Biochemical Pharmacology, Vol. 18, pp. 173–180. Pergamon Press. 1969. Printed in Great Britain

THE PREPARATION AND CHARACTERIZATION OF A SERIES OF ^{35}S-LABELLED ARYL SULPHATE ESTERS FOR METABOLIC STUDIES*

D. J. HEARSE,† A. H. OLAVESEN and GILLIAN M. POWELL

Department of Biochemistry, University College, Cardiff

(*Received* 7 *June* 1968; *accepted* 15 *August* 1968)

Abstract—In order to provide a series of suitable compounds for metabolic studies, methods were developed for the preparation of crystalline potassium salts of the ^{35}S-labelled sulphate esters of 4-hydroxybiphenyl, 4-cyclohexylphenol, 2-cyclohexylphenol, 2-naphthol and 5,6,7,8-tetrahydro 2-naphthol in good yield. Infrared spectrophotometric analysis of the products showed strong absorption bands in the regions 1280–1220 cm^{-1} and 1070–1040 cm^{-1} characteristic of the ester sulphate grouping. Ultraviolet absorption spectra revealed a characteristic spectral shift from the absorption region associated with the parent phenols (270–310 mμ) to that associated with sulphated phenols (240–280 mμ).

A SERIES of enzymes which are capable of desulphating many physiological and non-physiological aryl sulphate esters have been reported.[1] In spite of the fact that a great deal is known about the intracellular localization and mechanisms of action of these aryl sulphatases their biological significance remains obscure. Attempts have been made to assign a functional role to the enzymes by studying the metabolic fate of aryl sulphate esters administered to animals. As part of this study, and also as part of a more general investigation into the effect of various physico-chemical factors upon metabolism and modes of excretion, an investigation into the synthesis and characterization of a series of ^{35}S-labelled aryl sulphate esters (Fig. 1) suitable for such metabolic studies was undertaken.

MATERIALS AND METHODS

Reagents

4-Hydroxybiphenyl, m.p. 165–167° was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. and recrystallized from methanol before use. 4-Cyclohexylphenol, m.p. 122–128° and 2-cyclohexylphenol, m.p. 57–59°were obtained from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. and recrystallized from methanol. 2-Naphthol, m.p. 122–123° was obtained from British Drug Houses Ltd., Poole, Dorset. 5,6,7,8-Tetrahydro 2-naphthol was a gift from the National Gallery, London, and was purified by distillation at 120–122°. All solvents used were from fresh bottles

---

* D.J.H. is grateful to the Medical Research Council for a Research Studentship. The work was supported by U.S. Public Health Service Research Grant No. AMO1982 from the Arthritis and Metabolic Diseases Division.

† Present address: Department of Pharmacology, New York University Medical Center, 550, First Avenue, New York, N.Y. 10016, U.S.A.

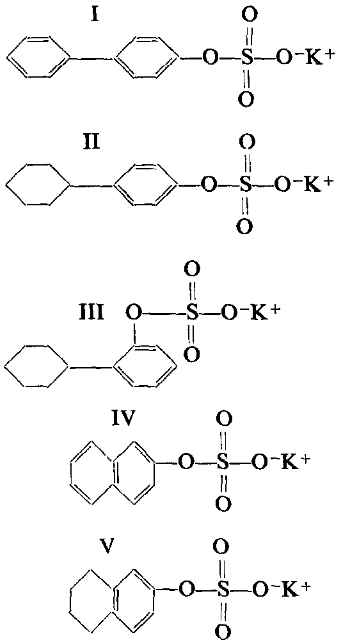

FIG. 1. Structures of :— I, potassium biphenylyl 4-sulphate; II, potassium cyclohexylphenyl 4-sulphate; III, potassium cyclohexylphenyl 2-sulphate; IV, potassium naphthyl 2-sulphate; V, potassium 5,6,7,8-tetrahydronaphthyl 2-sulphate.

of the respective 'ANALAR' reagent obtained from British Drug Houses Ltd. ^{35}S-Labelled chlorosulphonic acid was obtained from the Radiochemical Centre, Amersham, Bucks.

