Biochimica et Biophysica Acta, 525 (1978) 364—372
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BBA 68502

IV. SYNTHESIS AND ASSAY OF ANALOGS OF ADENOSINE 3′,5′-DIPHOSPHATE AS INHIBITORS OF BOVINE ADRENAL ESTROGEN SULFOTRANSFERASE *

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(Received January 30th, 1978)

Summary

Analogs of adenosine 3′,5′-diphosphate are described which aid in the characterization of the inhibition of estrone sulfurylation by 3′-phosphoadenosine 5′-phosphosulfate as mediated by bovine adrenal estrogen sulfotransferase (3′-phosphoadenylylsulphate:estrone 3-sulphotransferase, EC 2.8.2.4).

The facile conversion of ribonucleosides to 2′,3′-cyclic phosphate 5′-phosphate in neat pyrophosphoryl chloride is utilized to provide a reliable route to the requisite intermediates for enzymatic regiospecific conversion to ribonucleoside 3′,5′- and 2′,5′-diphosphates.

The importance of the 3′-phosphate ester to inhibition of estrone sulfurylation is confirmed by K_i measurements. Replacement of the 6-amino group by hydrogen or oxygen leads to considerable loss in affinity for the enzyme as does also dimethylation of the exocyclic amino group. Alterations in the pyrimidine ring are not well tolerated by the sulfotransferase but modifications in

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Abbreviations: 3′-AMP, adenosine 3′-phosphate; 2′:3′PAdo5′P, adenosine 2′,3′-cyclic phosphate 5′-phosphate; 2′:3′PGuo5′P, guanosine 2′,3′-cyclic phosphate 5′-phosphate; 3′PAdo5′P, adenosine 3′,5′-diphosphate; 3′P2Ado5′P, adenosine 3′-diphosphate 5′-phosphate; 3′PAdo5′P2, adenosine 3′-phosphate 5′-diphosphate, Ado5′S, adenosine 5′-sulfate; Ado5′PS, adenosine 5′-phosphosulfate; 3′PAdo5′PS, 3′-phosphoadenosine 5′-phosphosulfate; 3′PGuo5′P, guanosine 3′,5′-diphosphate; 3′PIno-5′P, inosine 3′,5′-diphosphate; 3′P8-BrAdo5′P, 8-bromoadenosine 3′,5′-diphosphate; 3′PAdo($N^1 \rightarrow O$)5′P, adenosine N^1-oxide 3′,5′-diphosphate; 3′PPuo5′P, nebularine (9-β-D-ribofuranosylpurine) 3′,5′-diphosphate; 3′P(7-deaza-Ado)5′P, tubercidin (4-amino-7-(β-D-ribofuranosyl)-7H-pyrrolo-[2,3-d]-pyrimidine) (7-deazaadenosine) 3′,5′-diphosphate; 2′P(7-deaza-Ado)5′P, tubercidin 2′,5′-diphosphate; 3′P(N^6-Me_2Ado)5′P, N^6-dimethylaminoadenosine 3′,5′-diphosphate; 3′PFo5′P, formycin (7-amino-3-(β-D-ribofuranosyl)-pyrazolo-[4,3,-d]pyrimidine) 3′,5′-diphosphate, 3′PCyd5′P cytidine 3′,5′-diphosphate.

the imidazole ring as in tubercidin (7-deazaadenosine) and 8-bromoadenosine 3′,5′-diphosphate lead to an enhanced affinity. The latter findings are discussed in terms of an hypothesis of stacking of the aromatic ring of the estrogen substrate and the purine moiety and its analogs.

Introduction

Sulfuric acid esterification (sulfurylation), as mediated by an appropriate sulfotransferase, is a principal synthetic adjunct to biotransformations such as hydroxylation and methylation [1,2] which together determine the metabolic fate of many endogenous and foreign substances. For example, the formation of sulfate conjugates is central to the metabolism and interconversion of steroids [3]. Similarly, important metabolic pathways for *N*-acetylarylamines include the intermediacy of corresponding *N*-hydroxy-*O*-sulfates [4].

