Biochimica et Biophysica Acta, 613 (1980) 85—94

BBA 68947

STUDIES ON BOVINE ADRENAL ESTROGEN SULFOTRANSFERASE

V. SYNTHESIS AND ASSAY OF ANALOGS OF 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULFATE AS COSUBSTRATES FOR ESTROGEN SULFURYLATION

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(Received April 11th, 1979)
(Revised manuscript received October 15th, 1979)

Key words: Estrogen sulfotransferase; Phosphoadenosine phosphosulfate analog; Sulfurylation; (Bovine adrenal)

Summary

Analogs of adenosine 3′-phosphate 5′-phosphosulfate (3′PAdo5′PS *) have been prepared by an extension of a method for the synthesis of 3′PAdo5′PS, itself. Reaction of the ribonucleoside 2′,3′-cyclic phosphate 5′-phosphate with triethylamine sulfonic acid leads to a ribonucleoside 2′,3′-cyclic phosphate 5′-phosphosulfate. The latter, on treatment with ribonuclease-T_2, provides the 3′-phosphate analog. Spleen phosphodiesterase, on the other hand, promotes opening of the 2′,3′-cyclic phosphate residue to a 2′-phosphate analog. In this manner, the 2′,3′-cyclic phosphates of 8-bromoadenosine-, nebularine-, inosine-, tubercidin-, and formycin-, 5′-phosphosulfates have been converted to corresponding 2′-, and 3′-phosphate analogs.

V for the utilization of 3′PAdo5′PS analogs in the sulfurylation of estrone, as mediated by bovine adrenal estrogen sulfotransferase (3′-phosphoadenylylsulfate : estrone 3-sulfotransferase, EC 2.8.2.4), is decreased more than 50% by the replacement of the 7-(ring)nitrogen atom by carbon or of the 8-H atom by bromine in the adenine moiety. However, binding of these analogs at the active site of the enzyme is still very efficient (small K_m and K_i values; competitive inhibition). The replacement of the 9-nitrogen atom of adenine by carbon lowers *V* even more, and the replacement of the 6-amino group by H, i.e., nebularine, or by 0 (inosine) results in 90 and 100% loss of enzyme activity,

* Abbreviations of the various compounds are listed in the Appendix on p. 93.

respectively. Shifting of the 3′-phosphate to the 2′ position in the ribose of adenosine is also detrimental and results in noncompetitive inhibition of 3′PAdo5′PS utilization.

Introduction

Studies with bovine adrenal estrogen sulfotransferase (3′-phosphoadenylyl sulfate:estrone 3-sulfotransferase, EC 2.8.2.4) indicated that 3′PAdo5′P blocks the sulfurylation of estrone by 3′PAdo5′PS [1,2]. On the basis of the sensitivity of the esterification to 3′PAdo5′P, a coproduct of all sulfotransferase reactions, efforts were made to identify some of the structural features essential to the reversible binding of this inhibitor to estrogen sulfotransferase [3]. This approach provided important guidelines in the design of corresponding analogs of 3′PAdo5′PS, which serve to characterize the cosubstrate specificity of the enzyme. The present report summarizes the results of modifications in the purine and sugar moiety of 3′PAdo5′PS and the corresponding enzyme kinetic data generated in the study of estrone sulfurylation by these analogs.

Materials and Methods

Estrone, estrone sulfate (recrystallized from methanol), ribonuclease T2 (EC 3.1.27.1), bovine spleen phosphodiesterase II, (orthophosphoric acid diester phosphohydrolase, EC 3.1.4.1), tubercidin and nebularine were purchased from Sigma Chemical Co. Formycin was obtained from Meija Seika Kaisha, Tokyo, Japan. [6,7-^{3}H]Estrone and [^{35}S]3′PAdo5′PS were purchased from New England Nuclear Corp. Bovine adrenal estrogen sulfotransferase was isolated and purified as described previously [2,4]. Pyrophosphoryl chloride was prepared by the method of Koransky et al. [5].

