Preparation and Characterization of Substrates Suitable for the Study of Stereospecific Secondary Alkylsulphohydrolases of Detergent-Degrading Micro-organisms

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During the course of the purification of novel stereospecific secondary alkylsulphohydrolases present in certain detergent-degrading micro-organisms, it became apparent that substrates prepared by sulphating secondary alcohols with H_2SO_4 are heterogeneous. Apart from the racemization that occurs if resolved alcohols are sulphated, evidence is provided to show that other isomers are produced in which the position of the ester sulphate group on the alkyl chain has been altered. These changes can be avoided if pyridine/ SO_3 reagent (prepared with SO_3) is substituted as sulphating agent. Experiments in which secondary alkyl sulphates prepared by both methods were tested as potential substrates for the two secondary alkylsulphohydrolase enzymes of *Comamonas terrigena* have provided initial information about the specificity of the enzymes.

Studies on the biodegradation of linear alkyl sulphate detergents by micro-organisms led to the discovery of alkylsulphohydrolase enzymes (Payne & Feisal, 1963; Hsu, 1963). Subsequently, it was shown that *Pseudomonas* C12B, an organism isolated from soil near a sewage outfall, could produce five distinct alkylsulphohydrolases when grown under specific culture conditions (Dodgson *et al.*, 1974). Two of these enzymes were active towards primary alkyl sulphates such as dodecan-1-yl sulphate (sodium dodecyl sulphate, SDS), whereas the remaining three were active towards preparations of 'potassium decan-5-yl sulphate' which had been obtained by treatment of racemic decan-5-ol with H_2SO_4 or chlorosulphonic acid at low temperatures (Dodgson *et al.*, 1974). These preparations were homogeneous according to the criteria available at the time.

A second micro-organism, *Comamonas terrigena*, was later shown to produce secondary alkylsulphohydrolases (Fitzgerald *et al.*, 1975). Two such enzymes were present in cell extracts of broth-grown cells and one of them has since been purified to homogeneity. This enzyme, designated CS2, exhibits relatively high specificity and appears to hydrolyse only the D-2-sulphate esters of secondary alcohols containing at least six carbon atoms (Matcham *et al.*, 1977a,b). The other enzyme, designated CS1, has not yet been purified to homogeneity but appears to hydrolyse only the L-2-isomers.

During the purification of enzyme CS2 it became apparent that secondary alkyl sulphate esters pre-

pared by sulphation with H_2SO_4 or chlorosulphonic acid exhibited considerable heterogeneity. Not only does the sulphation procedure lead to racemization of optically active isomers but also to the production of other isomers in which the position of the ester sulphate group on the alkyl chain has been altered. Clearly such substrate preparations, although useful for initial studies with crude cell extracts, are unsuitable for the study of purified individual enzymes.

To provide homogeneous optically active substrates for secondary alkylsulphohydrolases, an alternative procedure for the sulphation of secondary alcohols has now been developed that is based on the observations of Burwell (1949). Pyridine/ SO_3 (made from SO_3 and not from chlorosulphonic acid) is used as sulphating agent and allows resolved octan-2-ols to be converted into the corresponding sulphate esters without racemization or production of other positional isomers.

Materials and Methods

Unless otherwise stated, all chemicals were the purest available from BDH Chemicals, Poole, Dorset, U.K. or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Preparation of optically active octan-2-ols

Racemic octan-2-ol was resolved into the optically active isomers by the method of Kenyon (1922) as described by Vogel (1956). The method, which involves the formation of 2-octyl hydrogen phthalate and resolution of this by fractional crystallization of

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the brucine salt, was modified at the stage after the addition of brucine to the acetone solution of the 2-octyl hydrogen phthalate. Methanol was added to a final volume of about 800 ml to assist the dissolution of the brucine and the whole was then concentrated *in vacuo* at 35°C to a volume of about 300 ml. Acetone was added slowly to the viscous solution, during heating under reflux, until insoluble material began to appear. The dropwise addition of methanol redissolved this solid in the hot solution. Crystallization of the D-octan-2-ol component began within 30 min at room temperature and was complete after being left overnight. Subsequent procedures were as described by Vogel (1956).

