1157. *The Activation of Sulphuric Acid Esters by Use of Ethoxyacetylene in the Synthesis of Adenosine-5′ Sulphatophosphate*

By G. R. Banks and D. Cohen

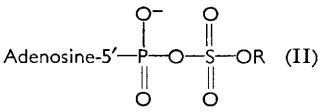
Benzyl and 2-cyanoethyl esters of sulphuric acid have been used to prepare adenosine-5′ sulphatophosphate by means of the reaction of the 1-ethoxyvinyl derivatives with adenylic acid and subsequent removal of protecting groups.

Adenosine-5′ sulphatophosphate, important biosynthetically as an analogue of "active sulphate," has been synthesised by Baddiley, Buchanan, and Letters, who allowed adenylic acid to react with a pyridine–sulphur trioxide complex,[1] and independently by Reichard and Ringertz who condensed adenylic acid and inorganic sulphate using dicyclohexylcarbodi-imide.[2]

[1] J. Baddiley, J. G. Buchanan, and R. Letters, *J.*, 1957, 1067.
[2] P. Reichard and N. R. Ringertz, *J. Amer. Chem. Soc.*, 1957, **79**, 2025.

In our studies on 1-alkoxyvinyl esters of various acids we have investigated the reaction of benzyl hydrogen sulphate [3] as the pyridinium salt with ethoxyacetylene in methylene chloride solution. After four hours at room temperature, the presence of the intermediate (Ia) was demonstrated by the observation in the infrared spectrum of the double peak

$CH_2{=}C(OEt){\cdot}O{\cdot}SO_3R$
(Ia); $R = CH_2Ph$
(Ib); $R = CH_2{\cdot}CH_2{\cdot}CN$



(II)

(1725, 1650 cm.$^{-1}$) which is characteristic of the 1-alkoxyvinyl esters of carboxylic, thiolic, and phosphoric acids.[4]

The crude product (Ia) was allowed to react with pyridinium 5′-adenylate and the resulting benzyl ester of the sulphatophosphate (II; $R = CH_2Ph$) was debenzylated catalytically to produce adenosine-5′ sulphatophosphate (II; $R = H$) in 35% yield, which was isolated by chromatography. The 2-cyanoethyl ester (II; $R = CH_2{\cdot}CH_2CN$) was prepared similarly. However, 2-cyanoethyl sulphate, isolated as the crude barium salt, could not be freed from inorganic contamination, although infrared and nuclear magnetic resonance spectroscopy confirmed its structure. Synthesis of the sulphatophosphate by this route was nevertheless preferred since the yield was higher and the starting material was easier to prepare.

The cyanoethyl group in (II; $R = CH_2{\cdot}CH_2CN$) was finally removed by treatment with alkali and the required sulphatophosphate isolated in 45% yield.

Experimental

Ethoxyacetylene was obtained from Koch–Light Laboratories and distilled before use.

Sodium benzyl sulphate was prepared by a modification of Bacon and Doggart's method.[3] The emulsion resulting from the reaction in chloroform of chlorosulphonic acid and benzyl alcohol in the presence of triethylamine was washed twice with water. The chloroform layer was then neutralised with 30% aqueous sodium hydroxide. The white precipitate was filtered and recrystallised twice from 95% ethanol to give silvery leaflets of sodium benzyl sulphate, m. p. 194—196° (lit., 183·5—184·5°) (Found: C, 40·1; H, 3·7; S, 14·7. Calc. for $C_7H_7O_4SNa$: C, 40·0; H, 3·4; S, 15·25%).

Barium-2-cyanoethyl Sulphate.—Chlorosulphonic acid (16·4 g.) and dry ether (150 ml.) were cooled to −12°, and ethylene cyanohydrin (10 g.) in pyridine (22·4 g.) was added, the temperature of the stirred solution being held below −10°. This temperature was maintained for 1 hr. after which the ether was decanted leaving a white sludge. Water was added to the latter until a clear solution resulted, and to this cooled solution barium hydroxide (12·1 g. as a saturated solution) was added. The resulting solution was evaporated to about 20 ml. under reduced pressure. The solution was filtered and the filtrate evaporated to dryness. The resulting white solid was recrystallised three times from aqueous ethanol and chromatographed on alumina with gradient elution in ethanol–water. The analysis figures were consistently lower than those calculated for barium 2-cyanoethyl sulphate, but infrared (ν_{as} SO_2 1450, ν_s SO_2 1200, ν CN 2270 cm.$^{-1}$) and nuclear magnetic resonance data (two triplets at τ 5·6 and 6·9) indicated its presence, probably contaminated by barium sulphate.

Adenosine-5′ Sulphatophosphate (*a*).—Sodium benzyl sulphate (415 mg.) was converted into it pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column. The eluant was evaporated to a gum under reduced pressure; this was dissolved in dry pyridine (10 ml.) and the solution evaporated once again to a yellow gum. This process was repeated, and the resulting material was dissolved in dry methylene chloride (3 ml.). This solution was added during 30 min. to a cooled (0°) and stirred solution of ethoxyacetylene (430 mg.) in methylene chloride (2 ml.). After 3 hr. at room temperature, excess of ethoxyacetylene and solvents were evaporated off under reduced pressure. An infrared spectrum of

[3] R. G. R. Bacon and J. R. Doggart, *J.*, 1960, 1332.
[4] H. H. Wasserman and P. S. Wharton, *Tetrahedron*, 1598, **3**, 321; *J. Amer. Chem. Soc.*, 1960, **82**, 661; H. H. Wasserman and D. Cohen, *ibid.*, p. 4435; G. R. Banks and D. Cohen, *Proc. Chem. Soc.*, 1963; D. Cohen and H. D. Springall, "Fifth European Peptide Symposium," Pergamon, Oxford, 1963, p. 73.

the remaining gum exhibited peaks at 1725 and 1650 cm.$^{-1}$ characteristic of (Ia). As attempts to purify this product by distillation under reduced pressure were unsuccessful, it was used in this crude state for the preparation of the benzyl adenosine-5′ sulphatophosphate.

