APPENDIX

An Improved Procedure for the Preparation of Alkyl Sulphate Esters Suitable for the Study of Secondary Alkylsulphohydrolase Enzymes

Graham F. WHITE,* Veronica LILLIS and Duncan J. SHAW
Department of Biochemistry, University College, P.O. Box 78, Cardiff CF1 1XL, Wales, U.K.

A simple, rapid and convenient method for the synthesis of secondary alkyl sulphate esters is described in which the sodium alkoxide of the parent alcohol is sulphated by using triethylamine–SO_3 complex. The procedure gives relatively good yields, even for the sulphation of long-chain alcohols and those in which the hydroxy group is remote from the terminal carbon atoms. Positional isomerization, arising from the migration of the hydroxy group along the carbon chain, is absent, and resolved enantiomers of alcohols react with complete retention of configuration.

During studies in these laboratories on stereospecific secondary alkylsulphohydrolases from certain detergent-degrading bacteria, the need has arisen for a method for preparing stereochemically pure isomers of secondary alkyl sulphate esters (Matcham & Dodgson, 1977). The classical methods of esterification of the parent alcohols with H_2SO_4 or chlorosulphonic acid were of limited use because they lead, not only to racemization during the sulphation of optically active isomers, but also to the formation of positional isomers. For example, treatment of decan-5-ol with H_2SO_4 leads to the formation of a heterogeneous mixture containing large amounts of decan-2-yl sulphate. These methods have been largely superseded by the use of the pyridine–SO_3 complex as a sulphating agent that, by eliminating acid conditions, avoids the danger of carbonium ion formation and the attendant isomerization. This procedure has enabled the synthesis of a number of secondary alkyl sulphates in a homogeneous state with respect to stereo- and positional isomerism. However, in practice the method presents some difficulties. The pyridine–SO_3 reagent is difficult to prepare in reproducible yield, and moreover its sulphating efficiency deteriorates significantly during storage. Yields of sulphate ester also tend to decrease considerably when alcohols are used that have long alkyl chains and/or the alcoholic hydroxy group near the centre of the carbon chain. Extension of work on the secondary alkyl sulphate sulphohydrolases to the S3 enzyme in *Pseudomonas* C12B (Shaw *et al.*, 1980), which has a more accommodating substrate specificity than the alkyl 2-sulphate sulphohydrolases studied hitherto, necessitated the synthesis of a number of secondary esters of various chain lengths and with the sulphate group at all possible positions from C-2 to the centre of the chain. In view of the practical difficulties encountered with the pyridine–SO_3 method, a search was made for an alternative.

The instability of the pyridine–SO_3 reagent arises from the low basicity of pyridine. Complexes of SO_3 with stronger Lewis bases, e.g. triethylamine, are considerably more stable (Gilbert, 1962). Thus triethylamine–SO_3 complex is readily prepared in pure form (Cherniak & Davidson, 1964), and can be stored for long periods without deterioration. The disadvantage of its use is that its increased stability is concomitant with lower reactivity as a sulphating agent. In the method described below, triethylamine–SO_3 complex was used as sulphating agent, and its lower susceptibility to nucleophilic attack was compensated by prior conversion of the alcohol ROH into the alkoxide RO^-Na^+, thereby increasing the latter's nucleophilicity.

Materials and Methods

Triethylamine–SO_3 complex was prepared by the method of Cherniak & Davidson (1964), and stored desiccated at 4°C. Sodium metal (99% pure) in the form of a 40% dispersion in mineral oil was purchased from the Aldrich Chemical Co., Wembley, Middlesex, U.K. Toluene and dioxan were of analytical grade and were stored over sodium metal. D- and L-Octan-2-ol and undecan-6-ol were Fluka products obtained through Fluorochem, Glossop, Derbyshire, U.K.; octan-3-ol was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and all other alcohols were from Aldrich Chemical

* To whom reprint requests should be addressed.

Co. Carbowax 400 on Chromosorb W AW-DCMS for g.l.c. was obtained from Jones Chromatography, Llanbradach, Glam., U.K. The sample of CS2 secondary alkylsulphohydrolase was prepared as described previously (Matcham & Dodgson, 1977*b*). All other materials were the purest available from either Sigma Chemical Co., Poole, Dorset, U.K., or BDH Chemicals, Poole, Dorset, U.K.