Thin-layer chromatography (TLC)

TLC was performed on silica gel (Kieselgel G, Merck Ltd., Darmstadt, W. Germany) using plates (20 × 5 cm) with a gel thickness of 0·25 mm. All plates were activated at 110° for 45 min before use. The plates were developed by ascending chromatography over a distance of 10 cm in a pre-equilibrated tank. Solvent systems used were either 2-propanol: chloroform: methanol: water (10:10:5:2 by volume) or 1-butanol:acetic acid: water (60:20:20 by volume).

Paper electrophoresis

Samples were subjected to horizontal paper electrophoresis on Whatman No. 1 paper for 3 hr at a potential gradient of 10 v/cm. Buffers used were either 0·1 M sodium acetate: acetic acid (pH 4·5) or 0·1 M citric acid: disodium phosphate (pH 2·2).

Detection of radioactive areas on paper electrophoresis strips and TLC plates

These were located and quantitated using a Packard Model 7200 Radiochromatogram Scanner. Alternatively, radioactive areas were located by autoradiography. Electrophoresis strips or TLC's were kept in contact with X-ray film (Ilford Industrial B) for periods of up to 7 days.

Analysis of products

Elemental analyses for C, H and S were carried out by Drs. F. and E. Pascher, Microanalytical Laboratories, Bonn, W. Germany. Potassium was assayed by flame photometry using an Eel flame photometer. Ester sulphate content was determined by Method A of Dodgson.[2]

Infrared spectroscopy

Infrared spectra were measured using the Perkin–Elmer 157 spectrophotometer. All spectra were obtained using Nujol mulls between sodium chloride plates.

Ultraviolet spectroscopy

Ultraviolet spectra was measured using the Optica CF4R recording spectrophotometer. All compounds were measured in solutions of 0·1 N NaOH over the range 230–320 mμ with a path length of 1 cm.

EXPERIMENTAL AND RESULTS

Preparation of potassium biphenylyl 4-^{35}*S-sulphate*

(a) *Preparation of triethylamine-sulphur trioxide complex.* A mixture of anhydrous chloroform (2·0 ml) and triethylamine 0·8 ml) was cooled to — 15° with constant stirring. A mixture of chloro-^{35}S-sulphonic acid (0·2 ml, 7·5 mc/mM) and chloroform (0·75 ml) was added dropwise to the cooled mixture. The reaction mixture was stirred for 30 min at — 15° and was then extracted with ice-cold water (2 × 1 vol.). Chloroform was then removed *in vacuo* at 20° and the resultant white crystalline solid dried *in vacuo* over P_2O_5 at room temperature. Yield 0·39 g, 71 per cent, m.p. 91–92°.

(b) *Sulphation of* 4-*hydroxybiphenyl.* 4-Hydroxybiphenyl (0·48 g) and KOH (0·18 g) were dissolved in water (1·65 ml) at 70°. Potassium bicarbonate (0·31 g) and ^{35}S-labelled triethylamine-sulphur trioxide (0·39 g) were added and the whole stirred for 4 hr at 47°. The mixture was allowed to cool to room temperature and extracted with ether (3 × 2 vol.). The remaining slurry was diluted with water (3·0 ml) and 5N KOH (0·86 ml) and was salted to 15% w/v with KCl. The precipitated product was collected and redissolved in the minimum volume of methanol and reprecipitated by the addition of ether (10 vol.); the product was filtered off and washed with ether. Initial crystallization was carried out by dissolving the product in the minimum volume of an acetone-water mixture (50%, v/v); rapid crystallization of the ester was induced by evaporation of the acetone at 80°. The white crystalline solid was twice recrystallized from the minimum volume of water at 80–90°. The product was isolated, washed with ice-cold water and dried *in vacuo* over P_2O_5 at room temperature. Potassium biphenylyl 4-^{35}S-sulphate (yield 0·48g, 56 per cent) had a sp. act. of 26 μc/mg. Analysis of the pure product revealed SO_4^{2-}, 33·30 and K, 13·5. $C_{12}H_9O_4SK$ requires SO_4^{2-}, 33·34 and K, 13·5.