The variegated esterification reactions that comprise enzymic sulfurylation all indicate a common dependence upon 3′-phosphoadenosine 5′-phosphosulfate (3′PAdo5′PS) as the source of (active) sulfate [5]. This requirement had been the subject of extensive study in connection with bovine adrenal sulfotransferase (3′-phosphoadenylylsulphate:estrone 3-sulphotransferase, EC 2.8.2.4), which sulfurylates natural estrogens but not simple phenols or steroid alcohols [3,6—8]. The pure enzyme has been isolated, its chemical and physical properties described [3] and the structural features of the (estrogen) substrate, pertinent to sulfurylation of the phenolic (3-)OH group, have been delineated [9].

Studies with a partially purified preparation of bovine adrenal sulfotransferase [6] indicated that certain nucleotides and in particular 3′PAdo5′P, block sulfyrylation of estrone. These findings have since been confirmed with highly purified enzyme [3].

Estrone sulfate, unlike 3′PAdo5′P, has little or no effect on the estrogen sulfotransferase reaction. In view of the sensitivity of the enzyme to 3′PAdo5′P, a coproduct incidentally, of all sulfotransferase reactions, attempts were made to identify the structural features essential to the reversible binding of this nucleotide to estrogen sulfotransferase. This approach in turn provided important guidelines in the design of appropriate analogs of 3′PAdo5′PS which serve to extend the characterization of the cosubstrate specificity of estrogen sulfotransferase [10]. The present communication summarizes the results of stepwise modification of 3′PAdo5′P and the corresponding enzyme kinetic data generated in a study of the inhibition of estrone sulfurylation by analogs of the nucleotide coproduct.

Materials and Methods

Bovine spleen phosphodiesterase II (ortho-phosphoric acid diester phosphohydrolase, EC 3.1.4.1) ribonuclease T_2 (ribonucleate nucleotide-2′-transferase (cyclizing), EC 2.7.7.7) tubercidin (4-amino-7(β-D-ribofuranosyl)-7H-pyrrolo-[2,3-d]pyrimidine) (7-deazaadenosine), nebularine (9-β-D-ribofuranosylpurine), N^6-dimethyladenosine, disodium 2′- and 3′PAdo5′P were purchased from Sigma Chemical Co. Formycin (7-amino-3-(β-D-ribofuranosyl)-pyrazolo-[4,3-d]-

pyrimidine) was purchased from Meija Seika Kaisha, Tokyo, Japan. Bovine adrenal estrogen sulfotransferase (EC 2.8.2.4) was isolated and purified as previously described [3]. [^{35}S]3'PAdo5'PS and [6,7-^{3}H]estrone were purchased from New England Nuclear Corp.

General. All evaporations were performed on a Buchi rotary evaporator at temperatures below 30°C. Thin-layer chromatography was carried out as described earlier [11] 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 De-Saga 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 [12].

Estrogen sulfotransferase was isolated from the bovine adrenals and purified by ammonium sulfate fractionation and column chromatography on a DEAE-cellulose or DEAE-Sephadex A-50 [3,9].

Standard enzyme assay: The incubation mixtures contained in a total volume of 0.2 ml: 0.1 mM estrone, 2—23 μM 3'PAdo5'PS; $5 \cdot 10^5$ dpm [6,7-$^{3}H_2$]estrone, 0.19 M Tris · HCl buffer (pH 7.5 at 37°C), 5 μg enzyme and 200 μM dithiothreitol. Following a 10 min incubation (in the presence of a constant amount of inhibitor and a variable amount of 3'PAdo5'PS), the assay followed a previously published procedure [13]. When non-substrate nucleotides were used as inhibitors, the procedure for the enzyme assay was performed as described earlier [9], utilizing 5 μg enzyme, [^{35}S]3'PAdo5'PS, unlabeled estrone and the corresponding inhibitor.