General

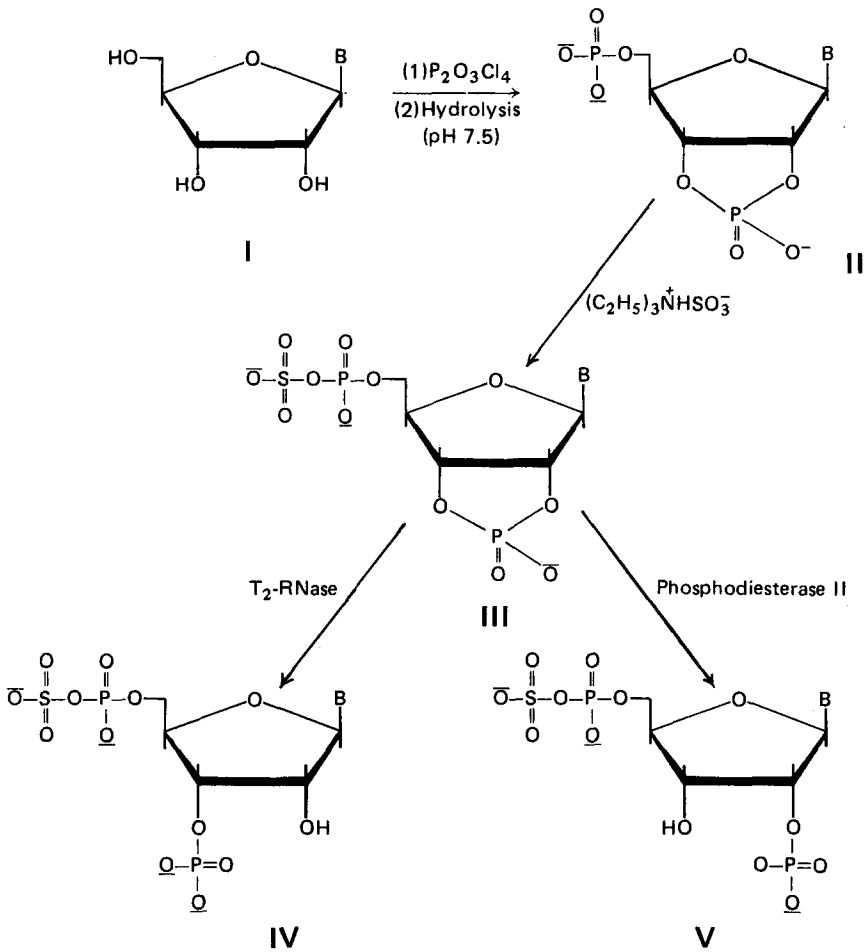
All evaporations were performed on a Büchi rotary evaporator at temperatures below 30° C. Thin-layer chromatography was carried out as described earlier [3,6] in solvent S_1: saturated $(NH_4)_2SO_4$/0.1 M ammonium acetate/2-propanol (79 : 19 : 2, v/v); solvent S_2: 1-propanol/conc. NH_4OH/H_2O (6 : 3 : 1, v/v) on precoated 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 for 1.5 h. Thin-layer electrophoresis was effected in a Brinkmann DeSaga apparatus on precoated cellulose F plates (E. Merck) in solvent E_2: 0.025 M sodium citrate (pH 5.4) at 15 V/cm for 2 h. R_F values and electrophoretic mobilities are summarized in Table I.

Ultraviolet absorption spectra were measured in a Model 14 Cary recording spectrophotometer. Phosphate content of the synthetic nucleotides was determined according to the method of Keleti and Lederer [7].