The resolved octan-2-ols were examined by i.r. spectrometry, g.l.c. and polarimetry. I.r. spectra of the resolved alcohols and racemic mixture were indistinguishable. The resolved and unresolved alcohols were identical on g.l.c., except that a minor contaminant had been eliminated during the resolution procedure. It was concluded that the D-octan-2-ol ($[\alpha]_{546.1}^{20}$, +11.89°; theory, +11.8°) was pure and that the L-octan-2-ol ($[\alpha]_{546.1}^{20}$, -10.8°; theoretical, -11.8°) was a minimum of 96% in the L(-) form with no contamination detectable by the other method used. The arguments for relating the octan-2-ols to D- or L-glyceraldehyde have been summarized by Bartholomew *et al.* (1977).

Preparation of secondary alkyl sulphates

All secondary alkyl sulphates were prepared as potassium salts by sulphation of the parent alcohol (Aldrich Chemical Co., Milwaukee, WI, U.S.A., or K & K Laboratories, Plainview, NY, U.S.A.) with either H₂SO₄ (Dodgson *et al.*, 1974) or pyridine/SO₃. Quotation marks have been used to draw attention to those esters prepared with H₂SO₄ and which are heterogeneous in consequence.

Pyridine/SO₃ reagent

Pyridine/SO₃ can be prepared by various methods, including direct reaction of pyridine with either SO₃ or chlorosulphonic acid. The reaction with chlorosulphonic acid gives pyridine/SO₃ and pyridinium chloride in equimolar amounts, and, in our hands, complete removal of contaminating Cl⁻ ions has never been achieved and the secondary alkyl sulphates prepared with this reagent have been heterogeneous. Accordingly, pyridine/SO₃ was always prepared as follows. Fuming H₂SO₄ (50–100 ml containing 25% SO₃) was warmed in a round-bottom flask and the SO₃ was collected over an ice/NaCl mixture. The all-glass apparatus was so constructed as to exclude moist air and the sublimate of SO₃ was stored under seal at -20°C until required.

Pyridine (120 ml, distilled from KOH and dried over CaH₂) and carbon tetrachloride (450 ml, distilled from CaCl₂) were vigorously stirred in an ice/NaCl mixture. The SO₃ (33 g) was slowly passed over this mixture by gently warming the sublimate, the whole operation again being conducted in an all-glass apparatus which excluded moist air. After all the solid SO₃ had disappeared, the stirring was continued for a further 10 min before collecting (filtration at the pump) the off-white solid which had formed in the mixing vessel. The material was not washed but was dried *in vacuo* over P₂O₅. The yield was 43 g (65% of theoretical value).

General sulphation procedure

Pyridine/SO₃ (0.05 mol), secondary alcohol (0.05 mol) and pyridine (0.5 ml, distilled from KOH) were vigorously stirred in an air-tight 50 ml round-bottom flask for 2 h at 65°C and then left to cool. The resulting cake was dissolved by adding hot saturated freshly filtered Ba(OH)₂ solution until the pH was slightly alkaline. Precipitated BaSO₄ was removed by centrifuging (2000 g for 5 min) the hot solution. The supernatant was transferred to a separating funnel and the pellet washed with hot water by resuspension and centrifugation. Unsulphated alcohol was then removed from the combined supernatant and washings by extraction with 3 × 200 ml of diethyl ether. The aqueous solution was concentrated *in vacuo* at 38°C and rediluted with water, successively, until there was no detectable smell of ether or pyridine. Finally, the concentrated solution (about 100 ml) was warmed, filtered through charcoal to remove any colour or BaCO₃ and then immediately passed through a column (300 ml) of Dowex 50W (H⁺ form). The acid eluate was collected in a round-bottom flask, the pH adjusted to neutral with KOH solution and the whole freeze-dried to a white fluffy powder.

The potassium salts of (±)-hexan-2-yl, (±)-heptan-2-yl, symmetrical heptan-4-yl, DL-, D- and L-octan-2-yl, (±)-octan-3-yl, (±)-octan-4-yl, symmetrical nonan-5-yl and (±)-decan-2-yl sulphates were prepared by this method. Yields varied (up to 70% of theoretical values) with the age of the sulphating agent and the complexity of the alcohol. For example, freshly prepared pyridine/SO₃ gave a 68% yield of DL-octan-2-yl sulphate whereas the same batch, 2 weeks later, gave a yield of only 42%. Yields also tended to decrease with increase in chain length and with the movement of the hydroxyl group towards the centre of the carbon chain. Attempts to prepare secondary alkyl sulphates of chain length greater than C₁₀ by this method have so far been largely unsuccessful. Indeed, yields of (±)decan-2-yl sulphate were generally of the order of 10% only.

