieved with the CF₃-derivative (0.43).

The beneficial effect of electron-deficient substituents in the meta position is nicely underlined by doubly 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 synthesize electron-rich derivatives, such as 1-(furan-2-yl)ethyl sulfate, 1-(thiophen-2-yl)ethyl sulfate, 1-(1H-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 H₂O at sulfur, along with release of the alcohol and HSO₄⁻ 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 SO₄²⁻ 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 H₂O by a binuclear Zn²⁺ cluster in the active site of the enzyme) is complemented by simultaneous protonation of the sulfate ester moiety through histidine 317 to generate HSO₄⁻. All of these processes basically proceed through *S*ₙ₁₂ mechanism via an intermediate benzylic carbenium ion. Our investigations on the mechanism of enzymatic hydrolysis 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 ¹⁸O-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).

To check the validity of the method, enzymatic hydrolysis of (R)-5a [enantiomter ratio (e.r.) >99:<1] by using inverting Pisa1 in ¹⁸O₂ was performed as a control experiment.[7] For handling purposes, the medium was composed of ¹⁸O₂-Tris-buffer (0.1 mL, 1 m, pH 8.0) diluted at a ratio of 1:10 with ¹⁸O-labelled H₂O (label 97:3). Addition of Pisa1

Scheme 2. Elucidation of retaining (*S*₂₂ at S), inverting (*S*₂₂ at C) and racemizing (*S*₁) pathways of non-enzymatic and enzymatic hydrolysis of (R)-5a through ¹⁸O-labeling (*k* values are stated as first order relative rate constants).
(2.6 mg) from 4.6 μL of 16OH2 stock solution led to a (calculated) 18/16O-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 18/16O-label of 79:21. These data confirm that Pisa1 hydrolyzed (R)-5a with complete inversion with concomitant incorporation of 18O 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 18/16O-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 S2 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 18O-labeling of (R)-5b can only take place through racemization, because retention retains the 18O-label. Because the ratio of 18/16O-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 S2 at C can be neglected and racemization through S1 at C through a benzylic carbocation strongly prevails.

(iii) Consequently, inversion (S2 at C) and racemization (S1 at C) are the major pathways. Their relative proportion can be estimated by taking the erosion of e.r. from (R)-5b to (S)-5b (e.r. from >99R:<1S to 81S:19R) 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 kinv (S2 at C) versus krac (S1 at C) are about 1:6:1.

Conclusions

The enantioselectivity of the enzymatic hydrolysis of benzylic sec-sulfate esters by using invertase alkalysulfatase Pisa1 could be significantly improved by suppressing the autohydrolysis of substrates by addition of DMSO as cosolvent. H218O-Labeling studies revealed that the major pathway of autohydrolysis proceeded through S2-type inversion at carbon. In contrast, nucleophilic attack at sulfur and the S1-type pathway through a benzylic carbocation 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 or anabolic properties and the stereo-complementary nucleophilic substitution of sulfur-based leaving groups.

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 Na2SO4 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 H2O (300 μL) with stirring for 3 h. After centrifugation for 3 min at 13,000 rpm, the organic phase was dried with Na2SO4 and directly measured with GC–MS. The enzymatic hydrolysis of substrates 1a, 2a and 7a is described elsewhere.[9]

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 N2 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, CM-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).

18O-Labeling Experiments: 18O-Enriched water (90 μL, 18O content 97%) was added to a buffer solution (16OH2 10 mM Tris/HCl pH 8.0) to obtain a final buffer concentration of 100 mM 18/16O-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 Na2SO4 and directly measured with GC–MS. GC–MS measurements were carried out with an Agilent 5975C GC connected to an Agilent 7890A GC fitted with a CTC Analytics PAL Autosampler by using a Chiralsal 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 16OH2).

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.

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