Sulphation of 4-*cyclohexylphenol*

Carbon disulphide (2·0 ml) and *N,N*-dimethylaniline (1·5 ml) were mixed and cooled to — 15° with constant stirring. Chloro-^{35}S-sulphonic acid (0·2 ml, 10·5 mc/mM) was added dropwise to the cooled mixture. The reaction vessel was then removed from the cooling bath and 4-cyclohexylphenol (0·43 g) was added. The mixture was stirred continuously for 1 hr and then kept at room temperature for a further

3 hr. The potassium salt of *cyclohexylphenyl* 4-^{35}*S-sulphate* was formed by the dropwise addition of 50% (w/v) KOH with stirring and cooling until the mixture remained alkaline. The precipitate was then isolated by centrifuging at 1000 *g* for 5 min and the liberated *N*,*N*-dimethylaniline was decanted. The pellet was resuspended in ether and after centrifuging the supernatant was discarded. This procedure was repeated twice. The potassium salt of the sulphate ester was dissolved in the minimum volume of anhydrous methanol at room temperature; any insoluble material was removed by centrifuging at 1000 *g* for 2 min. The supernatant was decanted and the ester precipitated from solution with ether (10 vol.). The product was separated by centrifuging at 1000 *g* for 2 min and washed with ether. This procedure was repeated twice and the product was dried *in vacuo* over P_2O_5 at room temperature. The ester was further purified by precipitation from the minimum volume of *N*,*N*-dimethylformamide with ether (10 vol.), washed with ether and dried. The material was then recrystallized twice from the minimum volume of hot water and the final product was dried over P_2O_5 *in vacuo* at room temperature. Potassium cyclohexylphenyl 4-^{35}S-sulphate (yield 0·42 g, 58 per cent) had a sp. act. of 36 μc/mg. Analysis of the pure product revealed C, 48·6; H, 5·1; S, 10·2; SO_4^{2-}, 32·6 and K, 13·4. $C_{12}H_{15}O_4SK$ requires C, 49·2; H, 5·1; S, 10·9; SO_4^{2-} 32·7 and K, 13·3.

Sulphation of 2-*cyclohexylphenol*

The procedure adopted was identical with that used for the sulphation of 4-cyclohexylphenol; using chloro-^{35}S-sulphonic acid (0·2 ml, 11 mc/mM) and 2-cyclohexylphenol (0·43 g). The isolation and purification procedure for *potassium cyclohexylphenyl* 2-^{35}*S-sulphate* was identical with that for the corresponding 4-sulphate ester. The product (yield 0·48 g, 73 per cent) had a specific activity of 37 μc/mg. Analysis of the crystalline product revealed C, 49·7; H, 5·1; S, 10·5; SO_4^{2-}, 32·8 and K, 13·3. $C_{12}H_{15}O_4SK$ requires C, 49·2; H, 5·1; S, 10·9; SO_4^{2-}, 32·7 and K, 13·3.

Sulphation of 2-*naphthol*

The procedure adopted was identical with that used for the sulphation of 4-cyclohexylphenol; using chloro-^{35}S-sulphonic acid (0·2 ml, 6·9 mc/mM) and 2-naphthol (0·43 g). The isolation and purification procedure was identical with that previously outlined except that it was not necessary to recrystallize the ester from *N*,*N*-dimethylformamide. Potassium naphthyl 2-^{35}S-sulphate (yield 0·49 g, 62 per cent) had a sp. act. of 27 μc/mg. Analysis of the pure product revealed SO_4^{2-}, 36·7 and K, 14·9. $C_{10}H_7O_4SK$ requires SO_4^{2-}, 36·6 and K, 14·9.