The specific optical rotations of aqueous solutions of the potassium salts of D- and L-octan-2-yl sulphates

were $[\alpha]_{5461}^D$, $+3.25^\circ$ and -3.25° respectively. Bartholomew *et al.* (1977) have established the relationship between these two esters and D- and L-glyceraldehyde respectively.

Analysis of alkyl sulphates

The K^+ content of the esters was determined by flame photometry and ester sulphate was estimated gravimetrically as $BaSO_4$ after hydrolysis of the ester by refluxing in 2M-HCl for 60 min. I.r. spectra were obtained by using a Perkin-Elmer Infracord spectrophotometer (cf. Lloyd *et al.*, 1961). The rate of acid hydrolysis was determined by refluxing 50 mg of ester in 25 ml of 0.25M-HCl and withdrawing 0.2 ml samples for neutralization and determination of liberated sulphate by the $BaCl_2$ /gelatin method (method A) of Dodgson (1961).

Mass-spectral analysis was performed with a Varian Mat CH5 double-focusing mass spectrometer with combined electron impact/field desorption source. Samples were dissolved in methanol and the wire current was increased until so-called 'soft ionization' occurred. Providing that there was not an excess of potassium in the sample, peaks were detected at molecular weights equivalent to $M+K$, $2M+K$, $3M+K$ etc., where M is the molecular weight of the potassium alkyl sulphate and K is the molecular weight of potassium. Potassium salts of the symmetrical esters nonan-5-yl sulphate and heptan-4-yl sulphate were analysed by n.m.r. (nuclear magnetic resonance) in a Perkin-Elmer R32 spectrophotometer at 90 MHz by using 2H_2O as solvent.

For g.l.c. analysis of alcohols liberated by acid hydrolysis, 50 mg of alkyl sulphate was dissolved in 25 ml of 2M-HCl, and the solution was boiled under reflux for 2 h. After cooling and neutralizing with 10 ml of 5M-NaOH the liberated alcohol was extracted with 3×15 ml of diethyl ether. The pooled ether extract was evaporated to 1 ml at room temperature ($20^\circ C$) under a stream of N_2 . Standards were also diluted into diethyl ether. Samples were then analysed in a Perkin-Elmer gas chromatograph, either F11 or F33, with flame ionization. Columns ($183 \text{ cm} \times 0.64 \text{ cm}$) were packed with either 15% (w/w) DEGS or 15% (w/w) EGSS-X on Supelcoport (80-100 mesh; Supelco, Bellefont, PA, U.S.A.). An injection temperature of $225^\circ C$ was used throughout. The oven temperature (115 – $185^\circ C$), attenuation and chart speed were varied according to the alcohol being analysed and are specified where appropriate.

Optical rotations were measured in a 1 cm-path-length cell at 5461 nm using the NPL Automatic Polarimeter type 243 (Thorn Automation, Nottingham, Notts., U.K.). Ethanol was used as a solvent for alcohols, and sulphate esters were dissolved in water. Optical rotations of less than 0.001 were not considered significant.

Enzymic hydrolysis of alkyl sulphates

The pure CS2 secondary alkylsulphohydrolase (active towards D-octan-2-yl sulphate) and partially pure CS1 secondary alkylsulphohydrolase (active towards L-octan-2-yl sulphate) were isolated from crude cell extracts of *C. terrigena* by the procedures described in the following paper (Matcham *et al.*, 1977b). The crude cell extract, containing equal amounts of the two enzymes, was prepared by the method of Fitzgerald *et al.* (1975).

For each substrate a 2.4 ml solution containing 6 μ mol of substrate, 0.1 M-Tris/maleate, pH 7.5, and suitably diluted enzyme was incubated at $31^\circ C$, and 0.2 ml samples were removed at various times. When crude enzyme was used each sample was rapidly mixed with 50 μ l of 15% (w/v) trichloroacetic acid and a 0.2 ml portion was removed from the supernatant after brief centrifugation. The liberated inorganic SO_4^{2-} ions were then measured by the $BaCl_2$ /gelatin method (method B) of Dodgson (1961). With the purer enzyme preparations, the initial 0.2 ml samples were added directly to the 1.3 ml of 4% (w/v) trichloroacetic acid specified in method B. In all cases the reaction was followed until no further sulphate was released.

