***Pseudomonas aeruginosa* arylsulfatase: a purified enzyme for the mild hydrolysis of steroid sulfates**

Short Title: **Steroid sulfate hydrolysis by *Pseudomonas aeruginosa* arylsulfatase**

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Abstract

The hydrolysis of sulfate ester conjugates is frequently required prior to analysis for a range of analytical techniques including gas chromatography-mass spectrometry (GC-MS). Sulfate hydrolysis may be achieved with commercial crude arylsulfatase enzyme preparations such as that derived from *Helix pomatia* but these contain additional enzyme activities such as glucuronidase, oxidase and reductase that make them unsuitable for many analytical applications. Strong acid can also be used to hydrolyse sulfate esters but this can lead to analyte degradation or increased matrix interference. In this work, the heterologously expressed and purified arylsulfatase from *Pseudomonas aeruginosa* is shown to promote the mild enzyme-catalysed hydrolysis of a range of steroid sulfates. The substrate scope of this *P. aeruginosa* arylsulfatase hydrolysis is compared with commercial crude enzyme preparations such as that derived from *H. pomatia*. A detailed kinetic comparison is reported for selected examples. Hydrolysis in a urine matrix is demonstrated for dehydroepiandrosterone 3-sulfate and epiandrosterone 3-sulfate. The purified *P. aeruginosa* arylsulfatase contains only sulfatase activity allowing for the selective hydrolysis of sulfate esters in the presence of glucuronide conjugates as demonstrated in the short three-step chemoenzymatic synthesis of 5 α -androstane-3 β ,17 β -diol 17-glucuronide (ADG, **1**) from epiandrosterone 3-sulfate. The *P.*

aeruginosa arylsulfatase is readily expressed and purified (0.9 g per L of culture) and thus provides a new and selective method for the hydrolysis of steroid sulfate esters in analytical sample preparation.

Keywords

Sulfate ester; Sulfatase; Steroid; Glucuronide; Sports drug testing; Chemoenzymatic synthesis

Introduction

Steroid metabolites from both exogenous and endogenous sources are typically excreted in biological fluids as phase II glucuronide or sulfate conjugates.^{[1][2]} They are of growing importance in fields such as sports drug testing,^[3] the detection of residues in agricultural produce,^[4] and medicine.^[5] In the context of sports drug testing, hydrolysis of these conjugates may be required prior to routine screening, confirmatory analysis, or more advanced techniques such as gas chromatography-isotope ratio mass spectrometry (GC-IRMS). The hydrolysis of phase II glucuronide conjugates can generally be achieved under mild conditions using the *Escherichia coli* β -glucuronidase enzyme (3.2.1.31).^{[3][6]} This approach has remained a mainstay of anti-doping laboratories where enzyme hydrolysis is followed by derivatisation and gas chromatography-mass spectrometry (GC-MS) analysis to screen the combined free and glucuronide conjugated steroidal fractions.^[7]

In contrast, no generally accepted method has been established for the hydrolysis of phase II steroidal sulfate conjugates, and they are not routinely included in human anti-doping screens.^{[3][8][9]} Steroid sulfates represent a significant fraction of the steroid metabolites and including them in analysis could improve the sensitivity and retrospectivity of sports drug testing. For example, the analysis of sulfate metabolites can afford greater retrospectivity for the detection of steroidal agents,^{[10][11][12]} and may also serve as markers to distinguish between steroids of exogenous and endogenous origin.^{[12][13][14]} Other non-steroidal

performance-enhancing drugs also afford important sulfate conjugates, including toremifene,^[15] andarine,^[16] and mesocarb.^{[17][18]}

Steroid sulfates can be chemically hydrolysed under strong acid conditions, but this can degrade acid-sensitive analytes and may also give rise to increased matrix-derived interference in the subsequent GC-MS analysis leading to reduced sensitivity.^{[3][19]} A range of commercial sulfatase enzymes (EC 3.1.6.1) may be employed, but these crude enzyme preparations typically have additional activities such as β -glucuronidase, oxidase or reductase that make them unsuitable for many applications.^{[3][19]} To address these issues a number of more elaborate protocols have been employed including, glucuronide hydrolysis and extraction of free steroids prior to sulfate hydrolysis, or separation of the sulfate fraction through solid phase extraction prior to strong acid or enzyme hydrolysis. The approaches and challenges associated with analysis of the sulfate fraction have been reviewed in detail.^[3]

In this work we report a study of the steroid sulfatase activity of the heterologously expressed and purified *Pseudomonas aeruginosa* arylsulfatase (PaS) including substrate scope and enzyme kinetic evaluation. These results are compared against four commercial sulfatase enzyme preparations. The PaS enzyme is shown to hydrolyse a range of steroid sulfates, with a substrate scope similar to that observed for *Helix pomatia* arylsulfatase preparation (HpS), the leading commercial crude enzyme preparation for the hydrolysis of steroid sulfates. Hydrolysis in a urine matrix is demonstrated for dehydroepiandrosterone 3-sulfate and epiandrosterone 3-sulfate. In contrast to HpS, which displays significant β -glucuronidase activity, PaS is a purified enzyme that allows for the selective hydrolysis of sulfate esters in the presence of glucuronide conjugates. This is illustrated by a short chemoenzymatic synthesis of 5 α -androstane-3 β ,17 β -diol 17-glucuronide (ADG) **1**.

Experimental

Materials

Unless otherwise stated, chemicals were purchased from Sigma–Aldrich (Castle Hill, Australia).