Sulphation of 5,6,7,8-*tetrahydro* 2-*naphthol*

The procedure adopted was the same as that for the sulphation of 4-cyclohexylphenol; using chloro-^{35}S-sulphonic acid (0·2 ml, 10·9 mc/mM) and 5,6,7,8-tetrahydro 2-naphthol (0·43 g). The isolation and purification procedure for *potassium* 5,6,7,8-*tetrahydronaphthyl* 2-^{35}*S-sulphate* was identical with that for potassium cyclohexylphenyl 4-^{35}S-sulphate. The product (yield 0·43 g, 56 per cent) had a sp. act. of 41 μc/mg. Analysis of the crystalline product revealed C, 44·3; H, 4·0; S, 11·6, SO_4^{2-}, 36·1 and K, 14·5. $C_{10}H_{11}O_4SK$ requires C, 45·2; H, 4·1; S, 12·0; SO_4^{2-}, 36·0 and K, 14·4.

TABLE 1. R_f VALUES OF ARYL SULPHATE ESTERS AS DETERMINED BY TLC IN TWO SOLVENT SYSTEMS

Aryl Sulphate Ester	R_f	
	Solvent A	Solvent B
Biphenylyl 4-^{35}S-sulphate	0·66	0·66
Cyclohexylphenyl 4-^{35}S-sulphate	0·63	0·68
Cyclohexylphenyl 2-^{35}S-sulphate	0·61	0·71
Naphthyl 2-^{35}S-sulphate	0·69	0·62
5,6,7,8-Tetrahydronaphthyl 2-^{35}S-sulphate	0·64	0·66

A—2-propanol:chloroform:methanol:water (10:10:5:2 by volume); B—1-butanol:acetic acid: water (60:20:20 by volume).

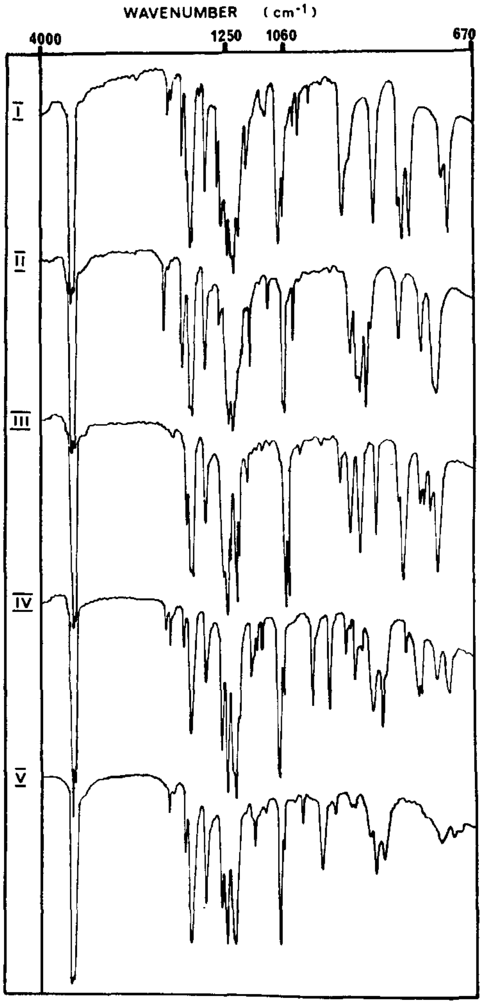

FIG. 2. Infrared absorption spectra of aryl sulphate esters. I, biphenylyl 4-^{35}S-sulphate; II, cyclohexylphenyl 4-^{35}S-sulphate; III, cyclohexylphenyl 2-^{35}S-sulphate; IV, naphthyl 2-^{35}S-sulphate; V,5,6,7,8-tetrahydronaphthyl 2-^{35}S-sulphate.

