Enzyme Inhibitors I

Inhibition of Adenosine Deaminase by Isosteric Nucleosides

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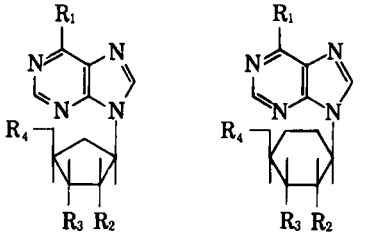
A series of isosteric nucleosides has been evaluated as inhibitors of adenosine deaminase in an attempt to determine which sites of adenosine are important for binding to the enzyme. The 6-amino group in the purine nucleus makes a strong contribution to binding, and the enzyme appears to be specific for amino since other groups (SH, OH, $NHNH_2$, Cl, H) at the 6-position were noninhibitory. For effective inhibition the purine must be substituted in the 9-position; the effect of the stereochemistry of a 2′-hydroxyl group on certain cyclic substituents at the 9-position of the purine nucleus has been measured.

THE CHEMISTRY and biochemistry of purine nucleosides and purine nucleotides have been studied extensively during the past 20 years, with the result that certain compounds have been prepared which are capable of inhibiting the growth of malignant cells (1). These studies have also resulted in an understanding of the *de novo* synthesis of purine nucleotides and of certain other purine transformations (2, 3). Little is known, however, about which of the atoms or functional groups in purine nucleosides and tides are required for binding to the active site of the various enzymes. Since it is well known that purine ribonucleosides and purine ribonucleotides are easily cleaved hydrolytically or enzymatically, certain purine nucleosides and nucleotides which may be effective agents in inhibiting the growth of malignant cells become ineffective *in vivo* because they are rapidly destroyed by cleavage into a purine and a carbohydrate moiety (4). In an attempt to circumvent this difficulty, we have synthesized a novel class of compounds which are hydrolytically and presumably enzymatically stable, but which should be capable of binding to an enzyme that normally employs a purine ribonucleoside as the substrate. These compounds, the isosteric nucleosides, are purines that contain at the 9-position a cyclopentyl or a cyclohexyl ring which is substituted in such a manner that it simulates sterically the sugar moiety of a nucleoside (I and II) (5, 6).

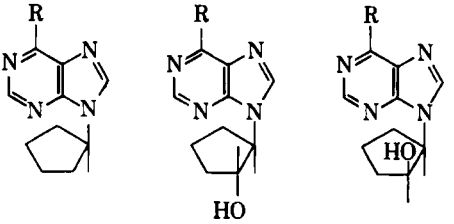
To obtain information about how a purine nucleoside binds to an enzyme, the authors have initiated a study of the effect of certain isosteric nucleosides on the enzyme, adenosine deaminase. This paper describes the effect on binding of certain substituents at the 6-position and at the 9-position of I and II. The compounds



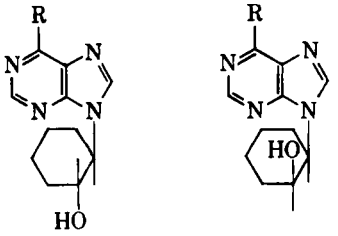
I II

[where R_1 may be NH_2, NHMe, NMe_2, SH, $NHNH_2$, H, or Cl; and R_2, R_3, and R_4 may be various combinations of H, OH, or NH_2.]

which were employed in this investigation fall into five types, depending upon the substituent at the 9-position of the purine nucleus, as shown by structures III, IV, V, VI, and VII.



III IV V



VI VII

[where the R group in each case = NH_2, SH, $NHNH_2$, H, and Cl.]

EXPERIMENTAL[1]

Reagents and Isosteric Nucleosides.—Adenosine and adenosine deaminase were purchased from the

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[1]The ultraviolet recordings were made on a Perkin-Elmer model 4000 A spectrophotometer.

TABLE I.—K_m OF ADENOSINE AND K_i OF ISOSTERIC NUCLEOSIDES WITH ADENOSINE DEAMINASE[a]

Compd.	$K_m \times 10^5 M$	$K_i \times 10^5 M$
Adenosine	7.3	
III (R = NH_2)		49.3
IV (R = NH_2)		13.5
V (R = NH_2)		17.4
VI (R = NH_2)		13.9
VII (R = NH_2)		21.1

[a] The values reported are at pH 7.6 and at 25° in 0.05 *M* phosphate buffer.

