Nonclassical Antimetabolites XIX

Simulation of 5′-Phosphoribosyl Binding III. Relative Contribution of Phosphate and the Other Oxygen-Containing Functions to the Binding of the 5′-Phosphoribosyl Moiety to Succinoadenylate Kinosynthetase

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The synthesis of 6-mercapto-9H-purine-9-yl-pentanol phosphate (VI) and 9H-adenine-9-yl-pentanol phosphate (VII) *via* 5-amino-4-chloro-6-(5′-hydroxypentylamino)pyrimidine (X) are described. The comparison of VI, VII, thioinosinic acid (III), 5′-adenylic acid (IV), 2′-deoxy-5′-adenylic acid (V), thioinosine (XIII), and adenosine as inhibitors of succinoadenylate kinosynthetase indicated that the twelvefold decrease in activity of VI and VII compared to the ribonucleotides, VII and IV, was due primarily to the removal of the 2′-hydroxyl group and not the 3′-hydroxyl or furanosyl oxygen group of the ribosyl moiety.

IN AN EARLIER study on the replacement of the phosphate moiety of a nucleotide by other groups that could simulate the binding of the phosphate (1), it was concluded that a systematic study on the relative contribution of phosphate and the other oxygen functions of 5′-phosphoribosyl moiety should be ascertained before proceeding further to the design and synthesis of compounds without a phosphate group that could bind to an enzyme in place of a nucleotide.[1] Such a study has now been completed with the enzyme succinoadenylate kinosynthetase, and the results are the subject of this paper.

DISCUSSION

Succinoadenylate kinosynthetase (3–5) is an enzyme that converts inosinate (I) and aspartate to adenylosuccinate (II) and requires guanosine triphosphate (GTP) as a cofactor. Both thioinosinate (III) (6–8) and 5′-adenylate (IV) (5) have been reported to be inhibitors of this enzyme; furthermore, 2′-deoxy-5′-adenylate (V) has been reported to be a weak, noncompetitive inhibitor of this enzyme (5), thus indicating that the 2′-hydroxy group of 5′-adenylate contributes to binding to this enzyme.

To determine the contribution of the oxygen functions at C-2′, C-3′, and C-4′ to binding, 6-mercapto - 9H - purine - 9 - yl - pentanol - 5′ - phosphate (VI) and 9H-adenine-9-yl-pentanol-5′-phosphate (VII) have now been synthesized. Reaction of 5-amino-4,6-dichloropyrimidine with 5-aminopentanol according to the method of Montgomery and Temple (9, 10) gave X in 92% yield. Ring closure with ethyl orthoformate in the presence of one equivalent of hydrochloric acid (11) afforded 6-chloro-9-(5′-formyloxypentyl)purine (XI) as a syrup which was not purified, but was converted directly to the higher melting 6-mercaptopurine derivative (VIII) with thiourea in ethanol in 69% over-all yield from X. (See Scheme I.) Treatment of X with polyphosphoric acid (12, 13) afforded the requisite phosphate (VI), isolated as its barium salt in 31% yield. For assay purposes, the barium salt was converted to the more soluble sodium salt with sodium sulfate in water.

Similar treatment of 9-(5′-hydroxypentyl)adenine (14) with polyphosphoric acid afforded the adenine analog (VII) in 45% yield isolated as its barium salt; this barium salt was readily soluble in warm 0.1 *N* hydrochloric acid and, on cooling, the crystalline zwitterion of VII separated from solution.

In Table I are listed the relative inhibitions of succinoadenylate kinosynthetase by the candidate compounds. 5′-Adenylic acid (IV) in the presence of 30.6 μM inosinate showed 50% inhibition of the enzyme reaction at a concentration of 68 μM; thus, the I/S ratio for 50% inhibition (I_{50}) was 2.3. Wyngaarden and Greenland have recorded an apparent enzyme-substrate dissociation constant (K_m) of inosinate as 5.4×10^{-5} *M* and an enzyme-inhibitor dissociation constant (K_i) for 5′-adenylic acid (IV) of 9.5×10^{-5} *M*, a ratio of 1.8.

2′-Deoxy-5′-adenylic acid (V) had an I_{50} of 13 (Table I); comparison with IV shows that removal of the 2′-hydroxyl causes a nearly sixfold reduction in binding. The effect of removing the phosphate group from 5′-adenylic acid (IV) can be gleaned by comparison with adenosine (XII); the latter had an I_{50} of 105, a 46-fold decrease in binding. Removal of all of the oxygen-containing functional groups of 5′-adenylic acid (IV), except the phosphate, as in VII, led to a fourteenfold decrease in binding, considerably less than the effect caused by removal of the phosphate group from IV. Of the fourteenfold loss in binding in proceeding from 5′-adenylic acid (IV) to VII, most of the loss is due to removal of the 2′-hydroxyl group (sixfold) as seen by comparison of IV, V, and VII.

