The stereochemical course of sulphuryl transfer catalysed by arylsulphatase II from *Aspergillus oryzae*

Christina L. L. CHAI,* Wendy A. LOUGHLIN and Gordon LOWE†

Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K.

Phenyl [(*R*)-¹⁶O,¹⁷O,¹⁸O]sulphate was synthesized and used to study the stereochemical course of sulphuryl transfer to *p*-cresol catalysed by arylsulphatase II from *Aspergillus oryzae*. The reaction was shown to proceed with retention of configuration at the sulphur atom, providing evidence for the involvement of a sulphyd-enzyme intermediate on the reaction pathway.

INTRODUCTION

Aspergillus oryzae, when grown under conditions of limiting sulphur availability, produces high levels of arylsulphatase activity. The arylsulphatase activity can be resolved into three components. The catalytic-centre activity of one of these enzymes, namely, arylsulphatase II, is greatly enhanced in the presence of tyramine. It has been demonstrated that this enhanced activity is due to tyramine behaving as an acceptor for the sulphuryl group, i.e. in the presence of tyramine, arylsulphatase II behaves as an arylsulphotransferase (Burns & Wynn, 1975). The enzyme is unusual among sulphotransferases in not requiring 3'-phosphoadenosine 5'-phosphate (Roy & Trudinger, 1970) as a cofactor, nor does it catalyse the transfer of the sulphuryl group from 3'-phosphoadenosine 5'-phosphosulphate to potential phenolic acceptors (Burns & Wynn, 1975). A kinetic investigation of the enzyme showed that it followed a Ping Pong kinetic mechanism, from which it was inferred that a covalent sulphyd-enzyme intermediate is involved in the reaction pathway, water being a much poorer acceptor than a phenol for sulphuryl transfer, so accounting for the sulphotransferase activity in the presence of a suitable acceptor (Burns *et al.*, 1977).

A Ping Pong kinetic mechanism is notoriously difficult to prove, since it depends on demonstrating that a set of parallel lines are obtained in a series of double-reciprocal plots of reaction rate versus substrate (e.g. donor) concentration, with different second substrate (e.g. acceptor) concentrations. Because of experimental error, it is not possible to prove that lines passing through a set of experimental points do not meet at some distant point which would indicate a sequential rather than a Ping Pong kinetic mechanism. The recognition of this problem, for example among the phosphokinases, led to the development of stereochemical methods to determine the stereochemical course of these reactions (Lowe, 1983). As a result of extensive studies it was found that phosphokinases (and other phosphotransferases) which adopt a sequential kinetic mechanism proceed with inversion of configuration at the phosphorus atom, whereas those which follow a Ping Pong mechanism occur with retention at the phosphorus atom. This clearly suggests that all enzyme-catalysed phosphotransfer steps proceed with inversion, which has proved to be a reliable guide to the mechanism of these enzymes (Lowe, 1983).

We have recently developed a general strategy for the synthesis of chiral [¹⁶O,¹⁷O,¹⁸O]sulphate esters of known absolute configuration (Lowe & Salamone, 1984; Lowe & Parratt, 1988) and

a method based on Fourier-transform infrared spectroscopy (FTIR) for their stereochemical analysis (Lowe & Parratt, 1988). In order to investigate the stereochemical course of arylsulphatase II from *Aspergillus oryzae*, phenyl [(*R*)-¹⁶O,¹⁷O,¹⁸O]sulphate was synthesized and the enzyme-catalysed sulphuryl transfer to *p*-cresol investigated.

MATERIAL AND METHODS

N.m.r. spectra were recorded on either a Varian Gemini 200 MHz or a Bruker AM 500 MHz FT spectrometer; the ¹³C n.m.r. spectra were broad-band proton-decoupled. All chemical shifts are reported as positive for resonances downfield from tetramethylsilane as internal reference. The i.r. spectra were recorded on a Perkin-Elmer 1750 Fourier-transform i.r. spectrometer with a Perkin-Elmer 7300 professional computer at a resolution of 1 cm⁻¹. The negative-ion electrospray mass spectra were measured on a VG Bio Q triple quadrupole atmospheric-pressure mass spectrometer equipped with a VG electrospray interface by Dr. R. T. Aplin. Samples (10 µl) were injected into the electrospray source via a loop injector (Rheodyne 5717) as 160 pmol/µl solutions in aqueous acetonitrile (1:1, v/v) at a flow rate of 2 µl/min. (Applied Biosystems model 140A dual syringe pump).

All chemicals and reagents were obtained from Aldrich, except for S¹⁸O₂ (99% ¹⁸O), which was obtained from Johnson Matthey G.m.b.H. Alfa Products, Karlsruhe, Germany, and [¹⁷O]water (45% ¹⁷O, 20.2% ¹⁶O, 34.8% ¹⁸O), which was obtained from EG&G, Mound Applied Technologies, P.O. Box 3000, Miamisburg, OH, U.S.A. The arylsulphatase II from *Aspergillus oryzae* was purified by the method of Burns & Wynn (1975) and had kinetic parameters with *p*-nitrophenyl sulphate and tyramine reported by Burns *et al.* (1977). The enzyme was generously given by Dr C. H. Wynn, Department of Biochemistry and Molecular Biology, University of Manchester, Manchester, U.K.