Synthetic

The preparation of the 3′-phosphoribonucleoside 5′-phosphosulfates (IV)



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(a) Adenine
(b) 8-Bromoadenine
(c) Purine
(d) Hypoxanthine
(e) 4-Aminopyrrole[2,3-*d*]pyrimidine (tubercidin)
(f) 7-Aminopyrazolopyrimidine (formycin)

Scheme I. Reaction sequence leading to analogs of 3'PAdo5'PS.

listed in Table I utilized an approach identical with that described recently [6] for the synthesis of 3'PAdo5'PS (IVa). Thus, treatment of a purine ribonucleoside (I) (Scheme I) with pyrophosphoryl chloride followed by neutralization of the reaction mixture with triethylammonium bicarbonate buffer (pH 7.5), provided the requisite intermediate nucleoside 2':3'-phosphate 5'-phosphate (II) in good yield (52—84%) after purification via column chromatography on DEAE-Sephadex A-25 and removal of the buffer [3]. Reaction of II with triethylamine sulfonic acid, as previously described [6], led to the correspond-

ing 2′ : 3′-phosphate 5′-phosphosulfate derivative (IIIb, c, d, e and f) in yields of 46—75%. The latter, on treatment with ribonuclease-T_2, gave the analogs of 3′PAdo5′PS i.e., IVb—f, in nearly quantitative yields following chromatography on DEAE-Sephadex A-25 with a linear gradient of 2 l each of 0.05—1.5 M triethylammonium bicarbonate, pH 7.5.

The positional isomers, 2′PTu5′PS (Ve) and 2′PFo5′PS (Vf) were obtained in 77 and 90% yield, respectively, by the action of spleen phosphodiesterase on IIIe and f in exactly the same manner as that described previously [6] for the conversion of IIIa to 2′PAdo5′PS (Va). Chromatography of Ve and Vf on DEAE-Sephadex A-25 (vide supra) provided products homogeneous on thin-layer chromatography in two systems (S_1 and S_2) and on electrophoresis (systems E_1 and E_2) (see Materials and Methods).

Biochemical

Assay of estrogen sulfotransferase. The incubation mixtures contained in a total volume of 0.2 ml: 0.19 M Tris-HCl buffer (pH 7.5 at 37°C), 200 μM dithiothreitol, 15 μg enzyme, 25—200 μM nucleotide, estrone at a saturation level (110 μM) and $5 \cdot 10^5$ dpm [6,7-^{3}H]estrone. The incubations were carried out in Kimax 2.5 ml glass-stoppered, conical, calibrated centrifuge tubes (±12% tolerance at the 0.4 ml volume). After a 10 min incubation at 37°C, followed by 1 min in a 100°C bath, the aqueous incubation mixtures were extracted with four 0.6-ml portions of diethyl ether. The residual ether was evaporated in a stream of N_2 and the volume of the aqueous sample was adjusted to 0.4 ml with methanol. A 20 μl sample of the latter was applied with 30 μg of a methanol-solution of marker-estrone sulfate to silica gel, instant thin-layer chromatography media (Gelman Instrument Co., Ann Arbor, MI) and developed with chloroform/acetone/acetic acid (110 : 35 : 6, v/v). After drying the sheet, the assay followed a previously published procedure [8].

In the inhibition experiments, the procedure for the enzyme assay was as described earlier (4), utilizing 15 μg enzyme, [^{35}S]3′PAdo5′PS, and unlabeled estrone.

Analysis of kinetic data. The initial velocity analysis was performed, maintaining the concentration of the estrogen at a saturation level. Only those nucleotides exhibiting the highest enzyme affinity were subjected to detailed kinetic analysis. Plots of data were made in the double reciprocal form ($1/v$ versus $1/[S]$) and in the form of a Hofstee plot ($v/[S]$ versus v). In the case of weak affinity nucleotides, the sulfate transfer potential was estimated in terms of a relative sulfurylation rate by measuring the reaction rate at one concentration (200 μM) and expressing it relative to the sulfate transfer potential of 3′PAdo5′PS at the same concentration. The relative sulfurylation rate of the latter is defined as unity (1.00) at 200 μM concentration.