Androsterone (A), boldenone (B), epiandrosterone (EA), etiocholanolone (EC), nandrolone (N) and testosterone (T) were obtained from Steraloids (Rhode Island, USA). Dehydroepiandrosterone (DHEA) was obtained from BDH (Poole, UK). Epiandrosterone-d₅ (2,2,3,4,4-d₅; EA-d₅) manufactured by CDN Isotopes Inc. (Pointe-Claire, Canada) was purchased from SciVac Pty. Ltd. (Hornsby, Australia). Epitestosterone (ET) was synthesised from T using literature methods.^[20] Milli-Q water was used in all aqueous solutions and in the liquid chromatography mobile phase. Liquid chromatography (gradient) grade methanol obtained from Merck (Kilsyth, Australia) was used for preparing the LC mobile phase and steroid standard solutions.

Synthesis of steroid sulfates

To explore the substrate range of the sulfatases investigated, steroid sulfates were synthesised by literature methods.^{[21][22][23]} All steroid sulfates were prepared in high purity following recrystallization with characterization by m.p., $[\alpha]_D$, ¹H and ¹³C NMR, LRMS and HRMS. An 'S' at the end of a steroid abbreviation denotes sulfation of a hydroxyl group.

Commercial enzymes

Four arylsulfatase preparations (EC 3.1.6.1) were purchased from Sigma–Aldrich for comparison with PaS. Three were derived from molluscs: *H. pomatia* sulfatase (HpS) (product number S9626), *Haliotis rufescens* (abalone) sulfatase (HrS) (S9754), and *Patella vulgata* sulfatase (PvS) (S8629). Stock solutions of each were prepared by dissolving 10 mg of solids per ml of 250 mM ammonium acetate at pH 6.9. The fourth preparation was *Aerobacter aerogenes* sulfatase, purchased as a solution in aqueous glycerol (S1629). The bacterium from which this fourth sulfatase is derived is also known as *Klebsiella pneumoniae*^[24] and since this name is used exclusively in current literature it is referred to here as *K. pneumoniae* sulfatase (KpS). The four commercial sulfatase preparations are reported to have β -glucuronidase activity in the Sigma–Aldrich product information. One other sulfatase, from *Flavobacterium heparinum* (*Pedobacter heparinus*), is commercially available. This is classified as a disulfoglucosamine-6-sulfatase (EC 3.1.6.11) and was not evaluated as part of this research.^[25]

PaS preparation

The protein sequence for PaS was obtained from Genbank (Accession No. AAG03573.1); derived from the *P. aeruginosa* genome sequence.^[26] The sequence was submitted to DNA2.0 (California, USA) for codon optimization (for expression in *E. coli*), gene synthesis, inclusion of a C-terminal His-tag (six His residues) and insertion into expression vector pJExpress404. This plasmid confers ampicillin resistance and placed PaS expression under tight control of the T5 promoter and *LacI* repressor. This construct was transformed into electro-competent *E. coli* DH5 α using a BioRad (Gladesville, Australia) electroporator according to manufacturer's instructions. Transformants were selected on Lysogeny Broth supplemented with 100 mg L⁻¹ ampicillin (LBA) agar plates and a single colony was selected to inoculate 2 mL of LBA media. This culture was incubated at 37 °C overnight and used to inoculate 0.5 L of Terrific Broth with 50 mg L⁻¹ ampicillin and 0.1 mM isopropyl β -D-1-thiogalactopyranoside in a 1 L conical flask. The flask was incubated at 25 °C with 200 rpm shaking for 44 hours before chilling on ice. The culture was centrifuged at 8000 $\times g$ to pellet the bacteria and the supernatant was discarded. The cells were resuspended in 10 mL of cold buffer A (50 mM Tris-sulfate at pH 7.5, 0.3 M NaCl, and 0.1 mM CaCl₂) per 1 g of wet cell pellet. The suspension was lysed using a French pressure cell at 12000 psi. Ammonium sulfate (0.2 g mL⁻¹) was dissolved in the lysate and then left on ice for 20 min to precipitate unwanted protein. The mixture was centrifuged at 30000 $\times g$ for 30 min and the supernatant was collected. Another 0.2 g mL⁻¹ of ammonium sulfate was dissolved in the supernatant to precipitate most of the PaS activity. The sample was centrifuged again at 30000 $\times g$ for 30 min and the pellet was dissolved in buffer A containing 30 mM imidazole. This sample was loaded onto a GE Healthcare (Silverwater, Australia) 5 mL HisTrap™ column with chelated Ni²⁺ and washed with 30 mL of buffer A containing 30 mM imidazole. PaS was then eluted from the column in buffer A containing 200 mM imidazole and fractions with *para*-nitrophenyl sulfate (PNPS) hydrolysis activity were pooled. For enzyme characterisation studies, the pooled fractions were extensively dialysed against 50 mM Tris-sulfate at pH 7.5, filter-sterilised (0.2 μ m membrane) and stored in aliquots at approximately 1 g L⁻¹ and -20 °C. Frozen aliquots were thawed immediately before use and then stored at 4 °C. The sulfatase activity remained stable during storage: for several months at 4 °C, or for more than a year at -20 °C.

Concentrated PaS for application in anti-doping labs was prepared as follows. The pooled fractions with PNPS hydrolysis activity had 0.5 g L⁻¹ ammonium sulfate added to precipitate most of the PaS at 4 °C for at least one hour. The mixture was centrifuged at 15000 × *g* for 15 min and the white pellet was then washed with 50 mL of 50 mM Tris-sulfate buffer at pH 7.4 with 0.5 g L⁻¹ ammonium sulfate. Centrifugation was repeated as before and the pellet was dissolved in 60% (v/v) glycerol, 50 mM Tris-sulfate at pH 7.4 (4 °C) for a final volume equivalent to 1% of the original culture volume for approximately 70 g L⁻¹. Concentration was determined by Bradford assay with bovine serum albumin as a standard. Samples were more than 95% pure based on SDS-PAGE stained by Colloidal Coomassie Blue G25 (BioRad, Gladesville, Australia) and reproduced in the Supporting Information (supplementary figure 1).