Epiandrosterone phenyl (*R*)-sulphite and epiandrosterone phenyl [(*R*)-¹⁸O]sulphite

To a solution of dry benzene (10 ml) containing dry pyridine (0.643 ml) was added freshly distilled thionyl chloride (0.192 ml). A solution of phenol (0.249 g) in benzene (10 ml) was added dropwise and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was cooled to 10 °C, and a solution of epiandrosterone (0.77 g) in benzene (30 ml) was added dropwise with stirring. After stirring for a further 1 h, the

Abbreviation used: FTIR, Fourier-transform infrared spectroscopy.

* Present address: Department of Chemistry, P.O. Box 600, Victoria University of Wellington, Wellington, New Zealand.

† To whom correspondence should be addressed.

reaction mixture was filtered. The filtrate was washed with saturated NaHCO_3 solution, followed by saturated NaCl , dried over anhydrous Na_2SO_4 and the solvent evaporated. The ^1H -n.m.r. spectrum of the crude reaction mixture showed that the required sulphite, along with another product, later identified as the bisepiandrosterone sulphite was present in the ratio of 2:1. The product was purified by flash silica-gel chromatography (diethyl ether/hexane, 1:1, v/v) to give *epiandrosterone phenyl (R)-sulphite* (0.647 g, 56% yield). The *epiandrosterone phenyl (R)-sulphite* was recrystallized from ether/hexane to give colourless crystals, m.p. 143–147 °C, (Found: C, 69.8; H, 8.2; S, 7.5%. $\text{C}_{25}\text{H}_{34}\text{O}_4\text{S}$ requires C 69.7, H 8.0, S 7.5%); δ_{H} (500 MHz; $[\text{D}_2]\text{chloroform}$): 0.6–2.5 (steroidal envelope), 4.92 (m, 1H, $\text{C}_3\text{-H}$), 7.1–7.4 (m, 5H, C_6H_5); ν_{max} (carbon tetrachloride): 1740 (C=O), 1488, 1186 (S=O) cm^{-1} .

$[\text{R}^{18}\text{O}]\text{Thionyl chloride}$ (533 mg) (Hepburn & Lowe, 1990) was dissolved in dry benzene (10 ml) containing pyridine (1.07 ml). A solution of phenol (415 mg, 4.41 mmol) in benzene (17 ml) was added dropwise, and the reaction mixture was stirred for 1 h at room temperature. This was cooled to 10 °C, and a solution of *epiandrosterone* (1.28 g) in benzene (51 ml) was added dropwise and the reaction mixture stirred for a further 15 min. The precipitate was filtered and the filtrate was washed successively with saturated NaHCO_3 and saturated NaCl solution, dried over anhydrous Na_2SO_4 and the solvent evaporated. The ^1H -n.m.r. spectrum of the crude reaction mixture showed that the required sulphite was formed along with bisepiandrosterone sulphite in the ratio of 3:1. The resulting residue was subjected to flash silica-gel chromatography with diethyl ether/hexane (1:1, v/v) to give *epiandrosterone phenyl [(R)- ^{18}O]sulphite* (825 mg, 43% yield). This was recrystallized from diethyl ether/hexane to give colourless crystals, m.p. 141–143 °C; δ_{H} (500 MHz; $[\text{D}_2]\text{chloroform}$): 12.18, 13.81, 20.51, 21.75, 28.34, 29.49, 30.81, 31.62, 35.12, 35.58, 35.76, 35.87, 37.03, 45.15, 47.71, 51.47, 54.42, 75.01, 121.44, 125.56, 129.75, 150.21, 220.45 p.p.m.; this confirmed it to be a single diastereoisomer.

Epandrosterone phenyl sulphate and epiandrosterone phenyl [(R)- ^{17}O , ^{18}O]sulphate

