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STUDIES ON BOVINE ADRENAL ESTROGEN SULFOTRANSFERASE

III. FACILE SYNTHESIS OF 3′-PHOSPHO- AND 2′-PHOSPHOADENOSINE 5′-PHOSPHOSULFATE

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Summary

A practical synthesis of 3′-phosphoadenosine 5′-phosphosulfate (IV) in yields of 68—72% from adenosine 2′,3′-cyclic phosphate 5′-phosphate (II) is described. Reaction of II with triethylamine-*N*-sulfonic acid affords adenosine 2′,3′-cyclic phosphate 5′-phosphosulfate (III) which, on treatment with ribonuclease-T_2, provides IV. Spleen phosphodiesterase, on the other hand, converts III to 2′-phosphoadenosine 5′-phosphosulfate (V).

The biological activity of IV, measured by sulfate transfer to [6,7-$^3H_2$]estrone as mediated by bovine adrenal estrone sulfotransferase (3′-phosphoadenylylsulfate:estrone 3-sulfotransferase, EC 2.8.2.4), is identical with that obtained with a sample of IV prepared by an established biochemical procedure. By contrast, V exhibits approximately one-third the activity of the natural isomer.

Introduction

A wide variety of sulfate esters are found in nature for which a corresponding spectrum of sulfotransferases of differing specificities are needed for sulfation of such diversified types of substrates as phenols, steroids, *N*-arylhydroxylamines and glycosides (for a summary of pertinent references see ref. 1). The source of active sulfate in these transfer reactions is in all cases 3′-phosphoadenosine 5′-phosphosulfate (IV) [1].

The pursuit of specificity studies of the sulfotransferases is handicapped by the lack of a suitable synthesis to provide an adequate supply of pure IV.

† Deceased.

Enzymic preparations of active sulfate from ATP are tedious, time-consuming and convenient for only small quantities [2]. On the other hand, the lengthy chemical procedure reported by Cherniak and Davidson [3] affords a product in 43% overall yield but contains approx. 5% of the isomeric 2'-phosphoadenosine-5'-phosphosulfate (V) in addition to a trace of adenosine 2'(3'),5'-diphosphate. The present communication describes an expeditious approach to pure IV and V, which serves, in addition, as a prototype for the synthesis of analogs of IV.

Materials and Methods

Bovine spleen phosphodiesterase II (EC 3.1.4.18) and ribonuclease T_2 were purchased from Sigma Chemical Co. Bovine adrenal estrogen sulfotransferase (EC 2.8.2.4) was isolated and purified as descibed by Adams and co-workers [4]. [^{35}S]PAPS, and [6,7-$^{3}H_2$]estrone were purchased from New England Nuclear Corp.

Thin-layer chromatography was performed in System S_1: saturated $(NH_4)_2SO_4$/0.1 M ammonium acetate/2-propanol (79 : 19 : 2, v/v), and in System S_2: 1-propanol/conc. NH_4OH/H_2O (6 : 3 : 1, v/v) on pre-coated cellulose sheets (Polygram Cel 300 UV_{254}, Machery Nagel). Paper electrophoresis was performed in a Savant high voltage electrophoresis apparatus on Whatman No. 1 paper in Solvent E_1: 0.02 M Na_2HPO_4, pH 7 at 30 V · cm^{-1} for 1.5 h. Thin-layer electrophoresis was performed in a Brinkmann Desaga apparatus on pre-coated cellulose F plates (E. Merck) in Solvent E_2: 0.025 M sodium citrate, pH 5.4 at 15 V · cm^{-1} for 2 h. R_F values and electrophoretic mobilities are summarized in Table I.