Data processing: The initial velocity analysis was carried out maintaining the concentration of estrone at saturation level, varying the 3'PAdo5'PS, concentration but maintaining the concentration of inhibitor at a fixed level. Expression of results in terms of Fractional Inhibition (*FI*) [13,14] provided a convenient screen of 3'PAdo5'P analogs as potential inhibitors of sulfurylation. For more detailed information, complete kinetic analysis was carried out. Plots of the data were made in the double reciprocal form ($1/v$ vs. $1/[S]$), and in the form of replots of slopes (and intercepts) vs. inhibitor concentration. On the basis of these plots, the proper inhibition pattern was chosen for each experiment. In addition, K_i can be derived from *FI* using the equations:

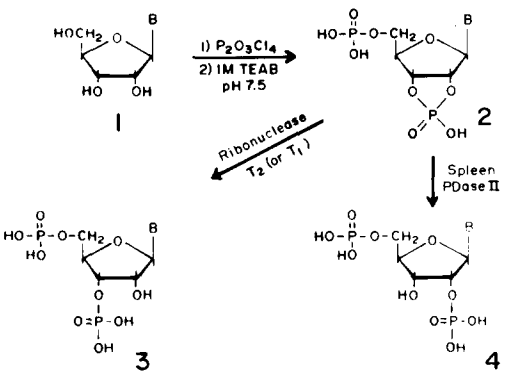
$$FI_c = \frac{1}{0.02K_i + 1} \tag{1}$$

for competitive inhibition, and

$$FI_{nc} = \frac{1}{0.01K_i + 1} \tag{2}$$

for non-competitive inhibition as shown earlier [13].

Synthetic. The ribonucleoside 3′,5′- and 2′,5′-diphosphates listed in Table II with the exceptions noted below, were prepared by the action of pyrophosphoryl chloride on the corresponding nucleoside according to the previously described methods [11,15,16]. Thus, the reactions of 1a, b, c, d, e, g, h, i and j with pyrophosphoryl chloride gave the corresponding 2′:3′-phosphate ribonucleoside 5′-phosphate (2) in yields of 52—83% following column chromatography and removal of the buffer as described in earlier work [11,15,16] (see Scheme 1).



Series	B
a	adenine
b	adenine N′ ⟶ oxide
c	8-bromoadenine
d	N^6-dimethyladenine
e	purine (nebularine)
f	hypoxanthine
g	guanine
h	cytosine
i	4-aminopyrrolo[2,3-d] pyrimidine (tubercidin)
j	7-aminopyrazolopyrimidine (formycin)

Scheme 1. Reaction sequence leading to analogs of 3′PAdo5′P.

All cyclic nucleotides (2), except 2g (see below), on treatment with ribonuclease T_2 gave the ribonucleoside 3′,5′-diphosphate (3) in near quantitative yields, after purification by column chromatography on DEAE-Sephadex A-25. The following example is considered typical.

Inosine 3′,5′-diphosphate (3f): A 2 ml aliquot (pH 6) containing 470 A_{248nm} units of 2f and 20 units of ribonuclease T_2 was maintained at room temperature overnight (18 h), at which time thin-layer chromatography in solvents S_1 and S_2 indicated complete conversion to 3f. Chromatography on DEAE-Sephadex A-25 with a linear gradient of 2 l each of 0.05 and 1 M triethylammonium bicarbonate (pH 7.5) yielded 3f in quantitative yield which was homogeneous in S_1, S_2, E_1 and E_2 and exhibited ultraviolet spectrum ($\lambda_{max}^{H_2O}$ 248 nm) characteristic of hypoxanthine. The product was dissolved in 1 ml 50% ethanol and stored at −15°C.

Guanosine 3′,5′-diphosphate (3g): To a 1 ml aqueous solution (500 A_{252nm} units) of guanosine 2′,3′-cyclic phosphate 5′-phosphate containing 200 units ribonuclease T_1 was added 1 ml 0.2 M triethylammonium bicarbonate and the buffered (pH 7.5) solution was incubated at 37°C for 28 h. Complete conversion to 3g was indicated by thin-layer chromatography in S_1 and S_2. The enzyme was denatured by heating the reaction mixture in a boiling water bath for 1 min, and the product, which was homogeneous in S_1, S_2, E_1 and E_2, exhibited a typical ultraviolet spectrum for guanine ($\lambda_{max}^{H_2O}$ 252 nm). The nucleotide was stored in 1 ml 50% ethanol at −15°C.

Tubercidin 2′,5′-diphosphate (4i): Bovine spleen phosphodiesterase (9 units) contained in 0.15 ml of water was added to a solution of 2i (740 A_{270nm} units) in 2 ml of water, pH 6.5. The reaction mixture was maintained at ambient temperature for 72 h after which thin-layer chromatography in S_1 and S_2 indicated complete conversion to 4i. The enzyme was denatured and the product characterized as described above for 3f.