In the inhibition experiments, the estrogen concentration was again set at a saturation level, whereas the cosubstrate concentration was varied and the inhibitor was maintained at several fixed levels. Plots of the data were made in the double reciprocal mode ($1/v$ versus $1/[S]$), in the form of replots of slopes or intercepts versus the inhibitor concentration and as Dixon plots, i.e., $1/v$ versus inhibitor concentration, to determine the inhibition pattern and the inhibition constant, K_i.

Results and Discussion

The synthetic approach to analogs of 3'PAdo5'PS (IVa) represents a successful extension of the methodology developed previously [6] for a practical synthesis of active sulfate; i.e., IVa. Thus, each ribonucleoside 2',3'-cyclic phosphate 5'-phosphate (II), derived from the action of pyrophosphoryl chloride on the precursory nucleoside (I) [3,6] was converted to the corresponding 5'-phosphosulfate derivative (III) by the action of triethylamine sulfonic acid. Regiospecific cleavage of the cyclic phosphate moiety in III to the desired ribonucleoside 3'-phosphate 5'-phosphosulfate (IV) was acchieved with ribonuclease-T_2.

The fact that the λ_{max} values for each of the ribonucleoside 3', [and 2'- (vide infra)] phosphate 5'-phosphosulfate derivatives (see Table 1) are identical with those of the corresponding nucleoside 3'(2'),5'-diphosphates [6] precludes any possibility of sulfurylation having occurred instead in the aglycon moiety.

When the cyclic phosphate intermediate was treated instead with bovine spleen phosphodiesterase (II), then in accord with our previous studies [3,6], which in turn are based upon a suggestion by Michelson [9], opening of the cyclic phosphate residue affords the positional isomeric ribonucleoside 2'-phosphate 5'-phosphosulfate. Accordingly, 2'PTu5'PS (Ve) was obtained from IIIe in a manner identical to that described for the conversion of IIIa to the isomer of active sulfate (Va) [6].

Certain modifications of the imidazole ring of IVa were tolerated by the enzyme. For example, replacement of the 8-proton by bromine, as in 3'P8-

TABLE I

PHYSICAL CONSTANTS OF SOME ANALOGS OF ADENOSINE 3'- AND 2'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE

Compound	Structure No.	pH 7					
		λ_{max} (nm)	Phosphate [a]/ Nucleoside	R_F		Electrophoretic mobility	
				S_1	S_2	E_1 [b]	E_2 [c]
8-Bromoadenosine 3'-phosphate 5'-phosphosulfate	IVb	265	1.92 ± 0.04	0.36	0.26	1.79	0.86
Tubercidin 3'-phosphate 5'-phosphosulfate	IVe	270	1.96 ± 0.06	0.60	0.21	1.75	0.83
Tubercidin 2'-phosphate 5'-phosphosulfate	Ve	270	1.98 ± 0.02	0.66	0.16	1.94	0.94
Formycin 3'-phosphate 5'-phosphosulfate	IVf	295	1.90 ± 0.01	0.56	0.15	2.13	0.80
Formycin 2'-phosphate 5'-phosphosulfate	Vf	295	2.03 ± 0.03	0.62	0.14	2.17	0.87
Inosine 3'-phosphate 5'-phosphosulfate	IVd	248	2.01 ± 0.04	0.75	0.23	1.92	1.07
Nebularine 3'-phosphate 5'-phosphosulfate	IVc	262	1.90 ± 0.01	0.61	0.34	1.84	1.06

[a] Phosphate was determined according to [7]. Inorganic phosphate was in each case $<<5\%$.
[b] Relative to 5'AMP = 1.00.
[c] Relative to adenosine 3'-phosphate 5'-phosphosulfate = 1.00.

