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for

Direct Evidence for ArO-S Bond Cleavage during Inactivation of *Pseudomonas aeruginosa* Arylsulfatase by Aryl Sulfamates

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Experimental Section (Chemistry)

General: Unless otherwise stated, all reagents used were of analytical grade. Melting points were obtained using an Electrothermal melting point apparatus and are corrected. IR spectra were recorded on a Perkin–Elmer Spectrum One FTIR spectrometer with a zinc selenide/diamond universal ATR sampling accessory as a thin film. ^{1}H and ^{13}C NMR spectra were obtained on a Varian UnityPlus 400 instrument (400 MHz for ^{1}H, 100 MHz for ^{13}C). Internal standards employed for NMR spectra were residual chloroform (^{1}H: d 7.24; ^{13}C: d 77.0 ppm) or internal tetramethylsilane (^{1}H, ^{13}C: d 0.00 ppm) for samples run in deuteriochloroform ($CDCl_3$); residual acetone (^{1}H: d 2.05; ^{13}C: d 29.9 ppm) for samples run in [D_6]acetone; residual methanol (^{1}H: d 4.87; ^{13}C: d 49.15 ppm) for samples run in [D_4]methanol and HOD (^{1}H: d 4.81 ppm) for samples run in D_2O. Dioxane (^{13}C: d 66.66 ppm) was used as an external standard for ^{13}C NMR spectra in D_2O. Elemental analysis was performed by CMAS, Belmont, Australia. Buffer A is 25 mM bis-tris propane/ 25 mM glycine/ 0.05% BSA, and buffer B is 50 mM citric acid/ potassium citrate/0.05% BSA.

Potassium 4-methoxyphenyl sulfate (1): This compound was prepared and characterized as described previously.[1]

Potassium 4-methylumbelliferone sulfate (2): This compound was prepared according to the procedure by Denehy et al.,[1] with minor modifications: sulfur trioxide pyridine complex (0.82 g, 5.16 mmol),

was added to a stirred solution of 4-methylumbelliferone (1.00 g, 5.7 mmol) in toluene (5 mL) under reflux. The solution was stirred under reflux overnight, during which time a thick, solid precipitate formed. TLC: butane-1-ol/ethanol/water, 4:1:5. Aqueous potassium hydroxide (0.5 M, 100 mL) was added until the precipitate dissolved and the mixture was extracted with ethyl acetate (3 × 80 mL). The aqueous phase was acidified to pH 5 using glacial acetic acid and then extracted with ethyl acetate (1 × 80 mL). The aqueous phase was re-basified to pH 10 using aqueous potassium hydroxide (0.5 M), and the solvent was evaporated under reduced pressure until a precipitate started to form. Recrystallization of the collected precipitate from boiling water yielded compound **2** as white solid (0.79 g, 52%). The qualitative test for the presence of free sulfate by dropwise addition of aq. $BaCl_2$ (0.5 M) to a solution of **2** yielded no precipitate. ^{1}H NMR (400 MHz, D_2O): d = 7.70 (d, J(H7,H8) = 8.4 Hz, 1H, H-8), 7.26 (d, J(H7,H8) = 8.4 Hz, 1H, H-7), 7.25 (s, 1H, H-5), 6.26 (s, 1H, H-3), 2.40 ppm (s, 3H, CH_3). ^{13}C NMR (100 MHz, D_2O): d = 164.2, 155.9, 153.9, 153.2, 126.6, 118.12, 117.7, 112.8, 112.8, 109.2, 18.0 ppm (11C, Ar). IR (KBr disc): n = 1700 (strong, CO); 1395 (C-O-C), 1230-1279 (broad, C-O-S), 1047 cm^{-1} (strong, SO_2 sym), which is consistent with the literature.[2]

Synthesis of sulfamates 3, 4, 6, 8-12 and 667COUMATE: Aryl sulfamates **3**, **4**, **6**, and **8-12**[1] as well as 667COUMATE[3] were prepared and characterized as described in our earlier work.

General procedure for the synthesis of sulfamates 5, 7, and FLAVOMATE: A solution of the respective alcohol (8 mmol) in *N*,*N*-dimethylacetamide (5 mL) was added to the chilled, freshly prepared solution of sulfamoyl chloride (24 mmol)[4] in acetonitrile (20 mL) and stirred overnight at room temperature under nitrogen. The reaction was quenched with water and the product was extracted into ethyl acetate (3 × 50 mL). The combined organic extracts were washed with water (3 × 100 mL), dried ($MgSO_4$), and the solvent removed under reduced pressure. Recrystallization from toluene afforded the title compounds.