General sulphation procedure

Dispersed sodium (0.35ml, containing 6mmol of sodium) was centrifuged at $670g_{av.}$ for 5min in a 10ml tapered glass tube. The supernatant was discarded (into ethanol or butanol to render safe traces of metallic sodium), and the pellet was washed by resuspension in 1ml of dry toluene. After centrifugation, the pellet was resuspended in dry toluene to produce a total volume of 1ml. The appropriate secondary alcohol (4mmol) was then added dropwise to the stirred sodium suspension, any heat generated being allowed to dissipate before the addition of the next drop. When the addition was complete, the tube was capped with aluminium foil and stirred until effervescence ceased. The mixture was centrifuged to remove unchanged sodium metal, and the supernatant containing the sodium alkoxide was transferred to a clean dry tube. A solution of triethylamine–SO_3 complex (4mmol) in 3–4ml of warm dioxan was added slowly to the stirred alkoxide solution, and stirring was continued for a further 1h at room temperature. The solution was evaporated almost to dryness by rotary evaporation, and residual toluene and dioxan were removed from the product by repeated addition and re-evaporation of 1ml portions of water. The resulting white slurry was dissolved in the minimum volume of water and run into a Dowex-50 (H^+ form) cation-exchange column (2.5cm × 10cm) to remove triethylamine and Na^+ ions. The column was eluted with water, and effluent fractions with pH 1–3 were collected. Pooled eluates were neutralized with KOH solution, extracted three times with equal volumes of diethyl ether to remove residual alcohol, and freeze-dried. The white fluffy solid was extracted into 100ml of hot methanol, and the solution was clarified by filtration if necessary. Methanol was removed by rotary evaporation, the residue dissolved in water and the resulting solution freeze-dried.

This routine procedure was amenable to a decrease in scale of up to 4-fold without loss of yield.

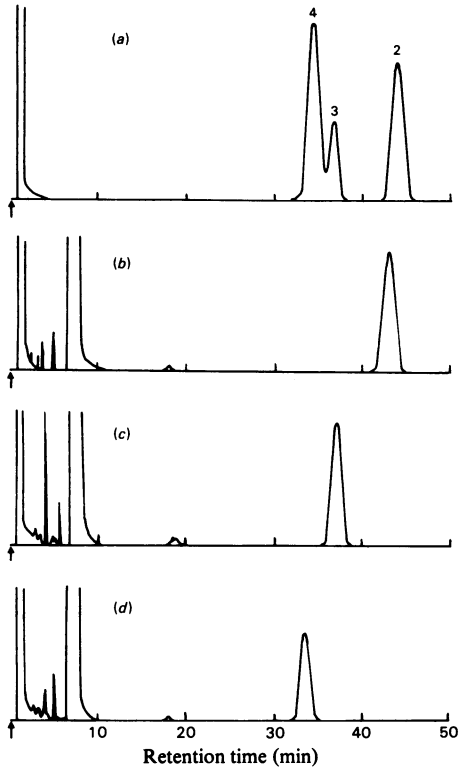
Analysis of alkyl sulphates

Chemical analysis. Ester sulphate content was measured by a gravimetric determination of the sulphate liberated by acid hydrolysis. The alkyl sulphate ester (50–100mg) was refluxed for 1h in 30ml of 2M-HCl, and excess of $BaCl_2$ (approx. 0.3g dissolved in warm water) was added. Precipitated $BaSO_4$ was collected on a pre-weighed glass sinter (no. 4 porosity), washed with warm water, acetone and ether, and dried to constant weight. K^+ content of the potassium alkyl sulphate esters was measured by flame photometry in an Evans Electroselenium flame photometer with potassium sulphate as standard.

Table 1. *Yields and chemical analysis of secondary alkyl sulphate esters prepared from the sodium alkoxides by using triethylamine–SO_3 complex*

See the text for details.