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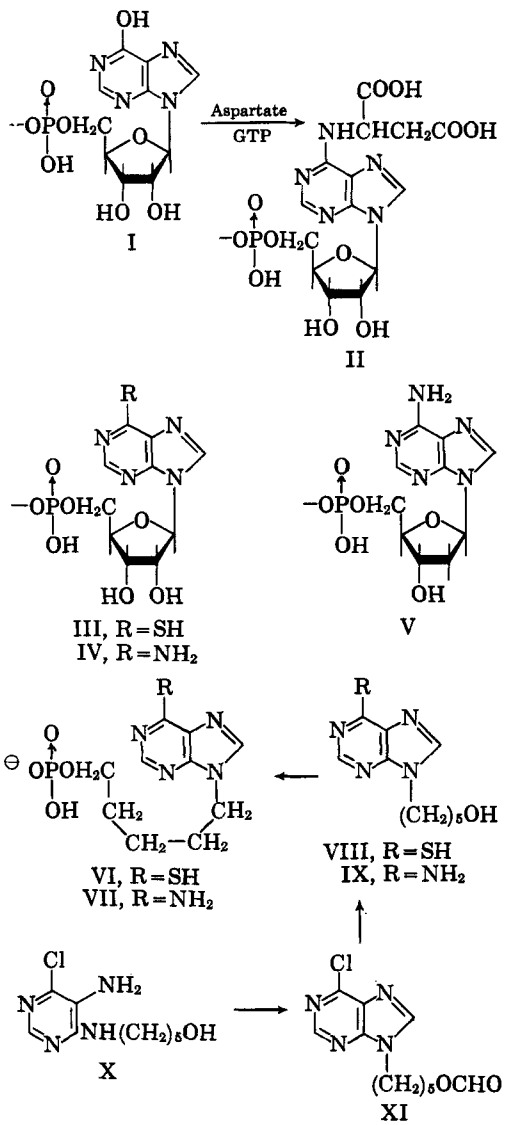
[1] The biochemical rational for and the chemotherapeutic need of simulation of phosphate binding have been discussed previously (2).



Scheme I

Quite similar results were obtained in the 6-mercaptopurine series. The ribonucleotide (III) had an I_{50} of 2.3, and the pentanol phosphate (VI) had an I_{50} of 27, but the riboside (XIII) appeared to be even less effective than adenosine (XII).

That the pentanol phosphate (VI) binds only about one-half as well as 2′-deoxy-5′-adenylic acid (V) does not necessarily indicate that the latter has an additional binding point not present in VI; the conformation written for VI in the formula diagram is probably necessary for binding. Such a conformation is energetically unfavorable compared to the normal staggered conformation of a hydrocarbon chain, and some net loss in binding might occur in order to supply the free energy necessary for an unfavorable conformation for binding. Thus, it would appear that only the 2′-hydroxyl of the C-2′, C-3′, and C-4′ oxygen-containing functions contributes to binding to this enzyme. A similar phenomenon has recently been observed with adenosine deaminase; 9-(2′-hydroxyethyl)adenine binds as well to the enzyme as the substrate, adenosine (15). Whether other enzymes using ribonucleotides or ribonucleosides as substrates will also lose five- to tenfold in binding when the 2′-hydroxyl group is removed is not easy to glean from the literature since usually only substrate properties are recorded.

The purine-9-pentanol phosphates (VI and VII) are sufficiently good inhibitors of succinoadenylate kinosynthetase that substitution of the phosphate of VI or VII by other groups that might simulate phosphate binding can now be studied. Such studies are well underway in this laboratory.

EXPERIMENTAL

Enzyme Measurements

Reagents.—Sodium guanosine triphosphate (GTP) and frozen *Escherichia coli* B (35% solids) were obtained from General Biochemicals. The sodium salts of 5′-inosinic acid (IMP), 5′-adenylic acid, and 2′-deoxy-5′-adenylic acid were obtained from Sigma Chemical Co. Streptomycin sulfate was purchased from Nutritional Biochemicals.

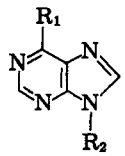
Succinoadenylate Kinosynthetase.—The method of Lieberman (3) was modified as follows.