3,5-Dichlorophenyl sulfamate (5): This compound was prepared according to the general procedure as fine beige needles (1.03 g, 53%); m.p. 149-151 °C (lit.[5] 146-147 °C). ^{1}H NMR (400 MHz, [D_4]methanol): d = 7.40 (t, J(H2,H4) = 2 Hz, 1H, H-4), 7.31 ppm (d, J(H2,H4) = J(H4,H6) = 2 Hz, 2H, H-2/H-6). ^{13}C NMR (100 MHz, [D_4]methanol): d = 153.0, 136.6, 127.9, 122.7 ppm (4C, Ar). IR (KBr disc): n = 3383 (strong, N-H), 3279 (strong, N-H), 1575 (strong), 1424, 1364 (strong, SO_2 asym), 1181 cm^{-1} (strong, SO_2 sym).

3-Cyanophenyl sulfamate (7): This compound was prepared according to the general procedure as colorless needles (0.94 g, 59%); m.p. 106-109 °C (lit. 104-107 °C[6] or 101-104 °C).[5] ^{1}H NMR (400 MHz, [D_4]methanol): d = 7.70-7.72 (m, 2H, Ph), 7.64-7.66 ppm (m, 2H, Ph). ^{13}C NMR (100 MHz, [D_4]-methanol): d = 152.4 (CN), 132.3, 131.6, 128.7, 127.2, 118.8, 114.7 ppm (6C, Ar). IR (KBr disc): n = 3344 (N-H), 3236 (N-H), 2244 (C≡N), 1482, 1385 (strong, SO_2 asym), 1196 (strong, SO_2 sym), 1126 cm^{-1}.

FLAVOMATE; flavone-3-*O*-sulfamate: This compound was prepared according to the general procedure, however, after quenching the reaction with water, the product was extracted into dichloromethane (2 × 80 mL), the combined organic extracts were washed with water (3 × 100 mL), dried ($MgSO_4$), and the solvent removed under reduced pressure. Recrystallization from ethyl acetate afforded the

title compound as colorless needles (0.77 mg, 30%); m.p. 177-179 °C. ^{1}H NMR (400 MHz, [D_6]acetone): d = 8.22 (dd, J(H5,H6) = 8.0 Hz, J(H5,H7) = 1.8 Hz, 1H, H-5), 8.12 (dd, J(H2`,H3`) = J(H5`,H6`) = 8.0 Hz, J(H2`,H4`) = J(H4`,H6`) = 2.4 Hz, 2H, H-2`/H-6`), 7.92 (td, J(H6,H7) = 8.0 Hz, J(H5,H7) = 1.8 Hz, 1H, H-7), 7.80 (d, J(H7,H8) = 8.0 Hz, 1H, H-8), 7.64-7.57 (m, 4H, H-3`, H-4`, H-5`, H-6), 7.39 ppm (s, 2H, NH_2). ^{13}C NMR (100 MHz, [D_6]acetone): d = 174.7 (1C, C=O), 159.0, 156.3, 135.9, 135.8, 132.6, 130.7, 129.9, 129.6, 126.7, 126.4, 124.5, 119.5 ppm (14C, Ar). IR (KBr disc): n = 3328 (N-H), 3241 (N-H), 1614 (broad), 1380 (strong, SO_2 asym), 1182 cm^{-1} (strong, SO_2 sym). $C_{15}H_{11}NO_5S$: calcd 56.78, H 3.49, N 4.41; found C 56.87, H 3.41, N 4.57.

COUMATE; 4-methylcoumarin-7-*O*-sulfamate: According to a published procedure,[7] 7-hydroxy-4-methylcoumarin (2.0 g, 11 mmol) and freshly prepared sulfamoyl chloride solution in acetonitrile (4.0 mL, 20 mmol) yielded the title compound as colorless rhombic crystals (0.9 g, 31%); m.p. 165-168 °C (lit.[7] m.p 165-167 °C). ^{1}H NMR (400 MHz, [D_4]methanol): d = 7.8 (d, J(H8,H9) = 9.6 Hz, 1H, H-9), 7.3 (m, 2H, H-6/H-8), 6.3 (s, 1H, H-2), 3.3 (br s, 2H, NH_2), 2.4 ppm (s, 3H, CH_3). ^{13}C NMR (100 MHz, [D_4]-methanol): d = 162.5 (1C, C=O), 155.2, 154.9, 154.4, 127.5, 119.7, 119.6, 115.0, 111.3 (8C, Ar), 18.7 ppm (s, CH_3). IR (KBr disc): n = 3351 (broad, N-H); 3217 (broad, N-H); 1700 (strong, CO); 1388 (strong, C-O-C), 1122 cm^{-1} (strong, SO_2 sym). $C_{10}H_9NO_5S$: calcd 47.06, H 3.55, N 5.49; found 47.13, H 3.58, N 5.51.