Sigma Chemical Co. Compounds of type III, where R = NH_2, SH, $NHNH_2$, H, and Cl, were prepared according to the procedure of Montgomery and Temple (7). Compounds of type IV, V, VI, and VII, where R = NH_2, SH, $NHNH_2$, H, and Cl, were prepared as previously described (5, 6).

trans - 2 - (6 - Methylamino - 9 - purinyl)cyclopentanol.—A 0.288-Gm. (1.23 mmole) sample of *trans*-2-(6-chloro-9-purinyl)cyclopentanol (6) was dissolved in 10 ml. of ethanol, and 10 ml. of 40% methylamine in water was added. The mixture was heated under reflux for 3 hours, and the volatile materials were removed *in vacuo;* the residue was extracted with hot benzene (4 × 30 ml.). The benzene extract on cooling gave 0.160 Gm. (59.3%) of the crude product, m.p. 194–195°. Three recrystallizations of the crude product from benzene gave the pure sample, m.p. 194–195°; ν in cm.$^{-1}$ (KBr); 3300 and 3150 (NH); 1640 (C=N).

Anal.[2]—Calcd. for $C_{11}H_{15}N_5O$: C, 56.63; H, 6.48; N, 30.03. Found: C, 56.88; H, 6.51; N, 29.92.

Assay Procedure

The general method of assay which was employed is described in Colowick and Kaplan (8) and involves measuring the rate of disappearance of the absorption band of adenosine at 265 mμ. All reactions were run in 0.05 *M* phosphate buffer at pH 7.6 at 25°. The stock solutions of all reagents were prepared in 0.05 *M* phosphate buffer at pH 7.6.

RESULTS

The initial testing for the ability of the isosteric nucleosides to inhibit adenosine deaminase was carried out at substrate concentrations which were approximately 20% below that concentration which was required to saturate the enzyme. The concentration of adenosine which was employed for the initial testing was 0.061 m*M*. Compounds that did not show significant inhibition of the enzymatic reaction at concentrations three to four times the concentration of the substrate were classified as noninhibitory. For those compounds which were significantly inhibitory, K_i's were determined by the method of Lineweaver and Burk (9). For each determination of K_i, two different concentrations of inhibitor were used, and each point on the plot was an average of at least two determinations. Finally, the total procedure was repeated, so that each K_i that is reported is an average of four evaluations. Table I gives the results of the determination of K_i for five different isosteric nucleosides.

[2] Galbraith Microanalytical Laboratories, Knoxville, Tenn.

Compounds III–VII, with R = SH, $NHNH_2$, H, or Cl, did not exhibit significant inhibition of adenosine deaminase when they were present at a concentration three to four times that of the substrate. *cis-* and *trans*-2-Aminocyclopentanol and *cis-* and *trans*-2-aminocyclohexanol did not inhibit the enzymatic reaction even when they were present in a concentration 30 times greater than adenosine. Adenine, inosine, and 5′-adenylic acid were neither substrates nor inhibitors at three to four times substrate concentration, whereas 2′-deoxyadenylic acid was a substrate.

The results of a typical experiment which was used to evaluate K_m for adenosine and K_i for VI (R = NH_2) are given in Table II.

DISCUSSION

In 1956, Feigelson and Davidson (10) studied the inhibition of adenosine deaminase by 8-azaguanine in an attempt to explain the carcinostatic effects of this compound and found that 8-azaguanine was a noncompetitive inhibitor with regard to substrate. They obtained indirect evidence that the inhibitor exhibits a higher affinity for the enzyme-substrate complex than for the free enzyme.