Synthetic

Adenosine 2',3'-cyclic phosphate 5'-phosphate. This compound was prepared from adenosine (compound I, 0.67 g, 2.5 mmol) by a modification of the method of Simoncsits and Tomasz [5,6]. After hydrolysis of the reaction mixture with 1.0 M triethylammonium bicarbonate in the manner described [6],

TABLE I

THIN-LAYER CHROMATOGRAPHY AND ELECTROPHORESIS OF COMPOUNDS

Compound	R_F		Mobility *	
	S_1	S_2	E_1 **	E_2 ***
Adenosine 5'-phosphate	—	—	0.58	0.19
II	0.33	0.32	0.82	0.72
III	0.33	0.48	0.99	—
Adenosine 3', 5'-diphosphate	0.50	0.15	0.85	0.59
IV	0.53	0.27	1.00	1.00
V	0.56	0.24	1.00	1.08
Adenosine 2', 5'-diphosphate	0.59	0.15	0.85	—

* Relative to mobility of compound IV.

** Paper electrophoresis.

*** Thin-layer electrophoresis.

the buffer was removed by concentration to a small volume, followed by several evaporations with ethanol under reduced pressure at 30°C. The white residue was dissolved in 37.5 ml of 0.05 M triethylammonium bicarbonate and stored at −15°C. A 6 ml portion of the crude product (5525 A_{259} units *) was chromatographed at 4°C on a 2.5 · 35 cm column of DEAE-Sephadex A-25 with a linear gradient of 2 l each of 0.05—1.0 M triethylammonium bicarbonate, pH 7.5. Fractions (approx. 20 ml each) 78—97 contained 3800 A_{259} units (62% yield **) of compound II. The buffer was removed in the usual manner, and the product was frozen for storage in 5 ml of H_2O.

Adenosine 2',3'-cyclic phosphate 5'-phosphosulfate (compound III). To a solution of 2890 A_{259} units of compound II in 9.1 ml of ethanol was added 0.34 ml of trioctylamine. The ethanol was removed under reduced pressure and the residue was rendered anhydrous by repeated evaporation from dimethylformamide at the oil pump. To the residue was added 3 ml of dimethylformamide, 3 ml of dioxane and 0.6 ml of pyridine and the mixture was shaken for 5 min. Then, 110 mg of triethylamine-*N*-sulfonic acid *** [3] was added, and the reaction mixture was sealed and shaken overnight at room temperature. A thin-layer chromatogram in S_2 indicated that the major product was compound III. The solvents were removed at the oil pump and the residue was suspended in 6 ml of ice-cold H_2O containing 0.2 ml of 1.5 M NH_4OH. The mixture was adjusted to pH 7 and applied to a Sephadex G-10 column (2.6 · 40 cm), which had been pre-equilibrated at room temperature with 0.1 M triethylammonium bicarbonate in 20% ethanol. Elution was continued until 60 ml had been collected. Six 20 ml fractions then were collected and fractions 1—4 were pooled to give 2880 A_{259} units. Buffer was removed in the usual manner. To the residue was added 5 ml of ice-cold H_2O and the pH was adjusted to 5.9 with 1.5 M NH_4OH. Crude compound III was used in the enzymatic transformations without further purification.

3'-Phosphoadenosine 5'-phosphosulfate, (compound IV). To the solution of compound III was added 0.2 ml (100 units) of an aqueous solution of ribonuclease T_2. After 20 h at room temperature a thin-layer chromatogram in System S_2 showed complete conversion of compound III to compound IV. Chromatography on DEAE-Sephadex A-25 in the manner described for compound II gave three major peaks. Peak 1 (fractions 104—122) contained 623 A_{259} units (22%) of adenosine 3',5'-diphosphate ($\lambda_{max}^{H_2O}$ = 259 nm). Peak 2 (fractions 140—159) contained 1932 A_{259} units (68%) of compound IV. Peak 3 (fractions 166—180) contained 196 A_{259} units (7%) of an unidentified compound, which exhibited an anomalous ultraviolet spectrum ($\lambda_{max}^{H_2O}$ = 262 nm).