Elemental analysis of sulfamates used in active site titration: The purity of compounds **4**, **9**, **11**, and 667COUMATE, which were used in active-site titration experiments, was confirmed by elemental analysis. Compound **4**, $C_7H_6N_2O_3S$: calcd C 42.42, H 3.05, N 14.13, found C 42.45, H 3.07, N 14.21; compound **9**, $C_6H_5INO_3S$: calcd C 24.09, H 2.02, N 4.68, found C 24.10, H 2.06, N 4.73; compound **11**, $C_7H_9NO_4S$: calcd C 41.37, H 4.46, N 6.89; found C 41.61, H 4.11, N 8.14; 667COUMATE, $C_{14}H_{15}$-NO_5S: calcd C 54.36, H 4.89, N, 4.53, found C 54.35, H 4.96, N 4.53.

Stability of sulfamates: Aryl sulfamates stock solutions were prepared in EtOH. Stabilities of inactiva-tors **3**, **4**, **11**, COUMATE, and FLAVOMATE were determined in buffer A pH 7 or 9 or buffer B pH 5 containing 5% EtOH at 37 °C. The following extinction coefficients (Δe, M^{-1}cm^{-1}) were determined un-der the above conditions (at pH 7 unless stated otherwise): 9730 (**3**; 400 nm), 4150 (**4**; 270 nm), 1040 (**11**; 277 nm at pH 5, 7, and 9), 9090 and 15050 (COUMATE; 340 nm at pH 7, and 362 nm at pH 9, respectively), 10900 and 9100 (FLAVOMATE; 344 nm at pH 7, and 410 nm at pH 9, respectively). At least two different concentrations of inactivator were incubated for at least 5 min and the rate of hydro-lysis was monitored continuously on a UV-Vis spectrophotometer. All experiments were performed in duplicates. Since sulfamate spontaneous hydrolysis is inversely proportional to the pK_a value of the parent phenol,[8] only the least stable inactivators **3** and **4** from the aryl sulfamate series were assayed. The rate constant of spontaneous decay, k_{uncat} (min^{-1}), was calculated from the dependence of the pseudo-first-order hydrolysis rate on the concentration of inactivator. A criterion of sufficient stability for inactivation experiments was k_{uncat} < 0.005 min^{-1}, i.e. less than 0.5% decomposition of inactivator in 1 min. The stability of phenyl sulfamate (**11**) was also investigated in the absence of BSA and in 100 mM tris buffer and no differences in the rate of spontaneous hydrolysis were noted.

Experimental Section (Biochemistry)

General: *PfuTurbo* DNA polymerase was obtained from Stratagene. Restriction enzymes, T4 DNA ligase, and PCR nucleotide mix were from Roche. DNA fragment and plasmid purification kits were obtained from Qiagen. Oligonucleotide primers were from Sigma Genosys. Buffer C is 100 mM potassium phosphate.

Construction of *Pa*AtsA expression vector: The gene encoding *Pa*AtsA (GenBank™ identifier 15595381) was amplified from *Pseudomonas aeruginosa* genomic DNA by PCR using the following forward 5'-GAGACCCGC**ATATG**AGCAAACGCCCCAACTTC-3' and reverse 5'-GTCGTG**CTCGAG**TTATTATCGCACCAGGAAAGGCGAAG-3' primers containing 5'-*Nde*I and 3'-*Xho*I restriction sites (shown in bold) and repeat stop codons (underlined), respectively. The amplicon was gel purified and restricted with *Nde*I and *Xho*I, and ligated into a similarly digested pET30b(+) vector using T4 DNA ligase. The reaction mixture was transformed into chemically competent *E. coli* DH5α and recombinant plasmid obtained from isolated kanamycin resistant colonies and confirmed by sequencing to afford pET30-*Pa*AtsA.