Analyte quantification

Spectrophotometry for para-nitrophenol (PNP). The hydrolysis of PNPS was monitored by measuring the absorption of 405 nm light by the PNP anion. Assays were performed in 96-well plate format with filled wells to obtain a 10 mm path length. Reactions or standards (300 µL) were mixed with 50 µL of 1 N KOH to ensure complete ionisation of PNP and to quench enzyme activity. The extinction coefficient for PNP with this method was 14,147 M⁻¹ cm⁻¹ determined with PNP standards from 0.3 to 40 µM (*r*² > 0.9995).

Liquid chromatography-mass spectrometry (LC-MS) for steroids and their derivatives. Steroids and steroid conjugates were separated using an Agilent (Mulgrave, Australia) 1260 UHPLC system with a Grace (Rowville, Australia) C18 column (50 mm with 5 mm guard column, 2 mm diameter, 1.8 µm particle size). The mobile phase consisted of 58% methanol and 42% aqueous ammonium acetate for analysis of steroid sulfates or 62% methanol and 38% aqueous ammonium acetate for steroid glucuronides. Both mobile phases had a final concentration of 10 mM ammonium acetate. Analytes were ionised by atmospheric pressure electrospray ionisation (AP-ESI) with an Agilent 6120 quadrupole mass spectrometer: estrone (E), the steroid sulfates and the steroid glucuronide were monitored in negative mode for the proton-loss species ([M-H]⁻), the steroid 3-sulfate, 17-glucuronide for the dianion ([M-2H]²⁻), and the remaining free steroids in positive mode for the proton adducts ([M+H]⁺). Negative and positive ions were monitored simultaneously with switching time optimised for analyte peak widths of 0.15 min. Capillary voltage and

fragmentation voltage were optimised for each analyte and are specified in the Supporting Information (supplementary table 1).

Enzyme assays. The mollusc enzyme preparations HpS, HrS, or PvS were assayed in 50 mM ammonium acetate at pH 5.0 (pH was selected according to the activity defined by Sigma–Aldrich and the buffer was selected for compatibility with LC-MS), KpS was assayed in 50 mM Tris-HCl at pH 7.4,^[27] and PaS in 50 mM Tris-acetate at pH 8.8.^[28] Each enzyme preparation was assayed for protein concentration using the Bradford assay with bovine serum albumin as a standard. The final enzyme concentrations used in the various assays are listed in the Supporting Information (supplementary table 2). Reaction components to be analysed by LC-MS were centrifuged at $16000 \times g$ for 3 min before transferring to clean vials for pre-incubation in a temperature controlled autosampler. All reactions were performed at 37 °C. Aliquots of enzyme and substrate were prepared and pre-incubated at 37 °C for 3 min before mixing enzyme with substrate to start reactions. Samples of the reaction were quenched by either addition of aqueous potassium hydroxide solution (1 M) for PNP detection or injection into LC mobile phase for analysis of steroid sulfate hydrolysis.

Substrate scope studies (Table 1) used 100 μM steroid sulfate. For kinetic analysis (Table 2; Supporting Information, supplementary figure 2), the substrate concentration range was prepared by serial two-fold dilution to cover 2.5 to 320 μM in reactions. Each enzyme-substrate combination was tested in three independent technical replicates (i.e. prepared and analysed at different times, but with the same samples of enzyme and substrate). Non-linear regression was used to fit concentration rate data to the Michaelis–Menten model or to a model with substrate inhibition included^[29] to estimate kinetic parameters.

Evaluation of PaS activity in a urine matrix

The collection of urine samples was conducted with approval of the Australian National University Human Research Ethics Committee.

Hydrolysis of steroid sulfates by PaS in a urine matrix. Aliquots of human urine (3 mL) were pipetted into test tubes (8 mL) and epiandrosterone- d_5 (EA- d_5 , 5 $\mu\text{g mL}^{-1}$) was added as internal standard. The samples

were adjusted to pH 9.0 ± 0.2 with the addition of aqueous ammonia solution (10% v/v) and mixing before the addition of PaS enzyme (50 μL , final concentration of 1 g L^{-1}) and overnight incubation at 37°C . Free steroids were isolated by solid phase extraction using an Agilent ABS Elut NEXUS[®] extraction cartridge (60 mg, 3 cc,) that was conditioned with methanol (3 mL) and water (3 mL), then loaded with the hydrolysis reaction and washed with aqueous sodium hydroxide solution (1 mL, 0.1 M) and water (3 mL), before elution with methanol (3 mL). The eluant was evaporated to dryness under nitrogen at 60°C before the liberated steroids were derivatised to form trimethylsilyl (TMS) ethers and TMS enol ethers using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide/ammonium iodide/dithiothreitol (50 μL , 1000:2:4 w/v/v) at 80°C for 60 min. The final extracts were dried under nitrogen at 60°C before being reconstituted in *n*-dodecane (50 μL) for GC-MS-MS analysis.

Detection of free steroids by GC-MS-MS of TMS derivatives. An Agilent 7000B GC-MS-MS was employed. The GC column was an Agilent J&W HP5-MS (30 m $\times$ 0.25 mm $\times$ 0.25 μm) with helium as carrier gas. Sample injections (1 μL) were made in pulsed-splitless mode with an injector temperature of 250°C . The column temperature was initially held at 182°C for 1 min, then was increased at 2°C min^{-1} to 220°C and held for 5 min, then increased to 310°C at $30^\circ\text{C min}^{-1}$ and held for 1 min. Head pressure was programmed to maintain a constant flow rate of 1.2 mL min^{-1} . The MS transfer line was set at 300°C and the ion source was operated in positive electron ionisation (+EI) mode with an ionization energy of 70 eV. Nitrogen was used for the collision gas at 1.5 mL min^{-1} . Instrument control and processing was performed using Agilent MassHunter[®] software. Multiple reaction monitoring (MRM) was used to identify derivatised DHEA and EA as specified in the Supporting Information (supplementary table 3), with limit of detection for both analytes at 0.1 ng mL^{-1} . Specificity for both steroids was evaluated following injection of neat standards to ensure there was no 'crosstalk' between the MRM transitions.