BrAdo5′PS (IVb), or of the 7-nitrogen by carbon, as in 3′PTu5′PS (IVe), reduced the V by about 50% relative to 3′PAdo5′PS (cf. Table II). Furthermore, the decrease in K_m for analogs IVb and IVe indicated that these are bound more tightly to the enzyme than active sulfate (3′PAdo5′PS). This conclusion is substantiated by the fact that the inhibition constants, K_i values for IVb and IVe, 8 and 8.5 μM, respectively, are lower than the K_m for 3′PAdo5′PS (cf. Table II).

Since it is now believed that most enzyme-catalyzed reactions involve two or three enzyme substrate complexes,

$$E + S \rightleftharpoons ES \rightleftharpoons EZ \rightleftharpoons EP \rightleftharpoons E + P$$

in which EZ is the true transition-state complex and EP an enzyme-product complex [10], the kinetic data can be explained by preferential formation of the ES complex by analogs IVb and IVe resulting in strong inhibition. By the same logic, the EZ complex is formed preferentially by IVa as indicated by its higher V_{max}.

Other structural variations in the imidazole ring, for example, as represented by C-nucleotide, 3′PFo5′PS (IVf), lead to a cosubstrate with a modest capacity to sulfurylate estrone as indicated in Table II. The decreased affinity of this analog for the enzyme is also reflected in the larger K_i value (270 μM).

The present findings indicate that 3′PTu5′PS and 3′P8-BrAdo5′PS are bound to the same enzymic site to which 3′PAdo5′PS and the corresponding desulfurylated nucleotides bind since all of these analogs are competitive inhibitors of the natural cosubstrate, as shown in Table II and Fig. 1. There is, however, some uncertainty in the interpretation of the inhibition data obtained with the weak competitor, 3′PFo5′PS, although there is indication that this nucleotide and its coproduct are non-competitive inhibitors of 3′Ado5′PS (cf. Fig. 2). In this connection, it is of interest that the inhibition of 3′PTu5′PS and 3′P8-BrAdo5′PS by the corresponding coproducts, i.e., 3′PTu5′P and 3′P8-BrAdo5′P, respectively, is also competitive (K_i = 55 and 40 μM, respectively, data not shown).

Contrary to the effect observed with changes in the imidazole ring, replacement of the 6-amino group of adenine by hydrogen as in 3′PPuo5′PS, or by oxygen as in 3′PIno5′PS, results in a substantial loss of enzyme affinity (cf. Table III). The utilization of these two cosubstrate analogs is 10 and 0%, respectively, relative to 3′PAdo5′PS at 200 μM nucleotide concentration.

As mentioned above, the cosubstrates and coproducts listed in Table II, possibly with the exception of the poor cosubstrate IVf and its desulfurylated coproduct, are bound to the same enzymic site. However, it remains unclear just how the enzyme can accommodate nearly equally well the dissimilar glycosyl-base torsion angles [3] subtended by the sugar-base moieties in the populations comprising IVa which is, on the one hand, predominantly *anti* whereas the population of IVb is, by contrast, principally *syn*. The same concern attends the corresponding coproducts, i.e., the nucleoside 3′,5′-diphosphates.

The positional isomer of active sulfate, 2′PAdo5′PS (Va) shows approximately one-third the activity of the natural donor [6]. This finding was somewhat surprising in view of the observation [3] that the coproduct 2′PAdo5′P is devoid of activity when assayed at a single low concentration (200 μM). How-