To a vigorously stirred suspension of ruthenium dioxide dihydrate ($\text{RuO}_2 \cdot 2\text{H}_2\text{O}$) (200 mg, 1.18 mmol) in carbon tetrachloride (tetrachloromethane) (25 ml) was added a solution of sodium *m*-periodate (600 mg) in distilled water (5 ml). The solution was stirred vigorously for 1 h, and as the reaction proceeded, the black suspension of ruthenium dioxide dihydrate reacted to give a clear yellow–green solution. The aqueous layer was separated and the organic layer dried over anhydrous Na_2SO_4 . The anhydrous solution of ruthenium tetroxide in carbon tetrachloride was cooled to 0 °C and a solution of recrystallized *epiandrosterone phenyl sulphite* (340 mg, 0.79 mmol) in dry carbon tetrachloride (3 ml) was added to it in one portion. The reaction was allowed to proceed for 5 min, then quenched with propan-2-ol (1 ml), followed by filtration through fluted filter paper. To maximize the yield, it was necessary to recover the precipitate from the filter paper and stir the black solid and ethyl acetate for 30 min. This was re-filtered and the pooled filtrate was evaporated under reduced pressure to give *epiandrosterone phenyl sulphate* (253 mg, 72%) as a white solid, m.p. 109–110 °C (decomp.) (Found: C, 67.3; H, 7.4, S, 7.3%. $\text{C}_{25}\text{H}_{34}\text{O}_5\text{S}$ requires C 67.2, H 7.6, S 7.2%); δ_{H} (200 MHz; $[\text{D}_2]\text{chloroform}$): 0.6–2.6 (steroidal envelope), 4.75 (m, 1H, $\text{C}_3\text{-H}$), 7.2–7.5 p.p.m. (m, 5H, C_6H_5); ν_{max} (carbon tetrachloride): 1740 (C=O), 1488, 1410 (SO_2 antisymmetric stretch), 1392, 1209 (SO_2 symmetric stretch), 1175, 1150 cm^{-1} .

To a vigorously stirred suspension of ruthenium dioxide dihydrate (100 mg, 0.59 mmol) in carbon tetrachloride (12.6 ml)

was added a solution of sodium *m*-periodate (300 mg) in water (2.5 ml). This was stirred for 1 h at room temperature until the reaction mixture became a transparent yellow–green colour. The organic layer was separated and dried over anhydrous Na_2SO_4 . To the anhydrous ruthenium tetroxide solution was added $[\text{R}^{17}\text{O}]\text{-water}$ (250 μl) containing anhydrous Na_2HPO_4 (the pH of the solution was adjusted to pH 9). This was stirred vigorously for 16 h. The aqueous layer was then separated off and the organic layer containing ruthenium $[\text{R}^{17}\text{O}_4]\text{tetroxide}$ was dried over anhydrous Na_2SO_4 . The anhydrous solution of ruthenium $[\text{R}^{17}\text{O}_4]\text{tetroxide}$ was cooled to 0 °C, and a solution of recrystallized *epiandrosterone phenyl [(R)- ^{18}O]sulphite* (145 mg, 0.335 mmol) in dry carbon tetrachloride (3 ml) added and was allowed to react for 10 min, before quenching the reaction with propan-2-ol (1 ml). The reaction mixture was filtered and the precipitate was washed several times by stirring with ethyl acetate. The pooled filtrate was evaporated under reduced pressure to give the *epiandrosterone phenyl [(R)- ^{17}O , ^{18}O]sulphate* (115 mg, 76%).

Synthesis of phenyl sulphate and phenyl [(R)- ^{18}O , ^{17}O , ^{18}O]sulphate

Tetrabutylammonium azide (159 mg, 0.559 mmol) (Brandstrom *et al.*, 1974) in dichloromethane (1 ml) was added to a solution of *epiandrosterone phenyl sulphate* (250 mg, 0.559 mmol) in dichloromethane (4 ml). The reaction mixture was stirred at room temperature for 16 h, after which it was evaporated under reduced pressure. The residue was triturated with methanol/water (v/v, 1:1) (4×2 ml). The aqueous methanol layer was pooled and concentrated *in vacuo* (to < 1 ml). This was loaded on to a Sephadex G-10 column (50 ml) and eluted with water. The fractions absorbing at 254 nm were pooled, and the solution was adjusted to pH 7 with 0.1 M-tetrabutylammonium hydroxide. The solution was frozen with liquid nitrogen and freeze-dried to give a powdery white solid in 50% yield.

Tetrabutylammonium azide (78 mg, 0.275 mmol) in dichloromethane (1 ml) was added to a solution of *epiandrosterone phenyl [(R)- ^{17}O , ^{18}O]sulphate* (115 mg, 0.256 mmol) in dichloromethane (2 ml). The reaction worked up as above to give *phenyl [(R)- ^{16}O , ^{17}O , ^{18}O]sulphate tetrabutylammonium salt* as a powdery white solid in 45% yield.

Kinetics of intermolecular sulphuryl transfer

The kinetics of sulphuryl transfer from the tetrabutylammonium salt of phenyl sulphate to propan-2-ol was examined in carbon tetrachloride solution using an excess of propan-2-ol under pseudo-first-order conditions. The transfer reaction was investigated at three concentrations of propan-2-ol.