The exceptions to these general synthetic procedures are outlined below.

Adenosine N^1-oxide 3′,5′-diphosphate (3b): The oxidation of 3a with *m*-chloroperbenzoic acid in sodium acetate/acetic acid followed the procedure described by Uno et al. [18]. Chromatography of the reaction mixture on DEAE-Sephadex A-25 using a linear gradient of triethylammonium bicarbonate gave 3b in 89% yield.

Alternative preparation of 8-bromoadenosine 3′,5′-diphosphate (3c). Bromination of 3a (0.19 mmol) in sodium acetate (pH 4) according to the method described by Ikehara and Uesugi [19] afforded 3c in 81% yield after chromatography on DEAE-Sephadex A-25. The product was identical in all respects with a sample of 3c obtained by treatment of 2c with ribonuclease T_2.

Adenosine 3′-phosphate 5′-diphosphate was prepared as described [17] by displacement of diphenyl phosphate with inorganic orthophosphate from adenosine 2′,3′-cyclic phosphate 5′-diphenylpyrophosphate followed by specific cleavage of the cyclic phosphate moiety with ribonuclease T_2.

Results and Discussion

The facile conversion of certain ribonucleosides to corresponding 2′,3′-cyclic phosphate 5′-phosphates (2) in pyrophosphoryl chloride [15,16], which was applied to the development of a practical (chemical) synthesis of 3′PAdo5′-PS [11], has been extended to include the 2′,3′-cyclic nucleotide 5′-phosphates of 8-bromoadenosine (2c), N^6-dimethyladenosine (2d), nebularine (2e), inosine (2f), guanosine (2g), tubercidin (2i), cytidine (2h) and formycin (2j). Purification was achieved in each case via column chromatography on DEAE-Sephadex A-25 at 4°C following neutralization of the reaction mixture. Elution of products was carried out with a linear gradient of triethylammonium bicarbonate (0.05—1.0 M) to provide the intermediates (2) in yields of 52—83%.

Regiospecific opening of the cyclic phosphate moiety in 2 to the ribonucleoside 3′,5′-diphosphate (3) was effected in all cases, save guanosine 2′,3′-cyclic phosphate (2g), with ribonuclease T_2 at pH 5.5—6.0. The same transformation with 2g required ribonuclease T_1 at pH 7.5 to afford 3g. Adenosine N^1-oxide

TABLE I

PHYSICAL CONSTANTS OF SOME RIBONUCLEOSIDE 3′,5′- AND 2′,5′-DIPHOSPHATES

Ribonucleotide	Structure (nm)	λ_{max}^{pH7}	Phosphate/nucleoside [b]	R_F [c]		Electrophoretic mobility [c]	
				S_1	S_2	E_1 [d]	E_2 [e]
3′PAdo5′P [a]	(3a)	259	—	0.50	0.15	1.47	0.75
2′PAdo5′P [a]	(4a)	259	—	0.59	0.15	1.47	0.79
3′PAdo($N^1 \rightarrow O$)5′P	(3b)	231	2.00 ± 0.04	0.77	0.15	1.62	0.87
3′P8-BrAdo5′P	(3c)	265	1.90 ± 0.01	0.36	0.24	1.45	0.65
3′P(N^6-Me_2Ado)5′P	(3d)	275	2.03 ± 0.26	0.44	0.34	1.59	0.67
3′PPuo5′P	(3e)	262	2.08 ± 0.04	0.68	0.26	1.66	0.77
3′PIno5′P	(3f)	248	2.11 ± 0.17	0.75	0.16	1.74	0.79
3′PGuo5′P	(3g)	253	2.17 ± 0.02	0.68	0.15	1.56	0.76
3′PCyd5′P [f]	(3h)	271	1.96 ± 0.08	0.85	0.18	1.66	0.73
3′P(7-deaza-Ado)-5′P	(3i)	270	2.00 ± 0.04	0.60	0.18	1.45	0.57
2′P(7-deaza-Ado)5′P	(4i)	270	2.02 ± 0.06	0.70	0.18	1.51	0.63
3′PFo5′P	(3j)	295	1.93 ± 0.01	0.63	0.13	1.65	0.62

[a] Sigma Chemical Co.