*Synthesis of 5α -androstane- 3β , 17β -diol 17-glucuronide (ADG) **1***^[30]

*5α -androstane- 3β , 17β -diol 3-sulfate (ADS) triethylammonium salt **2**.* Epiandrosterone 3-sulfate triethylammonium salt^{[21][22]} **3** (198 mg, 0.42 mmol) in distilled methanol (2 mL) was added drop-wise to solid sodium borohydride (86 mg, 2.27 mmol, 5.4 equiv.) at 0°C . After the vigorous reaction had subsided,

the reaction was allowed to warm to room temperature and stirred until complete by TLC. The reaction was quenched with saturated aqueous sodium bicarbonate solution (25 mL) and extracted with ethyl acetate (6 × 20 mL) and then 3:1 chloroform-isopropanol solution (6 × 20 mL). The combined organic layers were dried with magnesium sulfate and concentrated under reduced pressure to yield pure 5 α -androstane-3 β ,17 β -diol 3-sulfate triethylammonium salt **2** (157 mg, 79%) as an off-white solid. Copies of the ^1H and ^{13}C NMR spectra (supplementary figures 3–5) are provided in the Supporting Information. **R_f** 0.31 (7:2:1 EtOAc : MeOH : H₂O); **m.p.** 215–220 °C; **[α]_D²⁵** +32 (c 10, MeOH); **^1H NMR** (400 MHz, CD₃OD) δ 4.35 (1H, m, H3), 3.60 (1H, t, *J* 8.6, H17), 3.00 (6H, m, N(CH₂CH₃)₃), 2.50–0.85 (22H, m), 1.30 (9H, t, *J* 7.4, N(CH₂CH₃)₃), 0.85 (3H, s, H18), 0.80 (3H, s, H19); **^{13}C NMR** (100 MHz, CD₃OD) δ 82.5, 79.7, 55.9, 52.3, 47.8, 46.3, 44.1, 38.2, 38.0, 36.9, 36.5, 36.3, 32.8, 30.6, 29.7, 24.3, 21.9, 12.7, 11.7, 9.4, one carbon overlapping or obscured; **LRMS** (–ESI) *m/z* 371 ([M–Et₃NH][–], 100%), 97 ([HSO₄][–], 30%); **HRMS** (–ESI) *m/z* found 371.1892, C₁₉H₃₁O₅S ([M–Et₃NH][–]) requires 371.1892.

5 α -androstane-3 β ,17 β -diol 3-sulfate, 17-glucuronide (ADSG) 4. 5 α -androstane-3 β ,17 β -diol 3-sulfate triethylammonium salt **2** (1.00 mg, 2.11 μmol) was dissolved in *tert*-butanol (320 μL) and sodium phosphate buffer (2.05 mL, 50 mM, pH 7.5). Glucuronylsynthase enzyme solution (0.58 mL, 1.09 g L^{–1}, final concentration 0.2 mg mL^{–1})^[31] and α -D-glucuronyl fluoride **5** (2.3 mg, 10.8 μmol , 5 equiv.)^[31] dissolved in sodium phosphate buffer (220 μL , 50 mM, pH 7.5) were added and the reaction incubated without agitation at 37 °C for 2 d. The reaction was then subjected to solid-phase extraction to remove non-volatile salts and other reaction components.^[32] A Waters (Rydalme, Australia) Oasis WAX SPE cartridge (60 mg, 3 cc) was conditioned with methanol (1 mL) and milliQ water (3 mL). The crude reaction mixture was loaded onto the cartridge and washed with aqueous formic acid (3 mL, 2% v/v), milliQ water (3 mL), and finally with ammonium hydroxide in methanol (9 mL, 5% v/v). The appropriate fractions were combined and the solvent removed under reduced pressure at 40 °C to afford a mixture of 5 α -androstane-3 β ,17 β -diol 3-sulfate, 17-glucuronide (ADSG) **4** and 5 α -androstane-3 β ,17 β -diol 3-sulfate (ADS) **2** with a 31% conversion as determined by 600 MHz ^1H NMR integration of the H17 protons. A copy of the ^1H NMR conversion

spectrum (supplementary figure 6) is provided in the Supporting Information. The crude mixture was used directly in the subsequent enzyme hydrolysis step.

5 α -androstane-3 β ,17 β -diol 17-glucuronide (ADG) 1.^[30] The selective hydrolysis of 5 α -androstane-3 β ,17 β -diol 3-sulfate, 17-glucuronide (ADSG) **4** (1 mM) prepared above was performed at 37 °C with PaS enzyme preparation (final concentration of 0.8 g L⁻¹ in Tris-acetate buffer (50 mM, pH 9) with a final volume of 1 mL. After four to six hours incubation, 1 mL of 0.8 g L⁻¹ PaS in Tris-acetate buffer (50 mM, pH 9) was added. This step replenished enzyme activity and allowed complete hydrolysis after an additional four hours at 37 °C. The reaction was then quenched with methanol, 50% (v/v) final concentration, and then subjected to solid-phase extraction to remove non-volatile salts and other reaction components. A Waters Oasis WAX SPE cartridge (60 mg, 3 cc) was conditioned with methanol (1 mL) and milliQ water (3 mL). The crude reaction mixture was loaded onto the cartridge and washed with aqueous formic acid (3 mL, 2% v/v), milliQ water (3 mL), methanol (3 mL), and finally with ammonium hydroxide in methanol (9 mL, 5% v/v) to elute the steroid glucuronide. The appropriate fractions were combined and the solvent removed under reduced pressure at 40 °C to afford ADG **1** as a white solid. A copy of the 600 MHz ¹H NMR spectrum (supplementary figure 7) and low-resolution mass spectrum (supplementary figure 8) are provided in the Supporting Information. **R_f** 0.30 (7:2:1 EtOAc : MeOH : H₂O); **¹H NMR** (400 MHz, CD₃OD) δ 4.35 (1H, d, $J_{\text{H20-H21}}$ 7.8, H20), 3.81 (1H, t, $J_{\text{H17-H16}}$ 8.7, H17); 3.52–3.19 (5H, m), 2.07 (1H, m), 1.96 (1H, m), 1.77–1.50 (6H, m), 1.45–1.10 (11H, m), 1.03–0.90 (2H, m), 0.84 (3H, s, CH₃), 0.83 (3H, s, CH₃), 0.66 (1H, m); **LRMS** (–ESI) m/z 467 ([M–H][–]); **HRMS** (–ESI) m/z found 467.2641, C₂₅H₃₉O₈ ([M–H][–]) requires 467.2645.