The tetrabutylammonium salt of phenyl sulphate (11.5 mg) was weighed directly into each of three Reacti-Vials (2 ml). *t*-butylbenzene in carbon tetrachloride (350 μl , 12.3 mM) was added to each Reacti-Vial, followed by carbon tetrachloride (1370, 1510 or 1580 μl). Each Reacti-Vial was capped and equilibrated in a thermostatically controlled oil bath at 65 °C. To each Reacti-Vial was added a solution of propan-2-ol in carbon tetrachloride (5.67 M, 280 μl to the first, 140 μl to the second and 70 μl to the third), so that the final volume of each reaction mixture was the same but 0.8 M, 0.4 M and 0.2 M in propan-2-ol respectively. Aliquots (5 μl) were withdrawn at 5 min intervals and injected directly into a Pye 104 gas chromatograph (operating temperature 150 °C) fitted with a 1.52 m (5 ft) packed glass column of OV-17 (10% on Chromosorb A) and a 3390A Hewlett Packard integrator. A 30 min time range was selected for each kinetic run such that at least 15% reaction occurred in this period. An infinity reading was taken when at least 10 half-lives had elapsed. Before each kinetic run the top 5 cm of the column packing was replaced with Chromosorb column support

Scheme 1. Synthesis of phenyl [(*R*)- ^{16}O , ^{17}O , ^{18}O]sulphate

The ^{18}O site is 99% enriched, but the ' ^{17}O ' site contains 32.3% ^{16}O , 37.0% ^{17}O and 30.7% ^{18}O as determined by negative-ion electrospray mass spectrometry (Fig. 1). Φ , ^{17}O ; $\bullet$, ^{18}O ; PhOH, phenol; $\text{S}(\bullet\text{Cl}_2)$, [^{18}O]thionyl chloride; $\text{Bu}_4\text{N}^+\text{N}_3^-$, tetrabutylammonium azide; RuO_4 , ruthenium [^{17}O]tetroxide.

Fig. 1. Smoothed negative-ion electrospray mass spectra (a) of phenyl sulphate in water/acetonitrile (1:1, v/v) (160 pmol/ μl) (average of 20 scans) and (b) of phenyl [(*R*)- ^{16}O , ^{17}O , ^{18}O]sulphate in water/acetonitrile (1:1, v/v) (160 pmol/ μl) (average of 29 scans) as their tetrabutylammonium salts

The relative intensities of the peaks are shown in the insets. 'FS(%)' means full-scale percentage.

check the performance of the column. The data are presented as semi-logarithmic pseudo-first-order plots in Fig. 2 (below).

Intermolecular sulphuryl-transfer reaction

Phenyl [(*R*)- ^{16}O , ^{17}O , ^{18}O]sulphate tetrabutylammonium salt (22.4 mg, 0.054 mmol) was added to a solution of (1*R*)-3-benzoyloxy-1-methylpropan-1-ol (13.4 mg, 0.069 mmol) [prepared from (*R*)-butane-1,3-diol, $[\alpha]^{21}_{-31^\circ}$ ($c = 1$, ethanol), by the method of Kim *et al.* (1985)] in dry carbon tetrachloride (1.5 ml) in a Reacti-Vial. The vial was immersed in an oil bath at 100 °C for 16 h. The reaction mixture was then evaporated and the residue dissolved in water (1 ml), followed by the addition of 3 equiv. of 0.1 M-NaOH. This was allowed to stir for 16 h at room temperature, the reaction mixture diluted with water and the solution adjusted to pH 8 with dilute HCl. Phenol was extracted with diethyl ether, and the aqueous layer was separated and evaporated to a small volume under reduced pressure. This was loaded on to a pyridinium Dowex 50X8-100 column, and eluted with distilled water. The solution of the product was evaporated under reduced pressure and then co-evaporated with dry acetonitrile (4 $\times$ 2 ml). The dry residual (1*R*)-3-benzoyloxy-1-methylpropyl [^{16}O , ^{17}O , ^{18}O]sulphate was dissolved in dry acetonitrile (15 ml) and cooled to -15 °C (solid CO_2 /carbon tetrachloride) and sulphuryl chloride (37 μl , 10 equiv.) added. The reaction mixture was removed from the cooling bath and allowed to react for a further 30 min; the solution was carefully evaporated under reduced pressure (water pump). The residue and an approximately equal weight of unlabelled (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathiane were dissolved in a small volume of diethyl ether and purified by chromatography on a short column of silica gel (1 g) in a pasteur pipette eluting with 1:1 diethyl ether/pentane. After the u.v.-absorbing fraction (probably derived by reaction of sulphuryl chloride with benzoic acid) had been eluted, the eluting solvent was changed to 100% diethyl ether. The required cyclic sulphate can be detected by t.l.c. (eluting solvent: ether, blue spot with phosphomolybdate spray at $R_F = 0.5$). The required fractions were pooled and evaporated under reduced pressure (11 mmHg). It was necessary to co-evaporate the sample several times with carbon tetrachloride to

(mesh size 80-100) and the column allowed to re-equilibrate. Standard solutions of phenol and *t*-butylbenzene in carbon tetrachloride were injected before and after each kinetic run to