		SO_4^{2-} content (%)		K^+ content (%)	
Sulphate ester	Yield (%)	Theoretical	Found	Theoretical	Found
Pentan-3-yl sulphate	46	46.6	44.7	18.9	—
DL-Octan-2-yl sulphate	55	38.7	39.6	15.7	14.6
D-Octan-2-yl sulphate	65	38.7	42.0	15.7	15.2
DL-Octan-3-yl sulphate	35	38.7	40.0	15.7	14.5
DL-Octan-4-yl sulphate	20	38.7	37.1	15.7	15.5
DL-Nonan-2-yl sulphate	16	36.6	36.6	14.9	14.1
DL-Nonan-3-yl sulphate	29	36.6	37.3	14.9	14.2
DL-Nonan-4-yl sulphate	20	36.6	36.7	14.9	15.1
Nonan-5-yl sulphate	30	36.6	33.6	14.9	14.9
DL-Decan-2-yl sulphate	36	34.8	33.4	14.1	13.7
DL-Decan-3-yl sulphate	60	34.8	32.7	14.1	13.4
DL-Decan-4-yl sulphate	15	34.8	32.7	14.1	14.0
DL-Decan-5-yl sulphate	32	34.8	36.0	14.1	15.1
Undecan-6-yl sulphate	56	33.1	33.1	13.4	12.7
DL-Tetradecan-2-yl sulphate	60	28.9	28.9	11.8	11.3



G.l.c. analysis. For g.l.c. analysis, parent alcohols were first liberated from their esters by solvolysis in aqueous dioxan (Mayers *et al.*, 1969). This procedure is known to release alcohols from secondary alkyl sulphate esters under mild conditions with complete retention of configuration and without positional isomerization (Mayers *et al.*, 1969). The appropriate ester (5 mg) was refluxed in 2 ml of dioxan/water (99:1, v/v) for 15–20 min. The mixture was left to cool, mixed with 8 ml of water, and liberated alcohol was extracted with 3 × 4 ml of diethyl ether. Pooled ether extracts were washed with 2.5 ml of water, dried over anhydrous Na_2SO_4 and evaporated in a stream of N_2 to a final volume of about 1 ml. Standards were also diluted into diethyl ether. Samples (1 μl) were then analysed in a

Fig. 1. *G.l.c. analysis of alcohols liberated by dioxan solvolysis of isomeric octyl sulphates prepared from the sodium alkoxides and triethylamine–SO_3 complex*
(*a*) Separation of a standard mixture of DL-octan-2-ol, DL-octan-3-ol and DL-octan-4-ol (designated 2, 3 and 4 respectively). The procedure for dioxan solvolysis of (*b*) DL-octan-2-yl sulphate, (*c*) DL-octan-3-yl sulphate and (*d*) DL-octan-4-yl sulphate, and details of the chromatographic procedure, are described in the text.

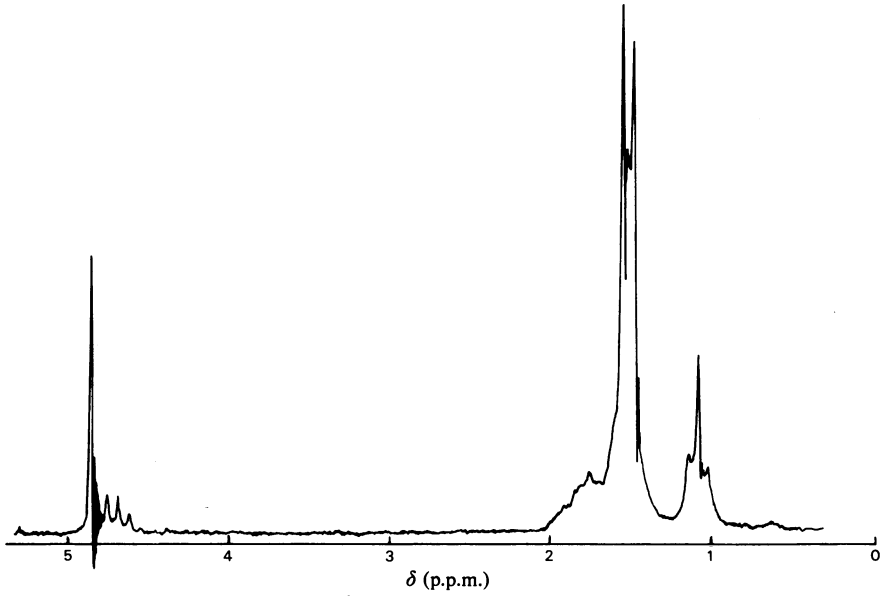


Fig. 2. *Complete proton n.m.r. spectrum of* DL-*octan-2-yl sulphate prepared from the alkoxide and triethylamine–SO_3 complex*
See the text for details. Chemical shifts are relative to a trimethylsilane reference standard.