Results

Sulfatase substrate range

Our initial research objective was to evaluate the four commercially derived sulfatase preparations alongside the heterologously expressed and purified PaS to establish substrate ranges for PNPS and steroid sulfate hydrolysis (Scheme 1). Activity, relative to total protein concentration, was measured by LC-MS or

spectrophotometry at a single 100 μM substrate concentration. Ten substrates, selected to encompass the structural diversity of commonly occurring steroid sulfates, were tested with each of the five enzymes and the resulting activity scored on a logarithmic scale (Table 1).

The commercial enzyme HpS was observed to hydrolyse the greatest number of sulfate substrates with only two steroid sulfates proving recalcitrant: androsterone 3-sulfate (AS) and epitestosterone 17-sulfate (ETS). The small aromatic substrate, PNPS, at 100 μM was hydrolysed most readily by HpS with a rate of $1.07 \text{ mmol min}^{-1} (\text{g protein})^{-1}$. In decreasing order of hydrolysis rates with 100 μM substrate concentration were: estrone 3-sulfate (ES, nine-fold slower than PNPS), dehydroepiandrosterone 3-sulfate (DHEAS), epiandrosterone 3-sulfate (EAS), boldenone 17-sulfate (BS), etiocholanolone 3-sulfate (ECS), testosterone 17-sulfate (TS), and nandrolone 17-sulfate (NS, 95,000-fold slower than PNPS).

The heterologously expressed and purified PaS showed a similar activity profile to HpS, except ECS was not significantly hydrolysed. Like HpS, the range of activity covered five orders of magnitude, from PNPS ($13.4 \text{ mmol min}^{-1} [\text{g protein}]^{-1}$) and ES (1.6-fold slower than PNPS) to BS (175,000-fold slower than PNPS). PaS was similar to HpS for hydrolysis of EAS per gram of protein. In comparing these enzymes with 100 μM substrate, PaS was faster with PNPS (13-fold), ES (69-fold), NS (46-fold), or TS (4-fold). In contrast, PaS was slower than HpS with DHEAS (3-fold) or BS (2-fold).

The other commercial sulfatases studied had narrower substrate ranges and lower activity per gram of protein. For example, HrS was capable of hydrolysing six of the ten substrates, showing an attenuated activity and substrate range relative to HpS and PaS. This enzyme preparation was unable to hydrolyse BS or NS and showed low activity with TS. Similarly, PvS could only hydrolyse four of the substrates but showed the least discrimination between phenolic and secondary hydroxyl-derived steroid sulfates, with similar rates observed for ES and EAS. Interestingly, DHEAS was hydrolysed ten-fold slower than EAS; these two similar substrates showed more consistent rates of hydrolysis with the other enzyme preparations. Finally, KpS exhibited the lowest sulfatase activity, with 240000-fold less activity per gram of protein than PaS for the model substrate PNPS. Furthermore, KpS showed no detectable hydrolysis activity for any of the steroid sulfates tested.

The two most active and promiscuous sulfatases PaS and HpS were selected for further analysis. The commercial sulfatase preparation HpS gave the broadest substrate range, hydrolysing eight of the ten substrates. The heterologously expressed and purified PaS was free of glucuronidase activity and had strong arylsulfatase activity on seven of the substrates tested (Table 1).

*Kinetic analysis of *P. aeruginosa* and *H. pomatia* sulfatases*

Substrate-saturation kinetics was measured in triplicate for PaS and HpS with four representative substrates: PNPS, ES, EAS and TS. Replicates gave consistent results. However, the replicates had greater variation with the poorest substrate, TS, and when substrate inhibition was observed (Supporting Information, supplementary figure 2). Michaelis–Menten substrate saturation kinetics explained the results for all except the hydrolysis of ES by PaS. This combination displayed substrate inhibition and an additional parameter (K_i) was included in the model giving an improved fit to the data.^[29] The Michaelis constant (K_M) was in the range of 7 to 90 μM for all substrate-enzyme combinations (Table 2) with PaS showing significantly lower K_M than HpS for PNPS, ES, and EAS, but no significant difference with TS (based on 95% confidence intervals).

An inspection of V_{max} values (Table 2) showed that PaS was faster per gram of protein than HpS for PNPS, ES, or TS, but not EAS. The greatest difference in V_{max} was seen with ES which was hydrolysed 65-fold faster by PaS. In contrast, EAS was hydrolysed by HpS two-fold faster than by PaS. Both PaS and HpS showed greater maximum hydrolysis rates for the aromatic sulfate esters PNPS and ES, although PaS had a greater bias in favour of the aromatic substrates than HpS. This can be seen when V_{max} is normalised for the model substrate, PNPS: PaS hydrolyses ES three-fold faster than HpS whereas HpS hydrolyses EAS 50-fold faster, or TS five-fold faster.