Scheme 2. Stereochemical course of chemical sulphuryl transfer

The mixture of isotopomers of (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathiane were analysed by FTIR spectroscopy in Fig. 3. Because the '¹⁷O' site contains ¹⁶O, ¹⁷O and ¹⁸O, nine isotopomers will be generated on cyclization of (1*R*)-3-hydroxy-1-methylpropyl [¹⁶O, ¹⁷O, ¹⁸O]sulphate which occurs with retention at the sulphur atom (Lowe & Parratt, 1988). The first set of isotopomers are those which are actually formed. The second set of nine isotopomers are those which would be formed if the (1*R*)-3-hydroxy-1-methylpropyl [¹⁶O, ¹⁷O, ¹⁸O]sulphate had the *R_s*, rather than the *S_s*, configuration. The two numbers below each isotopomer are the symmetric and antisymmetric >SO₂ stretching frequencies in cm⁻¹. ⊕ = ¹⁷O; ● = ¹⁸O; SO₂Cl₂, sulphuryl chloride; CCl₄, carbon tetrachloride; PhOH, phenol.

Fig. 2. Pseudo-first-order kinetic plots of the sulphuryl-transfer reaction between the tetrabutylammonium salt of phenyl sulphate and propan-2-ol in carbon tetrachloride solution at 65 °C

A_t is the concentration of phenol released at time t and A_∞ is the concentration of phenol released after at least ten half-lives. The second-order rate constant is $(0.39 \pm 0.05) \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$. □, 0.8 M-propan-2-ol; ◆, 0.4 M-propan-2-ol; ■, 0.2 M-propan-2-ol.

remove the diethyl ether. The FTIR spectrum of the isotopomeric mixture of (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathianes in carbon tetrachloride was obtained and the asymmetric and symmetric stretching frequencies assigned (Fig. 3 below) by comparison with the values obtained for each isotopomer (Lowe & Parratt, 1988).

Enzyme-catalysed sulphuryl transfer

An enzyme solution of arylsulphatase II from *Aspergillus oryzae* (0.75 ml, 8.1 units) was added to a solution containing Tris/HCl buffer (100 mM, pH 7.5, 0.5 ml), deionized water (1.75 ml), *p*-cresol (10.7 mg) and tetrabutylammonium phenyl [(*R*)-[¹⁶O, ¹⁷O, ¹⁸O]sulphate (21 mg). The mixture was kept at 37 °C in a constant-temperature bath for 16 h, after which the reaction was terminated by freezing the solution with liquid N₂ followed by freeze-drying. The phenyl [¹⁶O, ¹⁷O, ¹⁸O]sulphate/*p*-cresyl [¹⁶O, ¹⁷O, ¹⁸O]sulphate ratio was approx. 1.1:1, as de-

Fig. 3. FTIR spectrum showing the antisymmetric ($1414\text{--}1378\text{ cm}^{-1}$) and symmetric ($1201\text{--}1157\text{ cm}^{-1}$) $>\text{SO}_2$ stretching frequencies of the mixture of isotopomers derived by cyclizing (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate (obtained from phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate) with sulphuryl chloride

The scale is in cm^{-1} and the resolution was enhanced by Fourier deconvolution; for the antisymmetric stretching region a linewidth of 20 cm^{-1} and an enhancement factor of 2.0 was used, whereas for the symmetric stretching region a linewidth of 10 cm^{-1} and an enhancement factor of 1.5 was used. Isotopes in a bridging position do not influence the $>\text{SO}_2$ stretching frequencies (Lowe & Parratt, 1988). The (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate thus has the S_{r} -configuration. $\circ$, ^{17}O ; $\bullet$, ^{18}O ; $\ominus$, ^{16}O and ^{18}O ; $\odot$, $^{16}\text{O}, ^{17}\text{O}$ and ^{18}O .

terminated from reversed-phase h.p.l.c. [60% (v/v) 50 mM-triethylammonium carbonate (pH 7.5)/40% acetonitrile; du Pont 21.2 mm $\times$ 25 cm column; elution rate 4 ml/min]. *p*-Cresyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate was isolated by preparative reversed-phase h.p.l.c. under the same conditions. The buffer was removed by co-evaporation with methanol and 1 equiv. of 0.154 M-tetrabutylammonium hydroxide (154 μl) added and the solution freeze-dried to give tetrabutylammonium *p*-cresyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate.

p-Cresyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate tetrabutylammonium salt (10.3 mg, 0.024 mmol) was added to a solution of (1*R*)-3-benzoyloxy-1-methylpropan-1-ol (7 mg, 0.036 mmol) in dry carbon tetrachloride (0.9 ml) in a Reacti-Vial and the intermolecular sulphuryl transfer brought about as described for the tetrabutylammonium salt of phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate. Likewise the (1*R*)-3-benzoyloxy-1-methylpropyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate was cyclized as above and carrier (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathiane added. The FTIR spectrum of the isotopomeric mixture of (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathianes in carbon tetrachloride was obtained and the antisymmetric and symmetric stretching frequencies (Fig. 4 below) compared with those in the FTIR spectrum obtained from the product of the intermolecular chemical transformation (Fig. 3).