Isolation of 'enzyme-resistant substrate'

'Potassium octan-2-yl sulphate' (0.615 g prepared from racemic octan-2-ol with H_2SO_4) was dissolved in 200 ml of water and the pH was adjusted to 7.5 with a trace of KOH. The solution was kept at $31^\circ C$ and 40 units (see Matcham *et al.*, 1977b) of pure CS2 alkylsulphohydrolase was added. The pH of the solution was monitored as the reaction proceeded and maintained at 7.5 by the careful addition of dilute KOH to counteract the effect of protons liberated during the hydrolysis. When no further decrease in pH occurred (after 4 h) the reaction was considered to be complete and determination of liberated SO_4^{2-} ions showed that 40% of the 'octan-2-yl sulphate' had been hydrolysed. The incubation mixture was then extracted with 3×100 ml of diethyl ether to remove alcohol liberated during the incubation. The aqueous phase was concentrated to 50 ml *in vacuo* at $30^\circ C$ and then washed through a 50 ml column of Dowex 50W (H^+ form) to remove K^+ . The acid eluate was titrated to pH 9 with $Ba(OH)_2$ solution and precipitated $BaSO_4$ removed by centrifuging at 2000 g for 5 min. The clear supernatant was passed through a second column (50 ml) of Dowex 50W (H^+ form) to remove Ba^{2+} and the acid eluate was then carefully neutralized with a dilute solution of KOH. The neutral solution was then freeze-dried to give 358 mg of the enzyme-resistant substrate (theoretical yield 369 mg).

Results and Discussion

Enzymic hydrolysis and analysis of substrates prepared with H_2SO_4

Before the specificity of the CS2 secondary alkylsulphohydrolase became clear, several secondary alkyl sulphates, prepared by sulphation of racemic or symmetrical secondary alcohols with H_2SO_4 , were tested as substrates for the pure enzyme. Up to this point 'potassium decan-5-yl sulphate' (see Dodgson *et al.*, 1974) had been used as assay substrate. In each case, the enzymic liberation of sulphate tailed off at well below 50% of the theoretical end point (Table 1). In the case of 'octan-2-yl sulphate', the reaction was apparently complete at 40% of the theoretical extent of hydrolysis and, at this point, the incubation mixture was divided into two and either extra enzyme or extra substrate was added in concentrated form to avoid any significant dilution effect. Whereas the addition of more enzyme produced no further liberation of sulphate, the addition of more substrate resulted in a further release of sulphate (Fig. 1).

Collectively, these observations suggest that only 31–42% of each preparation could actually serve as a substrate for the CS2 enzyme. In preliminary attempts to determine the nature of the apparent contamination, the preparations were analysed for ester sulphate and K^+ content, and i.r. spectra were also determined. The analytical results were all consistent with theory and there was no obvious evidence of contamination from the i.r. spectra (Lloyd *et al.*, 1961). Attempts to use the rate of acid hydrolysis of 'octan-2-yl sulphate' as a means of detecting contamination by following the procedure developed by Rees (1963) for distinguishing carbohydrate sulphate esters, were unsuccessful. Liberation of SO_4^{2-} ions during the hydrolysis of 'octan-2-yl sulphate' with 0.25M-HCl at 100°C followed a first-order mechanism with no evidence of transitions in the rate curve.

All the sulphate esters were subjected to mass

spectrometry by the soft-ionization technique of field desorption. This results in the association of the molecular ion with potassium rather than fragmentation. For 'potassium octan-2-yl sulphate' a species of mol.wt. 287 (248+39) was produced together with a species of mol.wt. 289 resulting from the minor potassium isotope of atomic mass 41. With some alkyl sulphates, species corresponding to $2M+K$ (i.e. 535 for 'potassium octan-2-yl sulphate') were detected. However, in no sample was any contaminant with a distinct molecular weight detected within the range m/e —50–750. Attention was then turned to the enzyme-resistant component of 'potassium octan-2-yl sulphate'. Ester sulphate and K^+ contents, i.r. spectra and mass spectra of enzyme-resistant substrate were indistinguishable from those of the original substrate. Pure CS2 alkylsulphohydrolase was without activity towards the enzyme-resistant substrate, but the crude cell extract of *C. terrigena* released 67% of the ester sulphate content of the material as SO_4^{2-} ions. Since

Fig. 1. Effect of adding extra substrate or extra enzyme at the apparent end point of the hydrolysis of 'octan-2-yl sulphate' by the CS2 alkylsulphohydrolase

The enzymic hydrolysis of 'potassium octan-2-yl sulphate' (6 μ mol, prepared with H_2SO_4) by pure CS2 alkylsulphohydrolase was followed as described in the text. When no further hydrolysis occurred, the remainder of the incubation mixture (initial volume 2.4 ml) was divided into two equal portions of 0.6 ml and either extra substrate (+S, 1.5 μ mol in 15 μ l) or extra enzyme (+E, 0.8 unit in 10 μ l) was added at the time indicated by the arrow. Incubation was continued and any additional enzymically liberated sulphate was recalculated back to the initial starting volume of 2.4 ml.