Paper electrophoresis and TLC

All aryl sulphate esters were found to be radiochemically homogeneous on paper electrophoresis and TLC. R_f values from TLC's are shown in Table 1.

Characterization of prepared aryl sulphate esters.

Infrared spectroscopy. Infrared spectra of the aryl sulphate esters are shown in Fig. 2. Each ester exhibited clearly defined absorption bands throughout the wavelength range measured.

Ultraviolet spectroscopy. All ultraviolet spectra were measured in the range 230–320 mμ. The spectra of the aryl sulphate esters and the respective parent phenols are shown in Fig. 3. The wavelength of maximum absorption and molecular extinction coefficient for each aryl sulphate ester were measured in 0·1 N NaOH. The following respective values were obtained:—biphenylyl 4-sulphate, 249 mμ and 20600; cyclohexylphenyl 4-sulphate, 262 mμ and 420; cyclohexylphenyl 2-sulphate, 262 mμ and 360; naphthyl 2-sulphate, 275 mμ and 4500; 5,6,7,8-tetrahydronaphthyl 2-sulphate, 268 mμ and 275 mμ and 1020.

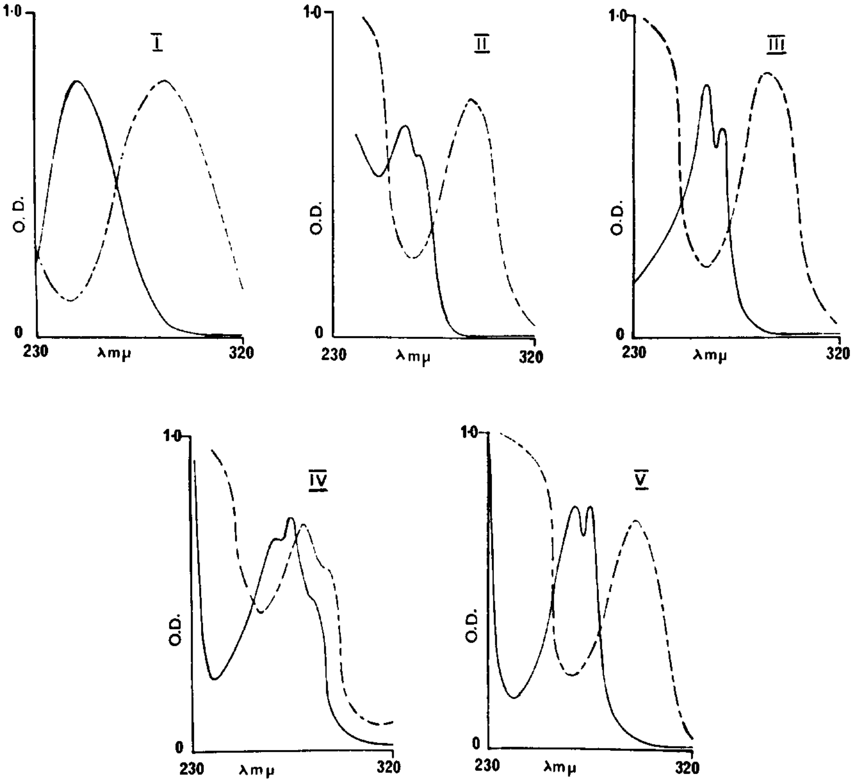

FIG. 3. Ultraviolet absorption spectra of a series of aryl sulphate esters (continuous line) and their parent phenols (broken line) measured in 0·1 N NaOH. I, biphenylyl 4-^{35}S-sulphate, II, cyclohexylphenyl 4-^{35}S-sulphate; III, cyclohexylphenyl 2-^{35}S-sulphate; IV, naphthyl 2-^{35}S-sulphate; V, 5,6,7,8-tetrahydronaphthyl 2-^{35}S-sulphate. The concentration of each solution was such that its optical density at the wavelength of maximum absorption was not in excess of 1·0. The curves are not quantitatively related to each other.