Fractions containing compound IV were pooled and the buffer was removed in the usual manner. The product was redissolved in approx. 3 ml of H_2O and the solution was adjusted to pH 7 with dilute NH_4OH. Treatment of a small aliquot of the latter with an equal volume of 0.2 M HCl at 37°C for 1.75 h gave adenosine 3',5'-diphosphate as the sole detectable product in Systems S_1 and S_2. Compound IV was homogeneous in S_1, S_2, E_1 and E_2. It exhibited a typical

* One A_{259} unit is that amount of material in 1 ml of solution that has an absorbance of 1.0 when it is measured with a 1.0 cm optical path at 259 nm.

** Yield based on adenosine and $\epsilon_{max}^{H_2O}$ = 15 400 for AMP. See ref. 7.

*** Triethylamine-*N*-sulfonic acid has high oral toxicity. See ref. 8.

adenine nucleotide spectrum ($\lambda_{max}^{H_2O}$ = 259 nm, $\lambda_{min}^{H_2O}$ = 227), and it could be frozen for storage or kept indefinitely in 50% ethanol at −20° C.

2'-Phosphoadenosine 5'-phosphosulfate (compound V). To a solution of 790 A_{259} units of crude compound III in 2 ml of H_2O, adjusted to pH 6 with 1.5 M NH_4OH, was added 0.4 ml (9 units) of a solution of spleen phosphodiesterase II in 0.05 M potassium acetate, pH 6. After 18 h at room temperature, chromatography in S_1 and S_2 showed approx. 40% formation of compound V. An additional 4.5 units of enzyme were added and the mixture was kept at room temperature for 18 h to complete the reaction. Compound V was isolated in 89% yield following chromatography on DEAE-Sephadex A-25 in the usual manner. It was homogeneous in S_1, S_2, E_1 and E_2 and exhibited a typical adenine nucleotide spectrum (λ_{max} = 259 nm, λ_{min} = 227 nm). It moved slightly faster than compound IV in S_1 and E_2 but had nearly the same mobility as compound IV in Systems S_2 and E_1. After adjustment of pH to 7, compound V was frozen for storage at −15°.

Biochemical

Enzyme assay. Sulfation of estrone with synthetic compound IV was determined using the procedure described by one of us [2].

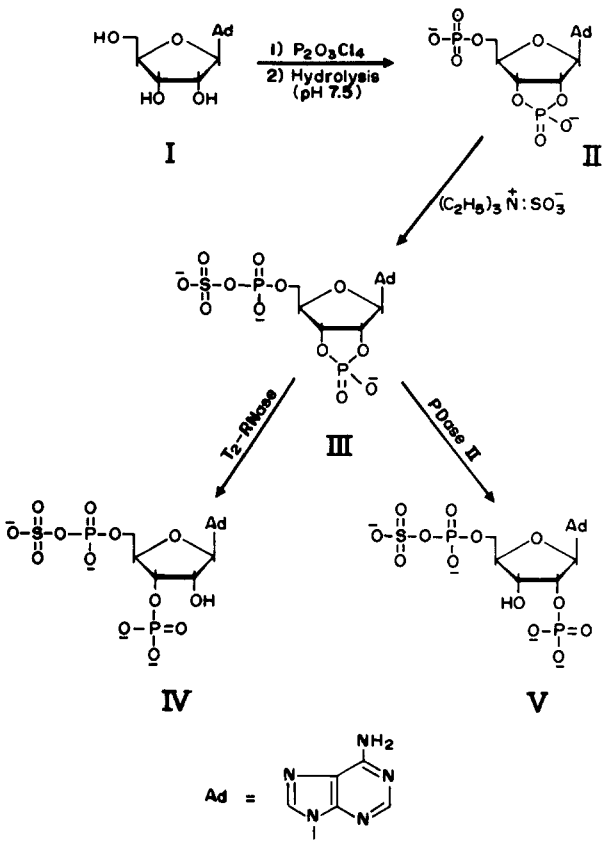
Sulfation by compound V. The ability of compound V to donate its sulfate to estrone was determined with [6,7-$^3H_2$]estrone ($1.3 \cdot 10^6$ dpm/4 nmol) by modification of the previously described method [2] in which compound V (0.11 mM) is now substituted for compound IV. After termination of the incubation, excess free [^{3}H]estrone was extracted three times with 0.3 ml of ethyl ether and the aqueous sample adjusted to 0.5 ml with redistilled methanol. An aliquot (25 µl) was applied, together with 15 µg of methanolic estrone sulfate marker, to type SG chromatography media (Gelman Instrument Co., Ann Arbor, Mich.) and developed with chloroform/acetone/acetic acid (110 : 35 : 6). Estrone sulfate was visualized by spraying with methylene blue [2], and the spot punched out with a cork borer and counted in 10 ml of Bray's dioxane scintillation solution [9]. Methylene blue did not affect counting efficiency. The amount of steroid sulfate ester produced was calculated utilizing the specific activity of [^{3}H]estrone.