Perkin–Elmer F11 gas chromatograph with flame-ionization detection. The column (3m × 3mm) was packed with 20% Carbowax 400 on Chromosorb W AW-DCMS and conditioned and operated at 110°C. This system is suitable for analysis of positional isomers of secondary alcohols containing up to nine carbon atoms/molecule (Castello & D'Amato, 1977).

Spectroscopic analysis. I.r. spectra were obtained in Nujol mulls between NaCl plates by using a Perkin–Elmer Infracord spectrometer. N.m.r. spectra were recorded in ^2H_2O by using a Perkin–Elmer R32 spectrometer operated at 90MHz. Optical rotations of alcohols (in ethanol) and sulphate esters (in water) were measured in a 1cm-path-length cell at 546nm in an NPL type 244 automatic polarimeter (Thorn Automation, Nottingham, Notts., U.K.).

Results and Discussion

All alcohols tested (Table 1) were capable of sulphation by the new method. Yields of sulphate esters varied, but mostly lay in the range 30–60%. This compares favourably with the pyridine–SO_3 method, particularly for the longer-chain alcohols, where the earlier method produced yields of only about 10% or less. Alcohols with hydroxy groups remote from the ends of the chain (e.g. nonan-5-ol, decan-5-ol, undecan-6-ol) were also sulphated more

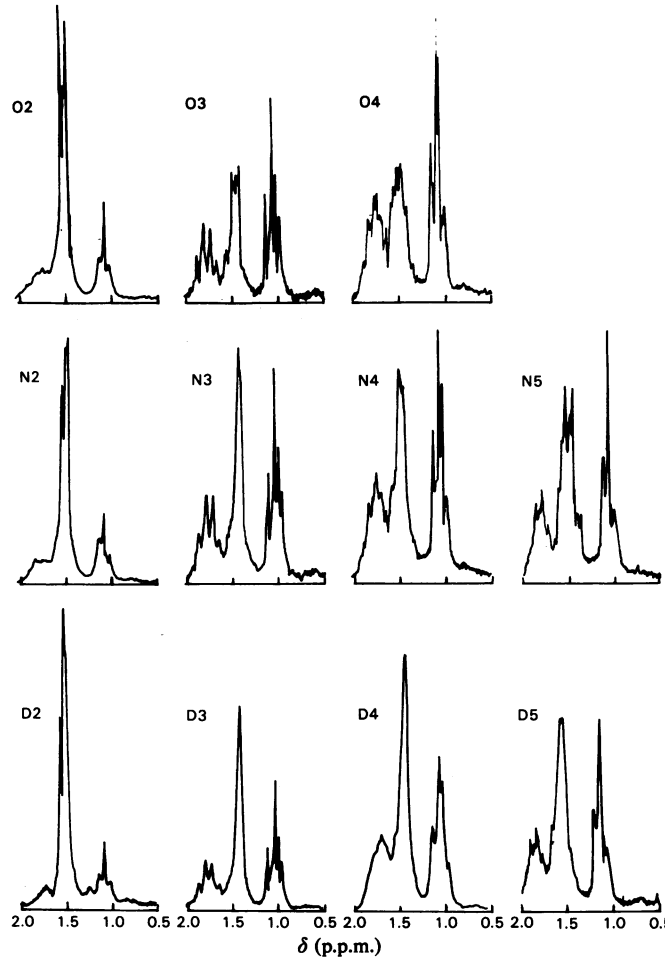


Fig. 3. *Partial proton n.m.r. spectra of secondary octyl, nonyl and decyl sulphate esters prepared from the sodium alkoxides and triethylamine–SO_3 complex*

See the text for details. O2, DL-Octan-2-yl sulphate; O3, DL-octan-3-yl sulphate; O4, DL-octan-4-yl sulphate; N2, DL-nonan-2-yl sulphate; N3, DL-nonan-3-yl sulphate; N4, DL-nonan-4-yl sulphate; N5, nonan-5-yl sulphate; D2, DL-decan-2-yl sulphate; D3, DL-decan-3-yl sulphate; D4, DL-decan-4-yl sulphate; D5, DL-decan-5-yl sulphate.

efficiently than hitherto. Furthermore, storage of triethylamine–SO_3 complex in a desiccator at 4°C did not lead to a decrease in its sulphating ability (as is the case with pyridine–SO_3 complex), thus improving the reliability of the method.