Expression and purification of *Pa*AtsA: *E. coli* BL21 (λDE3) cells were transformed with pET30-*Pa*AtsA and grown on LB agar containing 50 mgL^{-1} kanamycin. An isolated colony was grown aerobically in LB media containing 50 mgL^{-1} kanamycin (10 mL) at 37 °C on a rotary shaker (100 rpm), until noticeably turbid (18 h). Two such cultures (each 10 mL) were used to inoculate 2 × 500 mL of LB media containing 50 mgL^{-1} kanamycin in 2 L baffled flasks. The cultures were grown aerobically at 37 °C on a rotary shaker (100 rpm) to log phase (OD_{600} 0.5). The cultures were allowed to cool to room temperature (30 min) and protein production induced by the addition of isopropyl 1-thio-β-D-galactoside to a final concentration of 50 μM. The cultures were allowed to grow for 20 h at room temperature, after which the cultures were cooled on ice. Cells were harvested by centrifugation (6000 rpm, 15 min, 4 °C), and the cell pellet frozen in liquid N_2. The cell pellet (total wet weight 2.2 g) was defrosted on ice and resuspended in ice-cold 50 mM Tris.HCl buffer at pH 7.5 (30 mL). Hen egg white lysozyme was added to a final concentration of 1 $mgmL^{-1}$ and the mixture incubated on ice (30 min). The lysate was frozen in liquid nitrogen and allowed to defrost on ice. After defrosting, the lysate was sonicated (Branson Sonifier 250) using a microtip attachment (6 bursts × 10 s), with 60 s cooling on ice between bursts. The lysate was centrifuged (8000 rpm, 30 min, 4 °C) and the supernatant decanted and loaded onto a Ni-NTA (Qiagen) column pre-equilibrated with start buffer (10 mM imidazole in 50 mM sodium phosphate and 300 mM NaCl, pH 8.0), and eluted with start buffer (60 min), then wash buffer (20 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0, 15 min). Finally, protein was eluted with a gradient of imidazole (20–250 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0), with a major protein peak corresponding to *Pa*AtsA activity eluting at 40–160 mM imidazole. Fractions containing *Pa*AtsA were concentrated to a final volume of 400 μL and diluted to 20 mL by addition of 1.7 M $(NH_4)_2SO_4$ in buffer C, pH 7.5 for application onto a *tert*-butyl Sepharose (Amersham) column (resin volume = 20 × 60 mm) pre-equilibrated with 1.7 M $(NH_4)_2SO_4$ in buffer C, pH 7.5. The column was washed with the same buffer (2.0 $mLmin^{-1}$, 50 min), then with a gradient of decreasing ammonium sulfate 1.7–0 M $(NH_4)_2SO_4$ in this buffer over 100 min, with a major protein peak corresponding to *Pa*AtsA activity eluting at 0.86–0.34 M $(NH_4)_2SO_4$. The fractions were concentrated and dialysed three times

against buffer C, pH 7.0 at 4 °C and then diluted to a total final volume of 10 mL. The diluted protein was filtered (0.45 µm Millex filter, Millipore) and was stable for at least six months at 4 °C.

Assay of *Pa*AtsA concentration by gas chromatography-mass spectrometry (GC/MS): Enzyme concentration was determined after acid hydrolysis and GC/MS analysis of released amino acids. All analyses were performed in triplicate. Samples in buffer C, pH 7 containing approximately 1-5 nmol of protein were dried in a 100 µL glass vial insert together with 10 nmol of norleucine internal standard by evaporation at 50 °C (SpeedVac Plus SC110A, Savant Instruments). Samples were dehydrated by co-evaporation with MeOH (20 µL) and hydrolyzed in HCl vapor under a nitrogen atmosphere for 16 h at 110 °C (constant boiling 6 N HCl, Pierce Biotechnology). After cooling to room temperature, residual HCl was removed by co-evaporation with toluene (3 × 20 µL). Pyridine (20 µL) was added and the sample was allowed to stand for 3 h, before addition of *N*-methyl-*N*-trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) (20 µL, Pierce Biotechnology). After 1 h, the sample was analyzed by GC/MS (Agilent 6890N Gas Chromatograph coupled to 5973 Mass Selective Detector and 7683 Automatic Liquid Sampler) on a 30-m DB5-MS column with 250 µm inner diameter and 0.25 µm film thickness (J&W Scientific, Folsom, CA, USA). Samples (1 µL) were injected at a temperature of 270 °C, the interface was set at 250 °C and the ion source adjusted to 230 °C. The carrier gas was helium (flow rate 1 $mLmin^{-1}$). The temperature program was 1 min isothermal heating at 70 °C, followed by a 12.5 °C per min oven temperature ramp to 295 °C, then 25 °C per min to 320 °C and held for 1 min. Mass spectra were recorded at 3.2 scans per s (*m/z* 50–500). The chromatograms were analyzed by using HP ChemStation (Agilent). The contents of individual amino acids, referenced to norleucine, were compared to the calibration amino acid mixture. The calibration mixture consisted of equimolar amounts (10 nmol) of Gly, Ile, Leu, Lys, Pro, Thr, Tyr, Val, and norleucine as internal standard. Each of these amino acids was relatively abundant in the protein (with a minimum of 16 amino acids predicted from the gene sequence), showed no decomposition by acidic hydrolysis in time (after 2, 6, and 16 h) and possessed good sensitivity in the GC/MS. The calibration mixture was processed in the same way and at the same time as protein samples. Several TMS-derivatives were formed with Gly, Lys, Pro, Thr, and Tyr and their ratio was not identical in the protein and calibration samples. Therefore, only the single TMS-derivatives of Ile, Leu, and Val were used to calculate protein concentration. $[E]_{0,GC/MS}$ = 65 ± 11 µM (mean ± S.D.). This concentration value was used in all active-site titration experiments.