[b] Phosphate assayed according to a published procedure [12]. Inorganic phosphate was in each case $<<5\%$.

[c] See Materials and Methods for a description of solvent systems, etc.

[d] Relative to 5′AMP = 1.00.

[e] Relative to 3′PAdo5′PS = 1.00.

[f] Identification achieved by hydrolysis with 3′-ribonucleotide phosphohydrolase (rye grass 3′-nucleotidase, EC 3.1.3.6) to cytidine 5′-monophosphate [16].

3′,5′-diphosphate (3b) was derived directly from 3a by oxidation with *m*-chloroperbenzoic acid according to the method of Uno et al. [18]. Bromination of 3a in acetate buffer [19] provided an alternate route to 3c.

The identity and purity of the ribonucleoside 3′,5′-diphosphates were established from phosphate: nucleoside ratios, ultraviolet spectra, chromatographic and electrophoretic data (see Table I). The purity of 3 was confirmed in each case by conversion of the precursory cyclic nucleotides (2) to the isomeric nucleoside 2′,5′-diphosphates (4) with bovine spleen phosphodiesterase II (PDase II).

TABLE II

INHIBITION OF ESTROGEN SULFOTRANSFERASE BY SOME ADENINE NUCLEOTIDES

See Materials and Methods for experimental details.

Nucleotide	K_i (μM)
3′-AMP	3370
AMP	>5000
3′PAdo5′P	7
2′PAdo5′P	∞
2′ : 3′PAdo5′P	∞
3′P_2Ado5′P	46 *
3′PAdo5′P_2	116
Ado5′PS	1490
Ado5′S	>5000

* Calculated from Eqn. 1 (Materials and Methods, data processing).

In their kinetic studies of partially purified enzyme, Adams and Poulos [6] observed an inhibition of estrogen sulfurylation ranging from 14 to 84% with certain nucleotides at 100-fold excess. Subsequent work [3] with highly purified enzyme confirmed a prior result that ADP was a non-competitive inhibitor of 3'PAdo5'PS; the K_i being 2.8 mM. By contrast, ATP exhibited mixed inhibition whereas 3'PAdo5'P was a competitive inhibitor (K_i 0.056 mM). Their observations suggested that binding of the enzyme involved the 3'-phosphate group based upon the competitive inhibition shown by 3'-AMP (K_i 1.9 mM).

The findings with 3'-AMP and 3'PAdo5'P were confirmed in the present study (cf. Table II) in which it was also noted that AMP, Ado5'S and Ado5'PS, a biochemical precursor of active sulfate, all essentially fail to inhibit estrogen sulfotransferase. The importance of the 3'-phosphate ester of 3a to inhibition of estrone sulfurylation is further indicated by the fact that much like AMP, 2'PAdo5'P, a positional isomer of 3a, is devoid of inhibitory activity. In fact, of the several ribonucleoside 2',5'-diphosphates (4) examined, only 4i, as noted previously, shows any inhibition of the sulfurylation of estrone. Moreover, 2':3'PAdo5'P, the synthetic precursor of 3a (and its 2'-isomer) also lacks activity. It is noteworthy in this same connection that where the 3'-phosphate ester of 3a is replaced by a pyrophosphate moiety, as in $3'P_2$Ado5'P, the consequent increase in K_i (loss of activity) is greater than that observed for the corresponding interchange at the 5'-position, i.e. 3'PAdo5'P_2.

The results of the inhibition of estrogen sulfotransferase reaction by analogs of 3a are summarized in Table III. The data indicate that the replacement of the 6-amino group in 3a by hydrogen in 3'PPuo5'P (3e), and in particular by oxygen as in 3'PIno5'P (3f) or 3'PGuo5'P (3g) leads to a considerable loss of affinity for the enzyme. Similarly, dimethylation of the 6-amino group as in 3d causes a substantial reduction in enzyme affinity. Indeed, the results suggest that the exocyclic amino function comprises a point of binding in the formation of the ternary complex.

3'PCyd5'P (3h) showed no inhibition of sulfurylation at 100 μM concentra

TABLE III

EFFECT OF SOME RIBONUCLEOSIDE 3',5'- AND 2',5'-DIPHOSPHATES ON ESTROGEN SULFOTRANSFERASE

See Materials and Methods for experimental details.