Sulphate and K^+ contents of sulphate esters examined agreed with theoretical values, indicating the absence of gross contamination by other materials. I.r. spectroscopy of all esters showed bands typical of alkyl sulphates, and also indicated the absence of free alcohols.

The possibility that the esters prepared by the new method contained positional isomers was examined by g.l.c. of the alcohols liberated during dioxan solvolysis. Fig. 1(*a*) shows the separation of a standard mixture of octan-2-ol, octan-3-ol and octan-4-ol, each of which is clearly resolved. Alcohol fractions recovered from dioxan solvolysis of octan-2-yl sulphate (Fig. 1*b*), octan-3-yl sulphate (Fig. 1*c*) and octan-4-yl sulphate (Fig. 1*d*) gave a single peak in each case, corresponding to the parent alcohol from which the ester was originally derived. Similar results were obtained for the nonyl series. Evidently, no migration of alcoholic hydroxy and/or sulphate groups has occurred before, or during, sulphation.

A typical n.m.r. spectrum for DL-octan-2-yl sulphate prepared by the new method is shown in Fig. 2. The triplet centred at δ 1.1 p.p.m. (integrating for 3H) was assigned to the C-8 methyl group. The C-1 methyl resonance was shifted downfield by the proximity of the $-OSO_3^-$ group at C-2, and occurs as a sharp doublet superimposed on the methylene resonances for C-3 to C-7 (δ 1.4–2.0 p.p.m.). The C-2 hydrogen was shifted downfield to δ 4.5–5.0 p.p.m., but the splitting pattern here was frequently confused by the $^1H^2HO$ resonance and its spinning side-bands. This spectrum was indistinguishable from that for D-octan-2-yl sulphate prepared in the same way, or for the same compounds prepared by using pyridine–SO_3 complex. All spectra showed the absence of a –C*H*OH resonance, confirming the absence of parent alcohols. Spectra for the other esters in the δ 0.5–2.0 p.p.m. range (CH_3 and CH_2 protons) are collected in Fig. 3. Nonan-2-yl sulphate and decan-2-yl sulphate showed the same pattern as their octyl homologue, except that the signals for the methylene protons were enhanced, as expected. The alkyl sulphates with sulphate attached at the third carbon atom (C-3 esters) showed a pattern clearly distinguishable from that for the C-2 alkyl sulphate series, in that the triplet at δ 1 p.p.m. was replaced by a quartet integrating for six hydrogen atoms. Presumably, in the C-3 sulphate series the C-1 methyl group is only weakly de-shielded by the now more remote sulphate group, and it appears as a triplet nearly superimposed on that for the methyl distal to the sulphate group. The observed pattern of peaks in this region for the combined resonances of the terminal methyl groups was the same for octan-3-yl sulphate, nonan-3-yl sulphate and decan-3-yl sulphate, indicating that it is characteristic of C-3 alkyl sulphates. For the C-4 sulphate series the terminal methyl groups are even more alike in terms of chemical environment, and the merging of the two sets of triplets is more pronounced. Superimposition is almost complete, but the pattern for octan-4-yl sulphate, nonan-4-yl sulphate and decan-4-yl sulphate is still distinguishable from that for both the C-2 and C-3 alkyl sulphates, In the symmetrical esters, nonan-5-yl sulphate and undecan-6-yl sulphate (the latter not shown), the terminal methyl groups are identical, giving a triplet very similar to that observed for the C-8 methyl group of octan-2-yl sulphate, except that in the symmetrical esters the resonance integrates for six, rather than three, hydrogen nuclei. For decan-5-yl sulphate, which is not symmetrical, the difference between the terminal methyl groups is too small to produce different resonances and a spectrum similar to that of symmetrical esters (e.g. nonan-5-yl sulphate) was observed.

This n.m.r. analysis tends to support the g.l.c. evidence showing that the sulphation of sodium alkoxides with triethylamine–SO_3 complex is not accompanied by migration of functional groups along the carbon chain.