Hydrolysis of steroid sulfates in urine

DHEAS. Due to the endogenous contribution of urinary DHEA and DHEAS, buffer spikes were required to assess the hydrolysis efficiency of PaS ($61 \pm 4\%$, $n = 3$) from spiked DHEAS (100 ng mL^{-1} DHEA equiv.).

Method recovery without urine matrix contribution was assessed from pre- and post-extraction DHEA

spikes at 100 ng/mL to be 95%. In urine, hydrolysis with PaS demonstrated a more than 200-fold increase in DHEA when compared to control experiments not containing PaS (Supporting Information, supplementary figure 9). Selectivity was achieved for DHEA in urine with no matrix interferences observed for any of the 3 MRM transitions monitored. Compared with HpS and acid hydrolysis methods,^[33] PaS afforded 25% and 150% increased yields of DHEA in urine respectively. Matrix background observed from all three hydrolysis types was compared using peak areas normalised to DHEA that showed PaS to be equivalent to HpS. These were not as clean as the extract from acid hydrolysis, which was estimated to have one-quarter of the background based on the percentage of total peak areas. However, this result was not surprising, considering the acid hydrolysis procedure included an additional liquid-liquid extraction step.

EAS. The hydrolysis efficiency of PaS for EAS was estimated at $42 \pm 3\%$ ($n = 3$) from buffer spikes at 100 ng mL⁻¹ (EA equiv.). Method recovery without urine matrix was estimated at 92%. Poor selectivity for EA in urine was observed with the 434>169 and 434>239 transitions exhibiting co-eluting matrix interferences that allowed only partial resolution of the EA peak. Additional sample purification was attempted with C18 (Waters, Milford, MA, USA) solid phase extraction of urine samples prior to hydrolysis, but no improvement was found. The 434>239 transition was therefore used to monitor the efficacy of PaS. Hydrolysis with PaS increased EA from undetectable levels to a signal intensity of 2×10^4 in urine (Supporting Information, supplementary figure 10). Analysis of urinary EA was superior using PaS when compared to HpS, with the latter suffering from reduced sensitivity and selectivity. In comparison to the acid hydrolysis,^[33] the PaS extract provided similar signal intensity, though the matrix background determined from normalised peak areas was approximately half.

Chemoenzymatic synthesis of 5 α -androstane-3 β ,17 β -diol 17-glucuronide (ADG) 1

The commercially available sulfatase preparations are all reported to contain β -glucuronidase activity. In contrast, PaS is a purified enzyme, raising the prospect of selective sulfate ester hydrolysis in the presence of glucuronide conjugates. The *E. coli* strain used in PaS expression, DH5 α , is capable of β -glucuronidase expression, so the PaS preparation was tested for this activity by incubating 1 g L⁻¹ PaS with 5 mM *p*-nitrophenyl- β -D-glucuronide in 50 mM Tris-acetate at pH 8.8 at 37 °C. No hydrolysis to liberate the yellow

PNP anion was observed after overnight incubation, indicating that PaS was a suitable catalyst for the selective hydrolysis of sulfate ester conjugates.

To demonstrate this capacity for selective hydrolysis, a short chemoenzymatic synthesis of ADG **1** from EAS **3** was conducted (Scheme 2). Sodium borohydride-mediated reduction of EAS afforded 5 α -androstane-3 β ,17 β -diol 3-sulfate (ADS) triethylammonium salt **2**. This was then subjected to glucuronylation of the 17 β -hydroxy group employing a synthetically-derived 1- α -glucuronyl fluoride **5** donor in conjunction with the glucuronylsynthase catalyst derived from *E. coli* β -glucuronidase.^{[31][32][34]} This reaction afforded a mixture of ADS **2** and 5 α -androstane-3 β ,17 β -diol 3-sulfate, 17-glucuronide (ADSG) **4** with a 31% conversion as determined by 600 MHz ¹H NMR integration of the H17 protons. This mixture was used directly in the following hydrolysis experiments.

On a small scale, hydrolysis of the mixture containing ADSG **4** (~20 μ M) with PaS was monitored by LC-MS which showed the consumption of both ADS **2** and ADSG **4** and the accumulation of ADG **1** (Figure 1). Under the ESI conditions of this LC-MS analysis the saturated 5 α -androstane-3 β ,17 β -diol (AD) liberated from ADS **2** was not observed. In contrast to the selective PaS hydrolysis, the use of HpS resulted in hydrolysis of both sulfate and glucuronide conjugates such that no ADG **1** was produced (data not shown). After demonstrating that the final step in Scheme 2 was possible with the purified PaS, the reaction was scaled up to ~1 mM ADSG **4** (Scheme 2). This produced a mixture of hydrolysis products with ADG **1** remaining as the sole conjugated steroid, which could be readily purified by SPE using an Oasis WAX mixed mode polymeric/weak anion exchange cartridge.^[32] The data acquired for this compound was consistent with the proposed structure.^[30]

Discussion

The objective of this research was to discover a sulfatase enzyme capable of the mild and selective hydrolysis of steroid sulfates for analytical applications. In this study, four of the five enzymes tested displayed some steroid sulfatase activity (Table 1). The commercially available crude enzyme preparation

HpS showed the greatest substrate range, with detectable hydrolysis of seven of the nine steroid sulfates investigated. The heterologously expressed and purified PaS also showed broad scope with reasonable activity for six of the nine steroid sulfates. The remaining commercially-derived sulfatase preparations HrS, PvS and KpS showed reduced substrate scope and lower activity against this range of steroid sulfates. Both PaS and HpS showed useful activity as steroid sulfatases, but none of the five sulfatases tested here provided comprehensive sulfatase activity against the nine steroid substrates tested. Although not investigated as part of this work, we anticipate that PaS will prove useful in the hydrolysis of sulfate metabolites, particularly aryl sulfates, of non-steroidal performance-enhancing drugs, including andarine,^[16] and mesocarb.^{[17][18]}