Adenosine-5′ phosphoric acid (20 mg.) was converted into its pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column, and the eluant evaporated to dryness under reduced pressure. The product was dissolved as far as possible in dry methylene chloride (5 ml.) and dimethylformamide (5 ml.) and the crude ethoxyvinyl benzyl sulphate (40 mg.) in dry methylene chloride (4 ml.) was added. The yellow solution which resulted on shaking was left in the dark in a tightly stoppered flask for 5 days at room temperature. Solvents were then removed by evaporation under reduced pressure and the remaining residue was dissolved in water (20 ml.) and ethanol (1 ml.). The benzyl group was removed by catalytic hydrogenation with 10% palladium–charcoal catalyst. After the theoretical amount of hydrogen was taken up, the catalyst was filtered off and the filtrate evaporated to small volume. Chromatography and electrophoresis of this solution indicated formation of adenosine-5′ sulphatophosphate in 35% yield.

Estimation of the phosphorus content. The adenosine-5′ sulphatophosphate spot resulting from chromatographic separation using system *B* was cut out and the nucleotide eluted from the paper. The phosphorus content was estimated colorimetrically as phosphomolybdate,[5] the total nucleotide concentration being estimated by ultraviolet absorption (Found: P, 7·1. $C_{10}H_{16}N_5O_{10}PS$: P, 7·25%).

Adenosine-5′ Sulphatophosphate (*b*).—The crude barium-2-cyanoethyl sulphate (210 mg.) was converted into the pyridinium salt by passage through an Amberlite IR 120 (pyridinium form) ion-exchange column. Evaporation of the eluant under reduced pressure gave a yellow oil, which was dissolved in dry pyridine. The solution was again evaporated to an oil and this process was repeated, the oil being finally dissolved in dry methylene chloride (4 ml.). This solution was added during 30 min. to cooled (0°) and stirred ethoxyacetylene (470 mg.) in dry methylene chloride (3 ml.). The resulting orange solution was stirred at room temperature for 3 hr. after which solvents and excess of ethoxyacetylene were removed under reduced pressure. The remaining oil showed peaks in the infrared spectrum at 1740 and 1660 cm.$^{-1}$ characteristic of (Ib).

Adenosine-5′ phosphoric acid (21 mg.) as its pyridinium salt was dissolved as far as possible in dry methylene chloride (2 ml.) and dimethylformamide (2 ml.), and to this solution the above ethoxyvinyl ester (100 mg.) in dry methylene chloride (2 ml.) was added. The resulting solution was kept in the dark with exclusion of moisture for 5 days. Solvent was evaporated under reduced pressure and 0·5N-aqueous sodium hydroxide (5 ml.) was added to the remaining oil. The resulting solution was maintained at 100° for 4 min., samples (0·25 ml.) being extracted at one minute intervals. Each sample was immediately cooled in an ice-bath and neutralised with Amberlite IR 120 (H^+ form) ion-exchange resin. Chromatography in systems A and B followed by elution of $\frac{1}{4} \times 1$ inch strips of the paper parallel to the direction of development indicated formation of adenosine-5′ sulphatophosphate in 45% yield after treatment with the sodium hydroxide for 2 min.

Chromatography.—Ascending-front paper chromatography was carried out on Whatman No. 4 paper, and nucleotides were located by inspection under ultraviolet light. Adenosine-5′ sulphatophosphate was also prepared by the method of Baddiley *et al.*, and used as a marker. System A: n-propyl alcohol–ammonia (*d* 0·88)–water (6 : 3 : 1 v/v); System B: isobutyric acid–0·5N-ammonia (5 : 3 v/v).

	Chromatography		Electrophoresis at pH 5·5 3 hr./500v	
	System A	System B	cm. toward anode	cm. toward cathode
Adenosine-5′-phosphate	0·16	0·53	3·0	
Adenosine-5′ sulphatophosphate.....	0·25	0·38	5·5	
Adenosine	0·63	0·73		0·7
Adenosine-5′ sulphatophosphate (2′,3′)-sulphate (?) *	—	0·23	8·1	
Unknown cpd.	0·80	0·84		8·2

* The presence of two sulphate groups per nucleoside base was demonstrated by elution of the spots and quantitative precipitation of the sulphate released by acid hydrolysis.

[5] R. J. L. Allen, *Biochem. J.*, 1940, **34**, 858.

Electrophoresis.—This was done on Whatman No. 4 paper using 0·05M-ammonium acetate buffers at pH 5·5 and 8·8.

We thank the D.S.I.R. for a Research Studentship (G. R. B.) and Professor J. Baddiley for helpful suggestions.

UNIVERSITY OF KEELE, STAFFORDSHIRE. [*Received, April 26th,* 1965.]