A suspension of 10 Gm. of *E. coli* B cells (35% solids) in 10 ml. of 5 m*M* phosphate buffer (pH 7.2) was forced through a French press (American Instrument Co.) at 4000–6000 lb. gauge pressure. The thick exudate was caught in a small Waring blendor head cooled in an ice bath. After dilution with 45 ml. of 5 m*M* phosphate buffer (pH 7.2), the mixture was blended for 10 sec. at low speed and 25 sec. at high speed. The cell debris was removed by centrifugation at 5000 r.p.m. (Spinco Rotor 21) for 45 min. at 0–5°. The supernatant liquid (58 ml.) was magnetically stirred in an ice bath while 11.4 ml. of 5% streptomycin sulfate was added dropwise over about 3 min. After being stirred for an additional 12 min., 3 Gm. of analytical grade Celite was added, and stirring was continued for 5 min. The mixture was filtered by suction through a Celite pad on a 90-mm. Büchner funnel into an ice-cooled suction flask.

The filtrate was adjusted to pH 5.4 with 0.1 *M* acetic acid, placed in a 37° water bath for 15 min., then stirred in an ice bath for 15 min. with 2 Gm. of Celite. The mixture was filtered through a Celite pad on a 65-mm. Büchner funnel into an ice-cooled receiver. The filtrate was adjusted to pH 7.0 with 1 *M* potassium hydroxide; volume, 35 ml. After dilution with 17.5 ml. of 1 *M* glycine buffer (pH 9.6), the solution was cooled in an ice bath, and 18 Gm. of ammonium sulfate was added over a period of 3 min. with magnetic stirring. After being stirred an additional 10 min., 2 Gm. of Celite was added, the mixture was stirred 5 min. longer, then filtered through a Celite pad on a 50-mm. Büchner funnel into an ice-cooled suction flask; volume, 45 ml.

To the stirred and ice-cooled filtrate was added 4.2 Gm. of ammonium sulfate over a period of 2 min. After being stirred for 10 min., 2 Gm. of Celite was added and stirring continued for 5 min. The mixture was filtered through a Celite pad on a 50-mm. Büchner funnel. The Celite pad was quickly suspended in 20 ml. of deionized water, the lumps

TABLE I.—INHIBITION OF SUCCINOADENYLATE KINOSYNTHETASE BY



Compd.	R_1	R_2	mM Concn. of Inhibitor	% Inhibition	I/S[a]
IV	NH_2	Ribosyl-5′-phosphate	0.068	50	2.3
V	NH_2	2′-Deoxyribosyl-5′-phosphate	0.39	50	13
XII	NH_2	Ribosyl[b]	3.2	50	105
VII	NH_2	$-(CH_2)_5OPO(OH)_2$	0.97	50	32
III	SH	Ribosyl-5′-phosphate	0.070	50	2.3
XIII	SH	Ribosyl[b]	1.05	0	>140[c]
VI	SH	$-(CH_2)_5OPO(OH)_2$	0.83	50	27

Assays were performed with 30.6 μM IMP, 3.75 mM aspartate, and 100 μM GTP as described under *Experimental*. The technical assistance with these assays by Shirley Herrmann is acknowledged. [a] I/S is the ratio of the concentrations of inhibitor to inosinate showing 50% inhibition (I_{50}). [b] 9-β-D-Ribofuranosyl. [c] Since 20% inhibition is readily detectable, the concentration necessary for 50% inhibition is estimated at four times greater than the concentration measured; I/S is calculated accordingly.

broken up, then the mixture was magnetically stirred in an ice bath for 10 min. The Celite was removed by filtration through a 50-mm. Büchner funnel into an ice-cooled receiver and washed with 20 ml. of deionized water in three portions; final volume, 37 ml. The solution was stored at 3° and maintained its enzyme activity over several months.

When 50 μl. was assayed as described below, an absorbance change of about 0.004 units/min. at 280 mμ was observed.

Assay Method.—An assay mix was composed of 3.0 ml. of 0.1 *M* magnesium chloride, 3.0 ml. of 306 μM IMP, 3.0 ml. of 0.5 *M* glycine buffer (pH 8.0), and 0.36 ml. of 5% streptomycin sulfate. GTP (1 m*M*) was kept frozen at −20° and was sufficiently stable in the frozen state for 5 days; a 1.5-ml. vial was thawed and kept in an ice bath for the day's assays, but the remainder was rejected at the end of the day. Most of the early difficulties with this assay were due to the instability of GTP since GMP or GDP, its hydrolysis products, were inhibitory (3, 5).