RESULTS AND DISCUSSION

Synthesis of phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate

The route used for the synthesis of phenyl [(*R*)-

$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate is outlined in Scheme 1. Epiandrosterone was selected as the chiral alcohol for this purpose after the synthetic route had been explored with unlabelled materials and the single diastereoisomer of epiandrosterone phenyl sulphite isolated shown to have the R_{r} -configuration by an X-ray-crystal-structure analysis (Chai *et al.*, 1991). Although the epiandrosterone phenyl sulphite is much more chemically labile than aliphatic sulphite diesters, it is configurationally stable. Moreover, the sulphite could be oxidized to the corresponding sulphate with ruthenium tetroxide without oxidation of the epiandrosterone moiety. The method of oxidation using a catalytic amount of ruthenium dioxide from which ruthenium tetroxide is generated and regenerated with an aqueous solution of sodium *m*-periodate in a two-phase system (Lowe & Salamone, 1984) was not satisfactory for this aryl sulphate diester because of its lability in an aqueous environment, so instead a stoichiometric amount of ruthenium tetroxide in anhydrous solvent was used. The best way that we found to prepare larger amounts of anhydrous Ru^{17}O_4 was to exchange the oxygen atoms in RuO_4 with weakly alkaline [^{17}O] water, and then extract and dry the product in an organic solvent. The unconfirmed isotopic composition of the [^{17}O] water used was 20.2% ^{16}O , 45% ^{17}O and 34.8% ^{18}O (data provided by the supplier). Inevitably the exchange method led to dilution of the ^{17}O and ^{18}O content in the ' Ru^{17}O_4 ' compared with the [^{17}O] water used. Conditions were used for the exchange reaction which had been shown in a preliminary experiment with [^{18}O] water (99% ^{18}O) to allow virtually complete equilibration to be attained. Assuming that

Scheme 3. Stereochemical course of the sulphuryl-transfer reaction catalysed by arylsulphatase II from *Aspergillus oryzae*

The configuration of the *p*-cresyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate, obtained from the enzyme-catalysed reaction, is analysed by chemical transfer of the chiral sulphuryl group to (1*R*)-3-benzoyloxy-1-methylpropanol, followed by debenzoylation, cyclization and FTIR analysis. The FTIR spectrum of the mixture of isotopomers of (4*R*)-methyl-2,2-dioxo-1,3,2-dioxathiane is shown in Fig. 4, which indicates that they have the same composition as those formed by direct sulphuryl transfer from phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate to (1*R*)-3-benzoyloxy-1-methylpropanol (Scheme 2 and Fig. 3) and hence the enzyme catalysed reaction proceeds with retention of configuration at the sulphur atom. The two values below each isotopomer are the symmetric and antisymmetric $>\text{SO}_2$ stretching frequencies in cm^{-1} . ⊕, ^{17}O ; ●, ^{18}O ; SO_2Cl_2 , sulphuryl chloride; CCl_4 , carbon tetrachloride; PhOH, phenol.

equilibration had been achieved, the ' Ru^{17}O_4 ' would be expected to have an isotopic composition of 32.4% ^{16}O , 38.1% ^{17}O , 29.5% ^{18}O from the known molar ratio of RuO_4 to [^{17}O] water used in the preparation. [^{18}O]Thionyl chloride was prepared from sulphur [$^{18}\text{O}_2$]dioxide (Hepburn & Lowe, 1990) and used to prepare epiandrosterone phenyl [(*R*)- ^{18}O]sulphite. Having established that the epiandrosterone phenyl sulphite has the *R_s*-configuration (Chai *et al.*, 1991) and knowing that the oxidation of sulphites to sulphates with ruthenium tetroxide occurs with retention of configuration at the sulphur atom (Lowe & Salamone, 1983), the [$^{17}\text{O}, ^{18}\text{O}$]sulphate diester obtained by treating epiandrosterone phenyl [(*R*)- ^{18}O]sulphite with ' Ru^{17}O_4 ' must have the *R_s*-configuration. Epiandrosterone phenyl [(*R*)- $^{17}\text{O}, ^{18}\text{O}$]sulphate diester on treatment with tetrabutylammonium azide (Brandstrom *et al.*, 1974) gave phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate tetrabutylammonium salt. The absolute configuration is unambiguous, since none of the sulphur to oxygen bonds are perturbed in the final displacement reaction by azide ion. The negative-ion electrospray mass spectrum of phenyl sulphate as its tetrabutylammonium salt is shown in Fig. 1(a). The peak at m/z 172.923 is that of the molecular ion. The relative intensities of the peaks at m/z 173.985 and 174.923 (inset to Fig. 1a) are close to the calculated values expected from the contributions of ^{13}C and ^{34}S . The negative-ion electrospray mass spectrum of phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate as its tetrabutylammonium ion is shown in Fig. 1(b). The peaks at m/z 174.985, 175.923 and 176.986 correspond to the molecular ions of phenyl [$^{16}\text{O}_2, ^{18}\text{O}$]sulphate, phenyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate, and phenyl