TABLE II

KINETIC PARAMETERS OF SOME ANALOGS OF ADENOSINE 3′-PHOSPHATE 5′-PHOSPHOSULFATE IN THE ENZYMATIC SULFURYLATION OF ESTRONE

Sulfurylated nucleotides (Cosubstrates)	Structure	V (nmol/min per mg)	K_m (μM)	K_i (μM)/type of inhibition	Desulfurylated nucleotides (cosubstrates)	K_i [a] (μM)/type of inhibition
Adenosine 3′-phosphate 5′-phosphosulfate	IVa	5.0	44 ± 5 [b]	—	Adenosine 3′, 5′-diphosphate	7.0/Competitive
Tubercidin 3′-phosphate 5′-phosphosulfate	IVe	2.3	21 ± 13	8.5/Competitive	Tubercidin 3′, 5′-diphosphate	0.6/Competitive
8-Bromoadenosine 3′-phosphate 5′-phosphosulfate	IVb	2.0	18 ± 12	8.0/Competitive	8-Bromoadenosine 3′, 5′-diphosphate	1.7/Competitive
Formycin 3′-phosphate 5′-phosphosulfate	IVf	1.2	130 ± 28	270/Noncompetitive	Formycin 3′, 5′-diphosphate	43/Noncompetitive

[a] K_i values taken from Tables II and III of Ref. 3. The type of inhibition was determined in the present study.
[b] Means ± S.D.

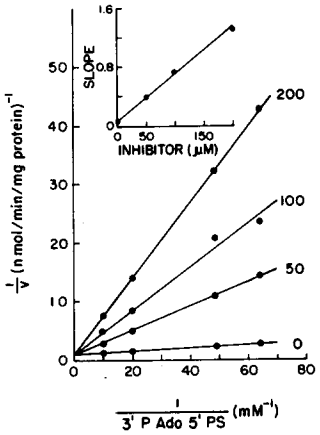

Fig. 1. Competitive inhibition of 3'PAdo5'PS by 3'P8-BrAdo5'PS. Standard enzyme assay employed, except that [^{35}S] 3'PAdo5'PS was used ($2.5 \cdot 10^5$ cpm) as the source of label. Numbers at the end of the lines represent inhibitor concentration (μM).

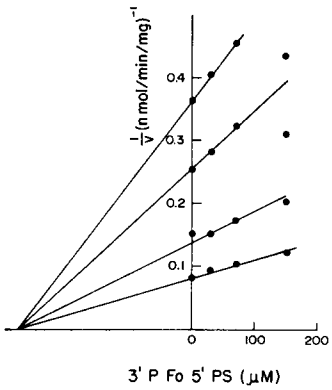

Fig. 2. Dixon plot for noncompetitive inhibition of 3'PAdo5'PS by 3'PFo5'PS. The inhibitor was at 30, 70 and 150 μM concentration. Standards enzyme assay employed, except that [^{35}S] 3'PAdo5'PS was used ($1.8 \cdot 10^5$ cpm) as the source of label.

ever, when tested at higher concentrations (2.4, 4.8 and 9.5 mM) weak inhibition of the noncompetitive type could be demonstrated (K_i = 12.9 mM) for the 2'-phosphate isomer. Of the several ribonucleoside 2',5'-diphosphates examined to date, only 2'PTu5'P has an appreciable effect on the estrogen sulfotransferase reaction and it is also a noncompetitive inhibitor. In fact, the K_i of the

TABLE III

RELATIVE SULFURYLATION RATES OF SOME ANALOGS OF ADENOSINE 3'-PHOSPHATE 5'-PHOSPHOSULFATE AND INHIBITION CONSTANTS OF COPRODUCTS

Sulfurylated nucleotides (cosubstrates)	Structure	Relative sulfurylation rate [a]	Desulfurylated nucleotides (coproducts)	K_i (μM)/-type of inhibition
Adenosine 3'-phosphate 5'-phosphosulfate	IVa	1.00	Adenosine 3', 5'-diphosphate	7 [b]/Competitive
Adenosine 2'-phosphate 5'-phosphosulfate	Va	0.29 ± 0.024	Adenosine 2', 5'-diphosphate	12 900/Noncompetitive
Tubercidin 2'-phosphate 5'-phosphosulfate	Ve	0.18 ± 0.013	Tubercidin 2', 5'-diphosphate	5 [b]/Noncompetitive
Nebularine 3'-phosphate 5'-phosphosulfate	IVc	0.19 ± 0.10	Nebularine 3', 5'-diphosphate	68 [b]/c
Formycin 2'-phosphate 5'-phosphosulfate	Vf	0.06 ± 0.01	Formycin 2', 5'-diphosphate	d
Inosine 3'-phosphate 5'-phosphosulfate	IVd	0	Inosine 3', 5'-diphosphate	800 [b]/c