Our data give information on the effect on binding to adenosine deaminase by various substituents at the 6-position and at the 9-position of the purine nucleus. Concerning the 6-position, it is apparent that an amino group must be present in order for a reversible complex to be formed, since compounds III–VII, with R = SH, $NHNH_2$, H, or Cl, were essentially noninhibitory. However, when a 6-amino group was present, all of the compounds were inhibitors of adenosine deaminase, and they exhibited competitive inhibition as shown by the reciprocal plot method of Lineweaver-Burk. In addition, it should be noted that binding by the substituent at the 6-position appears to be specific for an amino group and not just a basic group since the 6-hydrazino compounds (III–VII) were not inhibitory. It might be suggested that the 6-hydrazino compounds did not bind to the enzyme because the hydrazino group is larger than an amino group, and if a group smaller than amino were present, inhibition would occur. This reasoning, however, was incorrect since those compounds with a 6-hydrogen substituent were also noninhibitory. In addition, the 6-amino group may be secondary, but cannot be tertiary and still cause inhibition.

TABLE II.—INHIBITION OF ADENOSINE DEAMINASE BY *trans*-2-(6-AMINO-9-PURINYL)CYCLOHEXANOL (VI, R = NH_2)

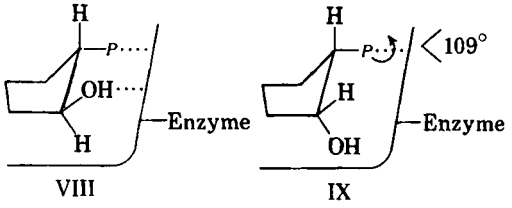
Run No.	m*M* Concn. of Adenosine	Rate[a,b]	m*M* Concn. of VI (R = NH_2)	Rate[c]	m*M* Concn. of VI (R = NH_2)	Rate[d]
1	0.085	0.98	0.082	0.78	0.123	0.69
2	0.077	0.94	0.082	0.74	0.123	0.65
3	0.070	0.90	0.082	0.68	0.123	0.61
4	0.062	0.84	0.082	0.63	0.123	0.55
5	0.054	0.77				

[a] All rates that are given are for the initial portion of the reaction in which the change in absorbance is linear with respect to time. [b] Change in O.D./minute for the various substrate concentrations shown in column 2. [c] Change in O.D./minute for the various substrate concentrations shown in column 2 in the presence of 0.082 m*M* concentration of VI (R = NH_2). [d] Same as *footnote c*, but in the presence of 0.123 m*M* concentration of VI (R = NH_2).

For example, IV and V (R = NMe_2) were both noninhibitory, whereas IV (R = NHMe) did inhibit the enzymatic reaction, but it was approximately one-tenth as effective an inhibitor as IV (R = NH_2). It appears, therefore, that steric factors may play a critical role in binding by the 6-position; but additional work is necessary before this question can be answered unambiguously. The result of such a study will be the subject of a future paper.

To assess the importance of substituents at the 9-position of the purine nucleus and of hydroxyl groups on the 9-substituent as binding points to adenosine deaminase, we determined the K_i of each compound (III–VII) where the 6-substituent was held constant (see Table I). It was anticipated that the hydroxyl groups in the ribose moiety of adenosine would make a contribution in binding to the enzyme; by studying those compounds in which the stereochemistry of the substituents at the 9-position is known, it should be possible to evaluate the approximate contribution by the 2′-hydroxyl group. Not unexpectedly, the unsubstituted cyclopentyl compound (III, R = NH_2) exhibited the largest K_i, thus, the enzyme-inhibitor complex is dissociated to a larger extent than with the other inhibitors. The *trans*-2-hydroxycyclopentyl (IV, R = NH_2) and the *trans*-2-hydroxycyclohexyl analogs (VI, R = NH_2) exhibited the smallest K_i's. and the two derivatives with the *cis* hydroxyl groups (V and VII, R = NH_2) exhibited K_i's which were similar and only slightly larger than the *trans* compounds. Since adenine is not inhibitory, it is apparent that a substituent is necessary at the 9-position of the purine for binding to the enzyme. The hydroxyl group at the 2-position of the cycloaliphatic ring contributes to binding, evidenced by the reduction in K_i in IV–VII (R = NH_2) relative to III (R = NH_2). That the K_i's of IV, V, VI, and VII (R = NH_2) are rather similar reveals that the stereochemistry of the hydroxyl group at the 2′-position of the cycloaliphatic ring does not appear to be so critical as might be presumed.