Ribonucleotide		K_i (μM)
3'PAdo5'P	(3a)	7.0
3'PAdo($N^1 \rightarrow 0$)5'P	(3b)	350 *
3'P8-BrAdo5'P	(3c)	1.7
3'PN^6-Me_2Ado5'P	(3d)	125 *
3'PPuo5'P	(3e)	68 *
3'PIno5'P	(3f)	800 *
3'PGuo5'P	(3g)	84 *
3'PCyd5'P	(3h)	∞
3'P(7-deaza-Ado)5'P	(3i)	0.6
2'P(7-deaza-Ado)5'P	(4i)	5.3 *
3'PFo5'P	(3j)	43 *

* Calculated from Eqn. 1, Materials and Methods.

tion which lends additional support to the conclusion [6] that the enzyme is specific for purine ribonucleoside 3′,5′-diphosphates. However, 3′PAdo($N^1 \rightarrow O$)5′P (3b) is also a very weak inhibitor (K_i 350) which indicates that alterations in the pyrimidine ring of 3a are not well tolerated by the enzyme. On the other hand, certain modifications in the imidazole ring of 3 may, in some instances, lead to an enhanced affinity for the enzyme. Thus, 3′P8-BrAdo5′P (3c) shows a 4-fold increase in affinity over 3a as estimated from K_i values. Moreover, the (7-) carbon isosteric analog of 3a [3′P(7-deaza-Ado)5′P], (3i) affords a K_i nearly one order of magnitude lower than that of 3a, and is, in fact, the most potent inhibitor of estrone sulfurylation among the analogs of 3a synthesized to date. By contrast, the C-nucleotide, 3′PFo5′P (3j) shows a 6-fold decline in affinity for the enzyme relative to 3a.

NMR studies indicate that, whereas AMP exists predominantly in the anti (glycosyl-base) conformation, the preferred conformation of 8-bromoadenosine 5′-monophosphate is syn [20]. By analogy, the glycosyl-base torsion angles of 3a and 3c are presumed to approximate those of the corresponding AMP, i.e. anti and syn, respectively. Accordingly, the findings with 3c are viewed with surprise in light of the fact that conformationally abnormal *syn*-nucleosides do not, in general, substitute for their *anti*-counterpart in enzyme-catalyzed reactions. It is, however, possible that the conformation of the bound ligand, i.e. 3c as suggested by Burgen et al. [21], is oriented by the macromolecule independently of its initial state. Alternatively, the enzyme selects the most favorable conformation of the free ligand for bonding.

Recently, Rozhin et al. [13] proposed that the transition state leading to estrone sulfurylation is achieved via enzyme-induced folding of the substrates into a stacked structure in which the adenine moiety of active sulfate is aligned to ring-A of the estrogen juxtaposed to the 18-(β) methyl group. Examination of space-filling models indicates that the proposed "β-stack" accommodates both anti and syn forms of the nucleotide equally well. Accordingly, when extended to the matter of product inhibition, the concept of the β-stack reconciles the presumed sharp conformational differences between 3a and 3c with the much less disparate kinetic data.

It remains unclear just how the stacking arrangement would facilitate the disposition of the 3′-phosphate for binding. A CPK model * of the stacked arrangement for the proposed transition state of estrone sulfurylation fails to indicate any restriction to the accessibility of the 3′-phosphate. However, the hypothesis must provide, in addition, some insight as to reason for the effective inhibition of 2P′(7-deaza-Ado)5′P, which is unique among the ribonucleoside 2′,5′-diphosphates and must be regarded for the present as an anomaly. It is worthy of note that 2′P- and 3′P(7-deaza-Ado)5′P, by analogy with tubercidin 5′-phosphate, which shows no definite preference for anti or syn conformation [22], can perhaps be accommodated with equal facility in the proposed stacked structure.

Acknowledgements

This investigation was supported in part by U.S. Public Health Service Research Grant No. CA-16716 and CA-16855 from the National Cancer Insti-

* Corey-Pauling-Koltum model.

tute and in part by an institutional grant to the Michigan Cancer Foundation from the United Foundation of Greater Detroit.

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