Inhibition by V. The effect of compound V as an inhibitor of estrogen sulfotransferase was determined by adding the inhibitor (0.1 mM) to an incubation mixture containing 22 µM compound IV and [6,7-$^3H_2$]estrone ($1.3 \cdot 10^6$ dpm/22 nmol). Results are expressed as fractional inhibition [10].

Results and Discussion

The phosphorylation of adenosine (I) with pyrophosphoryl chloride followed by neutral buffered hydrolysis, as described by Simoncsits and Tomasz [5,6] coupled with the improved method of isolation, provides a convenient route to II, (cf. Scheme 1). Byproducts, which were presumed to include a series of adenosine 2',3'-cyclic phosphate 5'-polyphosphates by analogy with the results obtained in the phosphorylation of guanosine [6], were observed in the elution pattern of the reaction mixture that provided compound II.

Introduction of the 5'-phosphosulfate anhydride moiety to form com-



Scheme 1. Reaction sequence leading to 3′-phosphoadenosine-5′-phosphosulfate (compound IV) and 2′-phosphoadenosine 5′-phosphosulfate (compound V). PDase II, spleen phosphodiesterase II.

pound III was effected with triethylamine-*N*-sulfonic acid. Treatment of compound III with ribonuclease T_2 gave compound IV in yields of 68—72% (based on compound II) following column chromatography on DEAE-Sephadex A-25.

Spleen phosphodiesterase hydrolyzed compound III specifically to compound V. The latter moved slightly faster in both Systems S_1 and E_2 than did compound IV (cf. Table I), which is in accord with previous reports [5,6] that, of the two isomeric ribonucleoside diphosphates, the 2′,5′-derivative generally exhibits the higher R_F value in System S_1.

In agreement with the assigned structures it was found that acidic treatment of compound IV gave adenosine 3′,5′-diphosphate with no chromatographic indication in either S_1 or E_2 of the presence of the 2′,5′-isomer. The same conditions of hydrolysis when applied to compound V gave adenosine 2′,5′-diphosphate as the sole product.

Biological activity of compounds IV and V was determined by sulfate transfer to estrone in the presence of estrogen sulfotransferase. The activity of compound IV was virtually identical to that obtained with a sample of active sulfate derived via the enzymatic procedure. Compound V exhibited approximately 33% the activity of the natural isomer. Moreover, compound V showed

fractional inhibition of estrone sulfation (by competition with compound IV) of 0.33 (unity = 100% inhibition).

The availability of the sulfated cyclic nucleotide (compound III) has provided ready access to compounds IV and V via cleavage of the 2′,3′-cyclic phosphate ester with the appropriate enzyme. Analogs of compound IV, which are required for structure-activity studies, are accessible by the same synthetic approach. This work will be the subject of a subsequent report.

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