The authors believe that this lack of steric requirements for the 2′-hydroxy group may be explained in the following way. Because the *trans* compound (IV, R = NH_2) is a close structural analog of adenosine which has the same stereochemistry of the 2′-hydroxyl group and because IV (R = NH_2) is a competitive inhibitor of adenosine deaminase it is reasonable to assume that the inhibitor and substrate possess a similar conformation and that they bind at essentially the same site on the enzyme. Thus one can postulate that the *trans* compound IV (R = NH_2) binds to the enzyme through the 2′-hydroxyl group and 6-aminopurine nucleus by means of the conformation represented by VIII. The *cis* derivative (V, R = NH_2) may be represented by the conformation shown in IX. Since the K_i's of the two compounds are nearly equal, it follows that the binding through the 2′-hydroxyl groups is almost equal. From a stereochemical viewpoint this result can be explained by assuming that the 6-aminopurinyl nucleus of the *cis* compound binds to the enzyme in exactly the same manner as it does in VIII. However in the case of IX a rotation about the C-N bond connecting the 1′-carbon of the cyclopentyl ring and the 9-position of the purine nucleus must occur. The result of this rotation in IX in which the purine nucleus is not rotated would allow the *cis*-2′-hydroxyl to bind to the same place on the enzyme that it does in the *trans* compound (VIII). However when the rotation does occur in IX the cyclopentyl ring must occupy a plane different from when VIII is bound to the enzyme. A similar argument can be presented to explain the binding of the *trans*- and *cis*-2′-hydroxycyclohexyl analogs (VI and VII, R = NH_2). Thus it appears that the active site of adenosine deaminase has a large bulk tolerance in the area where the 9-substituent of the adenosine analogs bind.[3] The study of the bulk tolerance and the steric requirements for binding to adenosine deaminase is continuing in this laboratory.



P = 6-Aminopurinyl

REFERENCES

(1) For a review, see Welch, A. D., *Cancer Res.*, **21**, 1475 (1961).

(2) Reichard, P., "Biosynthesis of Purines and Pyrimidines," and Schenk, F., "Biosynthesis of Nucleosides and Nucleotides," in "The Nucleic Acids," Vol. II, Chargaff, E., and Davidson, J. N., eds., Academic Press Inc., New York, N. Y., 1955, pp. 277, 309.

(3) Buchanan, J. M., "Biosynthesis of Purine Nucleotides," and Handschumacher, R. E., and Welch, A. D., "Agents Which Influence Nucleic Acid Metabolism," in "The Nucleic Acids," Vol. III, Chargaff, E., and Davidson, J. N., eds., Academic Press Inc., New York, N. Y., 1960, pp. 304, 453.

(4) Roll, P. M., *et al.*, *J. Biol. Chem.*, **220**, 439(1956).

(5) Schaeffer, H. J., and Weimar, R. D., Jr., *J. Am. Chem. Soc.*, **81**, 197(1959).

(6) Schaeffer, H. J., and Weimar, R. D., Jr., *J. Org. Chem.*, **25**, 774(1960).

(7) Montgomery, J. A., and Temple, C., Jr., *J. Am. Chem. Soc.*, **80**, 409(1958).

(8) Kaplan, N. O., in "Methods in Enzymology," Vol. II, Colowick, S. P., and Kaplan, N. O., eds., Academic Press Inc., New York, N. Y., 1955, p. 473.

(9) Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658(1934).

(10) Feigelson, P., and Davidson, J. D., *J. Biol. Chem.*, **223**, 65(1956).

[3] A different argument to explain that the binding of the compounds with *cis*- and *trans*-2′-hydroxy groups is nearly equal can be advanced if it is assumed that the *trans* compounds bind as in VIII by means of a hydrogen bond to a point on the enzyme that is close to the plane of the 5-membered ring. The *cis* compound (IX) could also form a hydrogen bond to the same place on the enzyme, even though the oxygen atom is above the plane of the 5-membered ring because of the free rotation of the C—O—H bonds. At present, it is impossible to differentiate between the two types of binding; however, studies on other compounds are planned to determine which of the modes of binding is important.