Table 1. End points for the enzymic hydrolysis of various substrates by pure CS2 secondary alkylsulphohydrolase. All substrates were prepared with H_2SO_4 as sulphating agent and each incubation mixture contained 6 μ mol of substrate. Further details are given in the text.

Substrate	Amount hydrolysed at end point	
	(μ mol)	(% of total)
Heptan-4-yl sulphate	2.11	35
Octan-2-yl sulphate	2.42	40
Nonan-2-yl sulphate	2.40	40
Nonon-5-yl sulphate	2.10	35
Decan-2-yl sulphate	2.50	42
Decan-5-yl sulphate	1.85	31

this substrate represents 60% of the 'potassium octan-2-yl sulphate' initially used for its preparation, the release of 67% of its ester sulphate content is approximately equivalent to the hydrolysis of a further 40% of the original sulphate ester.

These findings indicated the possibility that the CS2 enzyme was perhaps stereospecific and some support for this came from the observation that enzyme-resistant substrate had measurable optical rotation ($[\alpha]_{461}^{20}$, -2.2°) whereas 'potassium octan-2-yl sulphate' had not. However, this explanation would not account for the residual 33% of enzyme-resistant substrate which was not hydrolysed by either CS2 enzyme or the crude cell extract. Moreover, 'nonan-5-yl sulphate' and 'heptan-4-yl sulphate' are optically inactive, having symmetrical carbon chains, and yet were hydrolysed only to an extent of 35% by the pure CS2 enzyme. In the cases of these particular alkyl sulphates, their molecular symmetry makes them suitable for analysis by n.m.r. spectroscopy. For the 'heptan-4-yl sulphate' preparation the measured proportions of CH, CH₂ and CH₃ protons were not consistent with those predicted for homogeneous material, but were consistent with contamination by, for example, heptan-2-yl sulphate and/or heptan-3-yl sulphate (Table 2). Analogous results were obtained for 'nonan-5-yl sulphate'.

The possibility that all substrates prepared with H₂SO₄ might contain positional as well as optical isomers was confirmed by g.l.c. of acid hydrolysates of the substrates. In all cases the alcohol fraction recovered from acid hydrolysates of alkyl sulphates prepared originally from homogeneous alcohols, gave more than one peak of material on g.l.c. For example, the fraction recovered from the acid hydrolysate of enzyme-resistant substrate contained two peaks (Fig. 2), the major component corresponding to octan-2-ol. Similarly, the fraction recovered from the acid hydrolysate of 'decan-5-yl sulphate' gave two peaks of material on the chromatogram, the minor one corresponding to decan-5-ol and the major one to decan-2-ol (Fig. 2). Moreover, the fraction recovered after the enzymic (CS2) hydrolysis of 'decan-5-yl sulphate' showed only the peak corresponding to decan-2-ol, indicating that the sulphate group in the 5-position was not released by the enzyme (see also Matcham *et al.*, 1977b).

Fig. 2. Gas chromatography of alcohols liberated by acid or enzymic hydrolysis of secondary alkyl sulphates prepared with H₂SO₄.

Arrows indicate the times of injection and solvent peaks. See the text for further details. (a) DL-Octan-2-ol (1 μ l, injected without solvent); (b) acid hydrolysate of 'enzyme-resistant substrate'; (c) mixture of ($\pm$)decan-5-ol and ($\pm$)decan-2-ol (designated 5 and 2 respectively); (d) acid hydrolysate of 'potassium decan-5-yl sulphate'; (e) CS2 enzyme hydrolysate of 'potassium decan-5-yl sulphate'. In (a) and (b) oven temperature, 135°C; chart speed, 20mm/min; attenuations, 2×10^5 and 5×10^3 respectively. In (c), (d) and (e) oven temperature, 125°C; chart speed, 5mm/min; attenuation, 32×10^2 .

Analysis and enzymic hydrolysis of alkyl sulphates prepared with pyridine/SO₃

The sulphate esters prepared in this way all had K⁺ and ester sulphate contents that agreed with theoretical values, and i.r. analysis demonstrated the absence of any contaminating alcohol. Predicted mass spectra were also obtained and, for DL-, D- and L-octan-2-yl sulphates and ($\pm$)octan-3-yl and -4-yl sulphates, were all identical with that of 'octan-2-yl sulphates' (H₂SO₄ preparation).