Titration of *Pa*AtsA sulfhydryl groups using Ellman's reagent: Titration of sulfhydryl groups was performed according to Riener et al.,[9] with minor modifications. The enzyme in buffer C, pH 7 was diluted with this buffer to a final volume of 250 µL. Denaturing dilution buffer (250 µL of 137 mM NaCl/ 10.1 mM Na_2HPO_4/1.76 mM KH_2PO_4/8 M guanidine hydrochloride, pH 7.4) was added, followed by boric acid buffer (100 µL of 100 mM boric acid/0.2 mM EDTA, pH 9.6), so that the final pH was 8.0–8.2. The sample was vortexed and allowed to stand at room temperature for 10 min to ensure complete unfolding. After the addition of Ellmann`s reagent (10 µL of 10 mM 5,5`-dithio-bis(2-nitrobenzoic acid)/ 100 mM NaH_2PO_4/0.2 mM EDTA, pH 7.0), the sample was vortexed and, after 5 min at room temperature, the absorbance was read at 412 nm in 1-cm path-length methacrylate cuvettes. In the parallel reagent blank, the diluted enzyme was substituted by buffer C, pH 7 (250 µL). In the parallel protein

blank, Ellman's reagent was substituted by water (10 µL). Both blanks were measured (412 nm) after 5 min at room temperature. Extinction coefficient for 4 M guanidine hydrochloride (final concentration) was Δe_{412} = 18100 $M^{-1}cm^{-1}$, as determined from calibration by glutathione (5–40 µM). The concentration (M) of free sulfhydryl groups [SH] in undiluted denatured protein was calculated as per Riener et al.[9] Ellmann's reagent was kept frozen at –20 °C in aliquots. After thawing, it was kept on ice and was used within 2 h. BSA ($[SH]_{calcd}/[SH]_{theoretical}$ = 0.3) was used as a control. Each assay was performed in duplicate with three different enzyme concentrations. All samples were monitored for additional 2 h and no further increase in absorbance was observed.

The conditions for *Pa*AtsA unfolding were determined by monitoring fluorescence of 0.65 µM *Pa*AtsA in buffer C at pH 7 with 0–6 M guanidine hydrochloride (l_{ex} 295 nm, l_{em} 333 nm, slit width 5 nm).[10] The plateau of the sigmoidal plot of fluorescence intensity versus guanidine hydrochloride concentration indicates complete unfolding of *Pa*AtsA. In 4 M guanidine hydrochloride, *Pa*AtsA showed complete unfolding after 5 min.

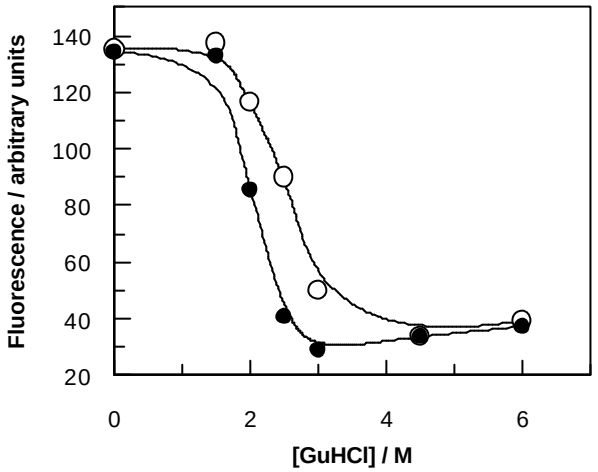

Figure S1. Unfolding titration of *Pa*AtsA in guanidine hydrochloride. Unfolding was monitored by fluorescence (l_{ex} 295 nm, l_{em} 333 nm, slit width 5 nm). Sample fluorescence determined after: 2 min incubation (○), 15 min incubation (●).

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