Previous research by *Cawley et al.* on mollusc-derived sulfatase preparations uncovered marked preferences for certain steroid sulfates substitution patterns and configurations.^[35] This work highlighted that HpS had a particularly broad substrate range when compared to other mollusc sulfatases such as PvS. The work also highlighted the dependence of HpS activity on the configuration of 3-hydroxyandrostane-17-ones: relatively high activity for DHEAS (3 β ,5-ene), EAS (3 β ,5 α), and ECS (3 α ,5 β); intermediate activity for 3 β -hydroxy-5 β -androstane-17-one 3-sulfate (3 β ,5 β); and no activity for AS (3 α ,5 α). Our measurements of HpS activity correlate well with this activity profile and include additional data for several steroid 17-sulfates: TS, BS, and NS (17 β) are hydrolysed whereas ETS (17 α) is not. Taken together, these results reveal significant differences in enzyme-substrate interactions and kinetic competence for the sulfatases studied. The bacterial sulfatase PaS appears to be slightly more constrained than the mollusc-derived HpS enzyme preparation given that it cannot hydrolyse ECS. However, in contrast to the recombinantly expressed PaS, it is not certain how many sulfatase isoforms are present in crude HpS preparation or how many contribute to the apparent steroid sulfatase activity. An attempt to isolate the genes responsible for HpS sulfatase activity indicated that at least three sulfatases are present, but only one was cloned as full-length complementary DNA. Attempted expression of this gene in either *E. coli* or *Saccharomyces cerevisiae* failed to afford functional enzyme,^[36] which serves to highlight the limits of our understanding of these mollusc-derived sulfatases.

There are subtle differences in activity and substrate range for PaS and HpS (Table 1). Greater activity per gram of protein is observed for PaS relative to HpS for TS and the 19-nor steroid, NS at 100 μ M substrate concentration. As noted above, HpS has activity for ECS, whereas PaS does not. The detailed kinetic analysis (Table 2) shows that PaS has lower K_M for most substrates, though there was no significant difference for TS. Given that sports drug testing analysis or medical research typically involves low concentrations of steroid compounds (pM– μ M), a lower K_M would be advantageous.

For both enzymes, the substrate range from PNPS to TS show a difference in V_{max} of five orders of magnitude (Table 2). The aryl sulfate esters can be hydrolysed more readily due to the conjugation afforded by the aromatic ring that provides increased stability for the leaving group.^{[37][38]} The uncatalysed hydrolysis of the S–O bond in near-neutral water for PNPS has been determined as $1.9 \times 10^{-10} \text{ s}^{-1}$, and estimated by extrapolation for the n-pentyl sulfate as $3 \times 10^{-26} \text{ s}^{-1}$.^[37] Therefore, even though the maximum rates of hydrolysis for the secondary alkyl sulfate TS is about four to five orders of magnitude slower than the aryl sulfates PNPS and ES, the rate enhancement afforded by HpS or PaS for TS may be significantly greater ($\sim 10^{11}$ -fold). It is worth noting that with reasonable catalytic rates for hydrolysis of an extremely stable S–O bond in alkyl sulfates, some alkyl sulfatases provide the greatest rate enhancements of the known enzymes.^[37]

Differences in maximum velocity were also observed with PaS displaying 25-fold greater V_{max} for PNPS than HpS. This reflects the enzyme purity, molecular weight and turnover (k_{cat}) for the pH and temperature used in the assays. It is likely that much of this difference arises due to the lower purity of HpS which is a crude enzyme preparation. In contrast, PaS is purified by affinity chromatography and given the effective post-translational modification of the active site cysteine to formyl glycine observed on expression in *E. coli*,^[39] the k_{cat} for PaS with PNPS measured in this work would be 26 s^{-1} at pH 8.8 and 37 °C. This is similar to previously determined k_{cat} of 14 s^{-1} for PNPS at pH 8 and 25 °C^[40] and suggests that the PaS preparation is of high purity with the majority of PaS molecules correctly modified with a formyl glycine residue.

In a urine matrix PaS demonstrated a superior ability to liberate DHEA and EA from their respective sulfate conjugates when compared to HpS, although analysis of the latter substrate was complicated by selectivity

issues arising from the urine matrix. Of particular importance, the yield of DHEA following hydrolysis with PaS was 25% greater than that observed for HpS and 150% greater than acid hydrolysis. In terms of the potential for translation to routine sports drug testing analysis, direct hydrolysis of steroid sulfates using PaS in urine appears satisfactory, without the need for prior extraction.

The PaS enzyme was selected as a candidate steroid sulfatase in part because whole cell cultures of *P. aeruginosa* derived from human faecal samples were reported to hydrolyse the sulfate ester of lithocholic acid sulfate.^[41] As an enteric microbe, this bacterium may rely on sulfatase activity in order to scavenge sulfate from bile acid salts.^[28] Thus it was our hope that this putative sulfatase activity would be extended to other steroid sulfates. Ironically, PaS did not hydrolyse lithocholic acid sulfate in our hands (results not shown) and this failure is consistent with the inability to hydrolyse ECS that also contains the 3 α ,5 β -configuration of the A-ring. It is possible that the activity observed for *P. aeruginosa* culture is due to one of the other reported sulfatases: AtsK or SdsA1 that are proposed to employ distinct C–O bond cleavage pathways.^{[42][43]}

The selective hydrolysis of sulfate conjugates in the presence of glucuronides presents a significant challenge to existing methods. Commercial sulfatase enzyme preparations such as HpS contain glucuronidase activity.^[3] Chemical methods of hydrolysis such as strong acid^[33] generally show poor discrimination for the hydrolysis of these phase II conjugates. In contrast the selective cleavage of glucuronides can be readily achieved through the agency of *E. coli* β -glucuronidase. The development of PaS as a purified enzyme allowed for the selective hydrolysis of steroid sulfate esters in the presence of glucuronide conjugates. This was exemplified by the short chemoenzymatic synthesis of ADG **1** (Scheme 2) involving the selective PaS-mediated hydrolysis of a sulfate ester protecting group in the presence of a steroid glucuronide. Thus PaS provides for the targeted investigations of sulfate esters in complex metabolite mixtures that contain glucuronide conjugates.