In a test tube were placed 0.62 ml. of assay mix, 0.20 ml. of GTP, 100 μl. of enzyme solution, and 0.89 ml. of water; when an inhibitor was used, the water plus inhibitor solution totaled 0.89 ml. To the top 1-ml. cell of a Cary 15 spectrophotometer was placed 0.90 ml. of the cell mix; the remainder of the cell mix was poured into the bottom cell. When the base line had become steady, the enzyme reaction was started by addition of 100 μl. of 37.5 m*M* L-aspartate (adjusted to pH 8.0). The change in absorbance at 280 mμ with time was recorded on 0–0.1 absorbance slide wire. The final concentrations in the upper cell were 10 m*M* magnesium chloride, 30.6 μM IMP, 100 μM GTP, 0.05 *M* glycine buffer, 3.75 m*M* L-aspartate, and 0.6 mg./ml. streptomycin sulfate. This procedure incorporates the Wyngaarden and Greenland (5) and Fromm (4) modifications of the original Lieberman (3) assay II.

By plotting V_0/V_1 against I for several concentrations of I, the concentration of I necessary to give 50% inhibition was readily determined ($V_0/V_1 = 2$), where V_0 = velocity of enzyme reaction with no inhibitor, V_1 = velocity of reaction in the presence of inhibitor, and I = concentration of inhibitor (2, 16).

Synthesis

Melting points were determined in capillary tubes in a Mel-Temp block, and those below 230° are corrected. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in water at the pH indicated with a Perkin-Elmer 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on Brinkmann Silica Gel G, and spots were observed by iodine vapor; solvents used were methanol (solvent *D*) or benzene–methanol (3:1) (solvent *E*). Paper chromatograms were run on Whatman No. 1 paper by the ascending technique with formic acid–ethyl acetate–water (2:7:1) (solvent *A*), aqueous 5% dipotassium hydrogen phosphate (solvent *B*), or *n*-butanol–acetic acid–water (5:2:3) (solvent *C*), and spots were detected by inspection under ultraviolet light.

5 - Amino - 4 - chloro - 6 - (5′ - hydroxypentylamino)pyrimidine (X).—A solution of 4.92 Gm. (30 mmoles) of 5-amino-4,6-dichloropyrimidine (Krishell Laboratories, Inc.), 3.4 Gm. (33 mmoles) of 5-aminopentanol, and 5 ml. (35.7 mmoles) of triethylamine in 100 ml. of *n*-butanol was refluxed with magnetic stirring for 22 hr., then spin-evaporated *in vacuo*. The residue was extracted twice with 100-ml. portions of boiling ethyl acetate, and the insoluble triethylamine hydrochloride was removed by filtration. On being chilled, the combined extracts deposited 6.4 Gm. (92%) of white needles, m.p. 70–75°, that were suitable for further transformations. Recrystallization from water gave white needles, m.p. 72–75°, $\nu_{max.}$ 3350, 3200 (NH, OH); 1660, 1580 cm.$^{-1}$ (NH, pyrimidine); $\lambda_{max.}$ (H_2O) 265, 292 mμ.

Anal.—Calcd. for $C_9H_{15}ClN_4O \cdot 0.5H_2O$: C, 45.0; H, 6.73; N, 23.3. Found: C, 44.7; H, 6.60, N, 23.4.

6-Mercapto-9H-purine-9-yl-pentanol (VIII).—A solution of 231 mg. (1 mmole) of X in 4 ml. of ethyl orthoformate and 0.11 ml. (1.3 mmoles) of 12 *N* aqueous hydrochloric acid was magnetically stirred for about 18 hr. at ambient temperature. Spin evaporation *in vacuo* gave XI as a syrup which showed ester absorption at 1720 and 1180 cm.$^{-1}$.

A solution of this syrupy XII and 84 mg. (1.1 mmole) of thiourea in 10 ml. of ethanol was refluxed

for 3 hr. with magnetic stirring. Most of the solvent was removed by spin evaporation *in vacuo*, then 10 ml. of water was added. The product was collected on a filter and washed with two 5-ml. portions of water; yield, 165 mg. (69%) of product, m.p. 257–259°, that was suitable for further transformation.

When the cyclization was performed with ethyl orthoformate–acetic anhydride (9), then the purine reacted with thiourea in butanol, crude 9-(5′-acetoxypentyl)-6-mercaptopurine was obtained in 41% yield, m.p. 225–235°, which showed *O*-acetate absorption at 1720 and 1250–1210 (broad) cm.$^{-1}$. Saponification with 1 *N* aqueous sodium hydroxide for 1 hr. at room temperature, gave on acidification a 31% yield (over-all from X) of VIII, m.p. 255–257°. Recrystallization from ethanol gave buff-colored crystals, m.p. 255–257°, $\nu_{max.}$ 3400 (OH); 1600, 1575, 1540 cm.$^{-1}$ (C=C, C=N); $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-3}$): pH 1, 327 (20.1); pH 7, 324 (22.6); pH 13, 313 (21.6).