[a] The relative sulfurylation rate [4] is a measure of utilization of cosubstrate analogs as compared to adenosine 3'-phosphate 5'-phosphosulfate (velocity of utilization of 3'PAdo5'PS at 200 μM concentration is taken as 1.00/). Means ± S.D.

[b] K_i Values taken from Tables II and III of Ref. 3. The type of inhibition determined in present study.

[c] Type of inhibition not determined.

[d] K_i not determined.

latter compares favorably with that of 3'PAdo5'P (cf. Table II). The relative sulfurylation rate of 2'PTu5'PS (Ve), on the other hand, is relatively low — approximately the same as that of Va.

In those cases where the relative binding of the cosubstrates and the corresponding coproducts was investigated (cf. Table II), the desulfurylated coproducts were found to bind more tightly than active sulfate or its analogs to estrogen sulfotransferase. It is significant, in this connection, that tighter binding of 3'PAdo5'P, relative to that of 3'PAdo5'PS, has also been observed with several other sulfotransferases for which this property has been investigated. Thus, 'preference' for 3'PAdo5'P has been reported for phenol sulfotransferase [11], 3-β-hydroxysteroid sulfotransferase [12] and bile salt sulfotransferase [13].

It is reasonable to expect that the specificity and inhibition properties of 3'PAdo5'PS analogs reported in this work will also be observed for other sulfotransferases. In fact, corresponding studies with β-hydroxysteroid sulfotransferase are currently in progress in our laboratory and the results will be the subject of a subsequent report.

Appendix

The following abbreviations have been used in the text:
2'PAdo5'P, adenosine 2',5'-diphosphate;
2'PAdo5'PS, adenosine 2'-phosphate 5'-phosphosulfate;
3'PAdo5'P, adenosine 3',5'-diphosphate;
3'PAdo5'PS, adenosine 3'-phosphate 5'-phosphosulfate;
2':3'PAdo5'P, adenosine 2',3'-cyclic phosphate 5'-phosphate;
2':3'PAdo5'PS, adenosine 2',3'-cyclic phosphate 5'-phosphosulfate;
3'P8-BrAdo5'P, 8-bromoadenosine 3',5'-diphosphate;
3'P8-BrAdo5'PS, 8-bromoadenosine 3'-phosphate 5'-phosphosulfate;
3'PFo5'P, formycin (7-amino-3-(β-D-ribofuranosyl)-pyrazolo-[4,3-*d*]-pyrimidine) 3',5'-diphosphate;
3'PFo5'PS, formycin 3'phosphate 5'-phosphosulfate;
3'PIno5'P, inosine 3',5'-diphosphate;
3'PIno5'PS, inosine 3'-phosphate 5'-phosphosulfate;
3'PPuo5'P, nebularine (9-β-D-ribofuranosylpurine) 3',5'-diphosphate;
3'PPuo5'PS, nebularine 3'-phosphate 5'-phosphosulfate;
3'PTu5'P, tubercidin (4-amino-7-(β-D-ribofuranosyl)-7H-pyrrole-[2,3-*d*]-pyrimidine) 3',5'-diphosphate;
3'PTu5'PS, tubercidin 3'-phosphate 5'-phosphosulfate;
2'PTu5'PS, tubercidin 2'-phosphate 5'-phosphosulfate;
Estrone, 1,3,5(10)-estratriene-3-ol-17-one.

Acknowledgements

This investigation was supported in part by U.S. Public Health Service Research Grant No. CA-16716, CA-16855 and CA-23079 from the National Cancer Institute and in part by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.

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