[$^{16}\text{O}, ^{18}\text{O}_2$]sulphate respectively. The relative intensities of the peaks are indicated in the insert to Fig. 1(b). In order to derive the correct ratio of the three isotopomers, allowance must be made for the ^{13}C and ^{34}S contribution from each isotopomer using the experimental determined ratios for the unlabelled phenyl sulphate (insert in Fig. 1a). The corrected relative intensities gave the following isotopomeric composition: m/z 174.985 (32.3%) phenyl [$^{16}\text{O}_2, ^{18}\text{O}$]sulphate, 175.923 (37.0%) phenyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate, 176.986 (30.7%) phenyl [$^{16}\text{O}, ^{18}\text{O}_2$]sulphate. These values are in good agreement with the expected isotopic composition of the ' Ru^{17}O_4 ' (see above).

Stereochemical course of chemical and aryl sulphatase II-catalysed sulphuryl transfer

Arylsulphatase II from *Aspergillus oryzae* in the presence of a phenolic acceptor is a much more effective sulphotransferase than a sulphatase (Burns *et al.*, 1977). In preliminary experiments it was shown that the enzyme would catalyse sulphuryl transfer from phenyl sulphate to *p*-cresol and that the *p*-cresyl sulphate formed could be separated from phenyl sulphate by reverse-phase h.p.l.c. The enzyme-catalysed reaction was now performed using phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate and *p*-cresol as the acceptor. In the initial experiment the reaction was only allowed to proceed until about 10% of the phenyl [(*R*)- $^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate had been used, the reaction being monitored by h.p.l.c. The reason for this was that if the reaction proceeded with inversion of configuration at the sulphur atom, since the product *p*-cresyl [$^{16}\text{O}, ^{17}\text{O}, ^{18}\text{O}$]sulphate would also be a donor, this would lead to

Fig. 4. FTIR spectrum showing the antisymmetric (1414–1378 cm^{-1}) and symmetric (1201–1157 cm^{-1}) $>\text{SO}_2$ stretching frequencies of the mixture of isotopomers derived by cyclizing (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate (obtained from *p*-cresyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate; Scheme 3) with sulphuryl chloride

The scale is in cm^{-1} and the resolution was enhanced by Fourier deconvolution; for the antisymmetric stretching region a linewidth of 20 cm^{-1} and an enhancement factor of 2.0 was used, whereas for the symmetric stretching region a linewidth of 10 cm^{-1} and an enhancement factor of 1.5 was used. Isotopes in a bridging position do not influence the $>\text{SO}_2$ stretching frequencies (Lowe & Parratt, 1988). This spectrum is essentially identical with that in Fig. 2 and so the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate has the S_{e} -configuration. This indicates that the arylsulphatase II catalyses sulphuryl transfer with retention of configuration at sulphur.

racemization. This preliminary experiment, however, indicated that the reaction proceeded with retention of configuration at the sulphur atom. In a subsequent reaction the conversion was allowed to proceed to 47% *p*-cresyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate.

In order to determine the configuration of the *p*-cresyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate it was necessary to transfer the chirally labelled sulphuryl group to (1*R*)-3-benzoyloxy-1-methylpropan-1-ol. A kinetic study of the transfer of the sulphuryl group from the tetrabutylammonium salt of phenyl sulphate to propan-2-ol in carbon tetrachloride under pseudo-first-order conditions showed that the reaction was first-order with respect to both phenyl sulphate and propan-2-ol (see Fig. 2). Next, the tetrabutylammonium salt of phenyl [(*R*)- $^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate was incubated with (1*R*)-3-benzoyloxy-1-methylpropan-1-ol at 100 °C (in a Reacti-Vial) for 16 h, by which time the transfer was essentially complete. The (1*R*)-3-benzoyloxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate was debenzoylated with aq. NaOH solution and the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate as its pyridinium salt cyclized with sulphuryl chloride (Scheme 2). The mixture of isotopomers so generated was investigated by FTIR; the spectrum of the symmetric and antisymmetric stretching frequencies are shown in Fig. 4. The distribution of isotopomers is that expected from the S_{e} -enantiomer of (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate (upper set of isotopomers in Scheme 2), indicating that the sulphuryl-transfer reaction has proceeded with inversion of configuration at the sulphur atom. The key peaks for the assignment of configuration are those containing ^{17}O , but some of these are not resolved. In the antisymmetric mode the cyclic [$^{16}\text{O}_{\text{a}},^{17}\text{O}_{\text{e}}$]sulphate ($\text{a} = \text{axial}$; $\text{e} = \text{equatorial}$; isotope in the bridging position does not effect the frequency) at 1401 cm^{-1} has the same frequency as the cyclic