Anal.—Calcd. for $C_{10}H_{14}N_4OS$: C, 50.4; H, 5.92; N, 23.5. Found: C, 50.3; H, 6.07; N, 23.3.

The compound moved as a single spot in solvents *A*, *B*, and *C* with R_f 0.65, 0.62, and 0.77, respectively. It moved as a single spot on TLC in solvents *D* and *E*.

Barium 6-Mercapto-9H-purine-9-yl-pentanol Phosphate (VI).—A mixture of 2.6 Gm. of phosphorus pentoxide and 3.5 Gm. of 85% phosphoric acid was cooled to room temperature, then 600 mg. (2.52 mmoles) of VIII was added. After being heated in a bath at 60° for 8 hr., the mixture was diluted with 40 ml. of water and heated in an oil bath at 100° for 20 min. to hydrolyze polyphosphates. The pH was carefully adjusted to 6.5 with hot saturated barium hydroxide solution. The barium phosphate was removed by filtration through a Celite pad and washed with four 25-ml. portions of hot water. Spin evaporation to a small volume *in vacuo* caused precipitation of the product, which was collected on a filter and washed with water (3 × 20 ml.), ethanol (3 × 20 ml.), and acetone (3 × 20 ml.); yield, 385 mg. (34%), m.p. > 300°. This compound moved as a single spot in solvents *A*, *B*, and *C* with R_f 0.23, 0.76, and 0.39, respectively. For analysis, a sample was recrystallized from water, then dried for 1 day at 100° over P_2O_5 in high vacuum; $\nu_{max.}$ 1590, 1570, 1530 (C=C, C=N); 1190 (P=O); 1080 (broad), 985 (P—O); no OH near 3400 cm.$^{-1}$.

Anal.—Calcd. for $C_{10}H_{13}BaN_4O_4PS$: C, 26.5; H, 2.89; N, 12.4. Found: C, 26.7; H, 3.59; N, 12.2.

For enzyme assay, 314 mg. (0.692 mmole) of the barium salt was dissolved in 200 ml. of hot water, then treated with a solution of 223 mg. (0.692 mmole) of sodium sulfate decahydrate in 30 ml. of water. The barium sulfate was removed by filtration through Celite and was washed with water. The combined filtrate and washings were spin evaporated *in vacuo*. The residual sodium salt readily dissolved in water. A trace of insolubles was removed by filtration through Celite. The filtrate (118 ml.) was assayed for content of VI by the absorbance at 324 mμ using the molecular extinction coefficient of 22,600 for VIII; this solution was 4.30 m*M* in VI and was used directly for assay. This solution showed one spot when run in solvents *A*, *B*, and *C* with R_f 0.27, 0.76, and 0.36, respectively, the same as the barium salt.

9H-Adenine-9-yl-pentanol Phosphate (VII).—Treatment of 533 mg. (2 mmoles) of IX hydrochloride hemihydrate (14) with 2.6 Gm. of phosphorus pentoxide and 3.5 Gm. of 85% phosphoric acid, as described for the preparation of VI, gave a filtrate from the barium phosphate which was spin evaporated *in vacuo* to about 10 ml. The barium salt of VII was collected on a filter and washed with water (3 × 10 ml.), then ethanol (4 × 10 ml.); yield, 164 mg. Further addition of ethanol to the filtrate gave an additional 230 mg. of barium salt (total 45%); $\nu_{max.}$ 3350 (NH); 1650, 1600, 1575 (NH, C=C, C=N); 1110 (P=O); 1070, 1000 cm.$^{-1}$ (P—O).

In 10 ml. of hot 0.1 *N* hydrochloric acid was dissolved 100 mg. of the barium salt. The filtered solution deposited white needles of the zwitterion of VII on being cooled; yield, 58 mg. (78% recovery), m.p. 244–245°. Recrystallization from water gave white needles, m.p. 245–247°; $\nu_{max.}$ 3400 (broad NH), 2800–2200 (acidic H); 1700 (C=NH$^+$); 1670, 1600, 1510 (NH, C=C, C=N); 1220 (P=O); 1030 (broad), 950 cm.$^{-1}$ (P—O).

Anal.—Calcd. for $C_{10}H_{16}N_5O_4P$: C, 39.9; H, 5.35; N, 23.3. Found: C, 39.8; H, 5.36; N, 23.1.

The compound moved as a single spot in solvents *A*, *B*, and *C* with R_f 0.18, 0.57, and 0.52, respectively.

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