Sulphation of D-octan-2-ol by the new method yielded D-octan-2-yl sulphate with a specific rotation

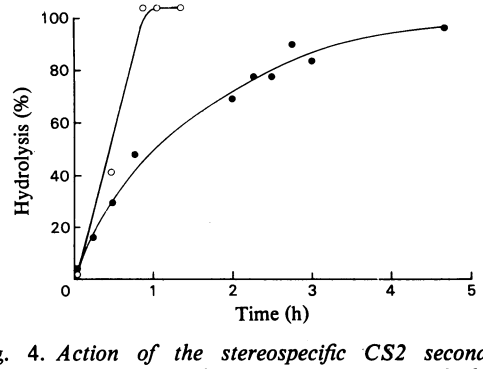


Fig. 4. *Action of the stereospecific CS2 secondary alkylsulphohydrolase of C. terrigena on octan-2-yl sulphate prepared by sulphation of the sodium alkoxide of D-octan-2-ol with triethylamine–SO_3 complex*

Octan-2-yl sulphate prepared from D-octan-2-ol was incubated in 0.1 M-Tris/HCl buffer, pH 8.2, with the CS2 enzyme preparation (see Matcham *et al.*, 1977) at 30°C. ○, 4.0 mM-Octan-2-yl sulphate and 0.14 unit of enzyme/ml; ●, 3.7 mM-octan-2-yl sulphate and 0.08 unit of enzyme/ml. Samples (50 μl) were withdrawn at intervals and assayed for liberated inorganic sulphate by the $BaCl_2$/gelatin method of Dodgson (1961).

$[\alpha]_{546}^{20}$ of +3.15° (*c* 0.05 in water). Within experimental error (±0.15°), this rotation agrees with the value of +3.25° for pure D-octan-2-yl sulphate produced by using the pyridine–SO_3 method (Bartholomew *et al.*, 1977). The established specificity of the CS2 alkylsulphohydrolase of *C. terrigena* for D-alkyl-2-sulphate esters (Matcham *et al.*, 1977) affords a useful method for confirming the stereochemical purity of the C-2 sulphate esters. D-Isomers are hydrolysed to completion, L-isomers not at all, and mixtures hydrolysed to an extent dependent on the degree of racemization. D-Octan-2-yl sulphate prepared by the new method, when treated with the CS2 enzyme, was completely hydrolysed (Fig. 4), showing the presence of only the D-isomer.

The consistency of yields of secondary alkyl sulphate esters achieved over a range of chain lengths and positional isomers in the present work suggests that this method may enjoy a wide application. Of particular value is the access it offers to longer-chain alkyl sulphates, and those with the ester group remote from the chain termini. Furthermore, the stereochemistry of the parent alcohol is preserved during sulphation, enabling the synthesis of optically pure secondary alkyl sulphate esters from resolved alcohols. Preliminary experiments have indicated that the method is also suitable for the sulphation of alcohol hydroxy groups in more complex compounds such as diols (e.g. diethylene glycol) and sterols (e.g. dehydroepiandrosterone).

We thank Dr. D. Wilson of the Department of Chemistry, University College, Cardiff, for providing, and assisting with the interpretation of, the n.m.r. spectra.

References

Bartholomew, B., Dodgson, K. S., Matcham, G. W. J., Shaw, D. J. & White, G. F. (1977) *Biochem. J.* **165**, 575–580

Castello, G. & D'Amato, G. (1977) *J. Chromatogr.* **131**, 41–55

Cherniak, R. & Davidson, E. A. (1964) *J. Biol. Chem.* **239**, 2986–2990

Dodgson, K. S. (1961) *Biochem. J.* **78**, 312–319

Gilbert, E. E. (1962) *Chem. Rev.* **62**, 549–589

Matcham, G. W. J. & Dodgson, K. S. (1977) *Biochem. J.* **167**, 717–722

Matcham, G. W. J., Dodgson, K. S. & Fitzgerald, J. W. (1977) *Biochem. J.* **167**, 723–729

Mayers, G. L., Pousada, M. & Haines, T. H. (1969) *Biochemistry* **8**, 2981–2986

Shaw, D. J., Dodgson, K. S. & White, G. F. (1980) *Biochem. J.* **187**, 181–190