Self-irradiation

Lloyd, Balazs, Embery and Wusteman[3] reported the degradation of certain radioactivity labelled sulphate esters by self-irradiation. All preparations described in this paper were tested for homogeneity after one and two half-life periods ($t_{\frac{1}{2}}$ = 87·2 days) and no evidence for degradation due to self-irradiation processes was observed.

DISCUSSION

Previous methods for the preparation of aryl sulphate esters, particularly those of Hardy and Scalera[4] and Hawkins and Young,[5] were investigated and were suitably modified to provide radiochemically labelled products for this and subsequent metabolic studies.

The preparation of three new aryl sulphate esters has been achieved, namely cyclohexylphenyl 4-^{35}S-sulphate, cyclohexylphenyl 2-^{35}S-sulphate and 5,6,7,8:tetrahydronaphthyl 2-^{35}S-sulphate. The yields of aryl sulphate esters were in the range of 56–73 per cent of pure product after full purification. The esters were found to be homogeneous on TLC using two solvent systems and also on paper electrophoresis in two buffer systems. Chemical analysis demonstrated that the products had the required empirical formulae.

Infrared studies with each product confirmed that the preparation contained no contaminating parent phenol since no strong absorption band was recorded in the 3600–3200 cm^{-1} region. Strong absorption bands were present in the regions 1280–1220 cm^{-1} and 1070–1040 cm^{-1}. Previous workers[6, 7] have assigned these absorption bands to S-O and C-O-S systems; it has been established[7-9] that absorption bands characteristic of these systems also appear in the range 900–800 cm^{-1}. Although strong absorption bands were detected in the latter region, since there was interference from absorption due to the aromatic nucleus, no attempt was made to assign these absorption bands to specific vibrational modes. Infrared absorption spectra have thus been used not only as criteria of purity but also as a means of verification of the structure assigned to the aryl sulphate ester.

Ultraviolet absorption spectra showed that all the aryl sulphate esters exhibited strong absorption in the range 240–280 mμ (see Fig. 3) with maxima between 250–275 mμ. The parent phenols absorbed strongly in the region 270–310 mμ with maxima in the range 280–295 mμ. This spectral shift coupled with the much greater molecular extinction coefficients of the free phenols allows the detection of trace amounts of contaminating parent phenol. Characteristic shifts of absorption maxima following the sulphation of a free phenol have been reported previously[10] for tyrosine and its *O*-sulphate ester, the shifts and absorption maxima falling within the limits outlined above.

The methods and purification procedures described provide suitable compounds for subsequent investigations into the metabolic fates and tissue distributions of aryl sulphate esters.

REFERENCES

1. K. S. Dodgson and F. A. Rose, *Nutr. Abstr. Rev.* **36**, 327 (1966).
2. K. S. Dodgson, *Biochem. J.* **78**, 312 (1961).
3. A. G. Lloyd, E. A. Balazs, G. Embery and F. S. Wusteman, *Biochem. J.* **98**, 34 P (1966).
4. W. B. Hardy and M. Scalera, *J. Am. Chem. Soc.* **74**, 5212 (1952).
5. J. B. Hawkins and L. Young, *Biochem. J.* **56**, 166 (1954).

6. L. J. BELLAMY, *The Infrared Spectra of Complex Molecules*, Methuen, London (1958).
7. A. G. LLOYD, N. TUDBALL and K. S. DODGSON, *Biochim. biophys. Acta* **52**, 413 (1961).
8. A. G. LLOYD, K. S. DODGSON, R. G. PRICE and F. A. ROSE, *Biochim. biophys. Acta* **46**, 108 (1961).
9. A. G. LLOYD and K. S. DODGSON, *Biochim. biophys. Acta* **46**, 116 (1961).
10. K. S. DODGSON, F. A. ROSE and N. TUDBALL, *Biochem. J.* **71**, 10 (1959).