[$^{18}\text{O}_{\text{a}},^{16}\text{O}_{\text{e}}$]sulphate (Lowe & Parratt, 1988), but if the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate has the R_{f} -configuration, a peak would be observed at 1407 cm^{-1} , which is clearly absent from the spectrum. The antisymmetric stretching mode for the cyclic [$^{17}\text{O}_{\text{a}},^{18}\text{O}_{\text{e}}$]sulphate is observable at 1384 cm^{-1} , whereas no peak is discernable for the cyclic [$^{17}\text{O}_{\text{e}},^{18}\text{O}_{\text{a}}$]sulphate at 1389 cm^{-1} , providing positive evidence for the S_{e} configuration of the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate. It has been recognized, however, that the symmetric stretching mode of the isotopomers provides the clearest evidence of the configuration, since the linewidths of the peaks are intrinsically narrower and the spectrum better resolved (Lowe & Parratt, 1988). Confirmatory evidence that the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate has the S_{e} configuration is provided by the peak at 1192 cm^{-1} , which is assigned to the cyclic [$^{16}\text{O}_{\text{a}},^{17}\text{O}_{\text{e}}$]sulphate. A peak at 1170 cm^{-1} should be present, arising from the cyclic [$^{17}\text{O}_{\text{a}},^{18}\text{O}_{\text{e}}$]sulphate, but this is never resolved from the cyclic [$^{18}\text{O}_{\text{a}},^{16}\text{O}_{\text{e}}$]sulphate at 1172 cm^{-1} ; it is, nevertheless, discernable by the broadening of the peak due to both these isotopomers being present. The absence of peaks for the cyclic [$^{17}\text{O}_{\text{e}},^{18}\text{O}_{\text{a}}$] and [$^{16}\text{O}_{\text{e}},^{17}\text{O}_{\text{a}}$]sulphates at 1163 and 1186 cm^{-1} respectively confirms the assignment. Thus the chemical sulphuryl-transfer reaction has proceeded with inversion of configuration at the sulphur atom.

p-Cresyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate tetrabutylammonium salt, isolated from the enzymic reaction was then similarly incubated with (1*R*)-3-benzoyloxy-1-methylpropan-1-ol in carbon tetrachloride at 100 °C for 16 h. The (1*R*)-3-benzoyloxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate was debenzoylated with aq. NaOH, and the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate, as its pyridinium salt, was cyclized with sulphuryl chloride (Scheme 3). The mixture of isotopomers so generated was investigated by FTIR; the spectrum of the symmetric and antisymmetric stretching frequencies are shown in Fig. 4. The distribution of isotopomers is consistent with the (1*R*)-3-hydroxy-1-methylpropyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate having the S_{e} -configuration and is very similar to that shown in Fig. 3. Thus the *p*-cresyl [$^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate must have the R_{f} configuration like the phenyl [(*R*)- $^{16}\text{O},^{17}\text{O},^{18}\text{O}$]sulphate from which it was derived, indicating that the enzyme-catalysed sulphuryl-transfer reaction has proceeded with retention of configuration at the sulphur atom. This result implies that the enzyme-catalysed sulphuryl transfer takes place by way of a sulpho-enzyme intermediate, the sulphation of the enzyme occurring with inversion and the transfer from the sulpho-enzyme intermediate to the acceptor substrate also occurring with inversion, leading to overall retention of configuration at the sulphur atom. Gratifyingly, this result is in accord with the conclusions drawn from a kinetic investigation (Burns *et al.* 1977), which indicated that the enzyme follows a Ping Pong-type mechanism, implying the involvement of a sulpho-enzyme intermediate on the enzyme reaction pathway.

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REFERENCES

- Burns, G. R. J. & Wynn, C. H. (1975) *Biochem. J.* **149**, 697–705
- Burns, G. R. J., Galanopoulou, E. & Wynn, C. H. (1977) *Biochem. J.* **167**, 223–227
- Brandstrom, A., Lamm, B. & Palmertz, I. (1974) *Acta Chem. Scand. Sect. B.* **28**, 699–701
- Chai, C. L. L., Humphreys, V., Prout, K. & Lowe, G. (1991) *J. Chem. Soc. Chem. Commun.* 1597–1598

- Hepburn, T. W. & Lowe, G. (1990) *J. Labelled Compd. Radiopharm.* **28**, 617–620
Kim, S., Chang, H. & Kim, W. J. (1985) *J. Org. Chem.* **50**, 1751–1752
Lowe, G. (1983) *Acc. Chem. Res.* **16**, 244–251
Lowe, G. & Parratt, M. J. (1988) *Bioorg. Chem.* **16**, 283–297

- Lowe, G. & Salamone, S. J. (1984) *J. Chem. Soc. Chem. Commun.* 466–467
Roy, A. B. & Trudinger, P. A. (1970) *The Biochemistry of Inorganic Compounds of Sulphur*, pp. 106–113, Cambridge University Press, Cambridge

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