The symmetrical esters, nonan-5-yl sulphate and heptan-4-yl sulphate, were shown by n.m.r. spectroscopy to be homogeneous, unlike the analogous preparations made with H₂SO₄, and the analysis of heptan-4-yl sulphates prepared by the two methods is compared in Table 2.

Analysis by g.l.c. of alcohols present in the acid hydrolysates of alkyl sulphates prepared by the pyridine/SO₃ method, showed that a single peak of material, corresponding to the parent alcohol, was present in each case.

Table 2. Proportions of CH/CH₂/CH₃ protons in different samples of potassium heptan-4-yl sulphate as determined by n.m.r. spectroscopy

	CH/CH ₂ /CH ₃
Sulphating agent	
H ₂ SO ₄	1:11:5
Pyridine/SO ₃	1:8:6
Theoretical value	1:8:6

Table 3. *Hydrolysis of DL-, D- and L-octan-2-yl sulphates by various secondary alkylsulphohydrolase preparations from C. terrigena*

Substrates (6 μ mol quantities, prepared with pyridine/SO₃ reagent) were hydrolysed by crude cell extract of *C. terrigena*, pure CS2 enzyme or partially purified CS1 enzyme until the reaction was complete. See the text for further details.

Octan-2-yl sulphate isomer	Enzyme preparation	Amount of substrate hydrolysed at end point	
		(μ mol)	(% of total)
DL	Cell extract	6.0	100
DL	CS2	3.07	51
DL	CS1	3.0	50
D	CS2	5.95	99
D	CS1	0	0
L	CS2	0.21	3.6*
L	CS1	5.82	97

* Probably reflects contamination of substrate with small amount of the D-isomer.

Further indication that secondary alkyl sulphates prepared with pyridine-SO₃ reagent were suitable for enzymological studies came from experiments in which they were tested as possible substrates for the pure CS2 enzyme, the partially pure CS1 enzyme and crude cell extracts [which contain both enzymes in roughly equal amounts (see Matcham *et al.*, 1977b)]. DL-, D- or L-Octan-2-yl sulphates were hydrolysed to 50%, 100% or not at all, depending on the particular combination of enzyme and substrate (Table 3). Although not included in Table 3, nonan-5-yl, heptan-4-yl, ($\pm$)octan-4-yl or ($\pm$)octan-3-yl sulphate were not hydrolysed by any of the enzyme preparations (see also Matcham *et al.*, 1977b).

Besides demonstrating homogeneity of substrates prepared by the pyridine/SO₃ reagent, the results of these enzyme experiments provide additional information about the specificity of the CS1 and CS2 alkylsulphohydrolases produced by *C. terrigena*. This specificity is discussed in greater detail by Matcham *et al.* (1977a,b). However, sufficient information has been presented here to show that sulphate release from 'decan-5-yl', 'nonan-5-yl' and 'heptan-4-yl sulphates' prepared with H₂SO₄, was possible only because the preparations were contaminated with isomers possessing ester sulphate groups at C₂ of the alkyl chain. Equally, sulphation of alkan-2-ols with H₂SO₄ leads to some shift of the ester sulphate group towards the centre of the chain. These shifts are not totally unexpected, even during sulphation at low temperatures, in the light of the reactions possible between secondary alcohols and H₂SO₄ (Burwell, 1942),

and a mechanism involving dehydrogenation and transient formation of a mobile centre of unsaturation is probably involved.

These undesirable transformations do not occur when pyridine/SO₃ serves as sulphating agent. Yields may also be greater with this reagent and may be related to the fact that H₂SO₄ reacts only to an equilibrium representing the relative affinity of SO₃ for water and the alcohol undergoing sulphation. SO₃ adducts of comparatively weak bases, such as pyridine, are less vigorous but more efficient sulphating agents. Burwell (1949) during studies on the preparation of butan-2-yl sulphates from resolved butan-2-ols, found complete retention of configuration when pyridine/SO₃ was used, whereas there was only 22% and 12% retention of configuration with H₂SO₄ and chlorosulphonic acid respectively. Similarly, in the present work, attempts to prepare D-octan-2-yl sulphate by treatment of D-octan-2-ol with H₂SO₄ led to complete loss of configuration (as well as to the production of positional isomers). In contrast, retention of configuration was observed when resolved octan-2-ols were sulphated with the pyridine/SO₃ reagent.

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