Conclusions

Forty-five years ago, Cawley *et al.*^[35] highlighted the need to develop a general sulfatase preparation for clinical analysis of steroid sulfates; a need that has remained essentially unmet to this day.^[3] Although sulfate ester hydrolysis can be achieved by a range of methods,^[3] and the requirement for sulfate hydrolysis has been circumvented to some extent by the direct detection of steroid sulfates using LC-MS-MS methods, mild and selective steroid sulfatase activity would be beneficial for a range of applications including routine screening, confirmatory analysis, GC-IRMS and research.

The recombinantly expressed and purified PaS offers several advantages over HpS and other mollusc-derived sulfatase preparations in that it has relatively high activity for 3-keto, 17 β steroid sulfates and is easily purified. The latter trait allows application in selective sulfate ester hydrolysis, exploited in this work for the synthesis of ADG **1**. The PaS enzyme does not meet the requirements of a general steroid sulfatase as it hydrolysed only six of the nine steroid sulfates investigated: no detectable activity was observed for the α -configured sulfates ECS, AS and ETS. Even considering this limitation, access to steroid sulfatase activity without additional enzyme activities is likely to prove beneficial for range of analytical applications. Further, PaS is amenable to improvement because its amino acid sequence and crystal structure are known and, in contrast to the mollusc-derived counterparts such as HpS, it can be easily expressed in the laboratory. Further engineering will be directed to increasing the substrate scope and improving the catalytic efficiency of the PaS enzyme.

Supplementary Information

Parameters for analyte detection by LC-MS and GC-MS-MS; enzyme concentrations used in assays; SDS-PAGE gel for the PaS preparation; substrate saturation plots for HpS or PaS with PNPS, ES, EAS and TS; ¹H and ¹³C NMR spectra for ADS; ¹H NMR conversion spectrum for ADSG; ¹H NMR and LRMS for ADG; and GC-MS-MS chromatograms for DHEA and EA liberated by PaS.

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Table 1. Substrate range for five sulfatase preparations with 100 μM substrate at 37 °C. The specific activity ($\mu\text{mol min}^{-1} [\text{g protein}]^{-1}$) is represented on a logarithmic scale with: 0, no detectable activity; 1, 0.005–0.05; 2, 0.05–0.5; 3, 0.5–5; 4, 5–50; 5, 50–500; 6, 500–5000; and 7, 5000–50000.

Substrate	PNPS	ES	DHEAS	EAS	AS	ECS	TS	BS	NS	ETS
Sulfate position	aromatic	aromatic	3 β	3 β	3 α	3 α	17 β	17 β	17 β	17 α
HpS	6	5	3	3	0	2	1	2	1	0
PaS	7	7	3	3	0	0	2	2	3	0
HrS	5	3	3	2	0	2	1	0	0	0
PvS	3	2	1	2	0	0	0	0	0	0
KpS	2	0	0	0	0	0	0	0	0	0

Table 2. Kinetic parameters estimated by non-linear regression for PaS or HpS with four substrates. The values are presented with 95% confidence intervals ($1.96 \times$ standard error of estimate, rounded to one significant figure). Activity was measured in 50 mM ammonium acetate at pH 5.0 for HpS, or in 50 mM Tris HCl at pH 8.8 for PaS, and at 37 °C for both.

Enzyme	Kinetic constant	PNPS	ES	EAS	TS
PaS	$V_{\max}$ ($\mu\text{mol min}^{-1} [\text{g protein}]^{-1}$)	25000 ± 1000	17000 ± 2000	0.63 ± 0.05	0.09 ± 0.01
	K_M (μM)	7 ± 1	14 ± 5	26 ± 8	50 ± 20
HpS	$V_{\max}$ ($\mu\text{mol min}^{-1} [\text{g protein}]^{-1}$)	1180 ± 40	270 ± 30	1.53 ± 0.07	0.024 ± 0.007
	K_M (μM)	17 ± 3	90 ± 30	61 ± 8	60 ± 50

* PaS exhibited substrate inhibition with ES, $K_i = 600 \pm 400 \mu\text{M}$

Scheme 1. Hydrolysis of testosterone sulfate (TS) to testosterone (T) by PaS.

Scheme 2. Chemoenzymatic synthesis of 5 α -androstane-3 β ,17 β -diol 17-glucuronide (ADG) **1**.

Figure 1. Hydrolysis of 5 α -androstane-3 β ,17 β -diol 3-sulfate, 17-glucuronide (ADSG) **4** and 5 α -androstane-3 β ,17 β -diol 3-sulfate (ADS) **2** by PaS. The reaction was run at 37 °C and pH 8.8 with 0.9 g L⁻¹ PaS and ~20 μ M ADSG. An autosampler was used to periodically inject 1 μ L samples for analysis by LC-MS to measure the relative concentrations of ADSG **4** (x), ADS **2** (Δ), and the desired product, 5 α -androstane-3 β ,17 β -diol 17-glucuronide (ADG) **1** (o). The 5 α -androstane-3 β ,17 β -diol (AD) product derived from the hydrolysis of ADS **2** could not be detected by this LC-MS method.