Nonclassical Antimetabolites XXIII

Simulation of 5′-Phosphoribosyl Binding VI. Relative Contribution of the Oxygen Functions of the 2′-Deoxy-5′-phosphoribosyl Moiety of 2′-Deoxyuridylate to Thymidylate Synthetase

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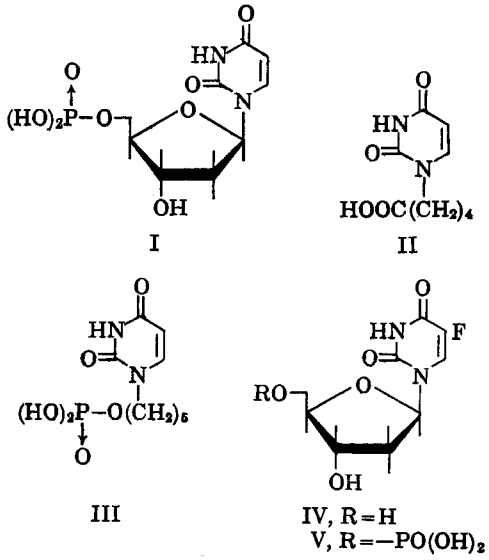
1-(5′-Hydroxypentyl)uracil (XI) was synthesized by alkylation of uracil with 5-chloropentyl *p*-nitrobenzoate (XIII), followed by removal of the *p*-nitrobenzoyl blocking group with methanolic *n*-butylamine. The phosphate ester (XIV) was prepared from XI with polyphosphoric acid at 60°. When assayed as an inhibitor of thymidylate synthetase, XIV required a concentration 60 times that of the substrate, 2′-deoxyuridylate, to give 50 per cent inhibition. Combined with the previous results that 5-fluoro-2′-deoxyuridylate (V) is a 90,000-fold better inhibitor than 5-fluoro-2′-deoxyuridine (IV), these current results show that the phosphate group contributes considerably more to binding to thymidylate synthetase than do the other two oxygenated functions of the 2′-deoxyribosyl moiety.

In the first study on simulation of phosphate binding of nucleotides, evidence was presented that 9-(4′-carboxybutyl)adenine could simulate the inhibitory binding of 5′-adenylic acid to lactic and glutamic dehydrogenase (1). However, in two cases where the nucleotide was used as a substrate, the corresponding heterocycle with an ω-valeric acid side chain in place of the sugar phosphate moiety could not simulate the phosphate binding of the nucleotide; for example, uracil-1-valeric acid (II) failed to simulate the binding of 2′-deoxyuridylate to thymidylate synthetase (2) and 6-mercapto-9H-purine-9-ylvaleric acid failed to simulate the binding of thioinosinate to succinoadenylate kinosynthetase (3).

A posteriori it was concluded that a more systematic study on the relative contribution of the oxygen functions *versus* the phosphate function of the (deoxy)ribotide moiety would have to be made (2). Such a study was performed with thioinosinate and 5′-adenylate, two inhibitors of succinoadenylate kinosynthetase; synthesis of compounds with the 2′, 3′- and 4′-oxygens of these nucleotides absent, namely, the purine-9-pentanol phosphates, gave compounds that were bound to the enzyme about one-thirteenth as well as the corresponding nucleotide—a loss of about 1.5 Kcal./mole in binding energy (4). An even greater loss in binding occurred when the phosphate group was removed from the nucleotide (4), thus confirming for this enzyme the earlier prediction that the ionized phosphate group of a nucleotide should confer more binding energy than the remaining three oxygen functions of the ribotide moiety (1). In order to show the apparent generality of this finding, a similar study has now been completed with 2′-deoxyuridylate (I), the substrate of thymidylate synthetase, and the results are the subject of this paper.

In a previous paper of this series (5) it was reported that the nucleoside, 5-fluoro-2′-deoxyuridine (IV), was less effective than the nucleotide, 5-fluoro-2′-deoxyuridylate (V), as an inhibitor of



I II III IV, R=H V, R=—PO(OH)2

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thymidylate synthetase by a factor of 9×10^4. In contrast, it has now been found that 1-(5′-hydroxypentyl)uracil phosphate (XIV) required only 60 times the concentration of substrate, 2′-deoxyuridylate (I), to give 50% inhibition; thus, removal of the phosphate function of V gave a loss of 6.4 Kcal./mole in binding energy, whereas removal of the other oxygen functions of the sugar (comparison of I and XIV) gave a loss in binding of 2.3 Kcal./mole, qualitatively in agreement with our earlier prediction (1).

Since the fluoro group of V causes 1000-fold better binding to thymidylate synthetase than the substrate (I) (6, 7), it can be estimated that 5 - fluoro - 1 - (5′ - hydroxypentyl)uracil phosphate should bind to thymidylate synthetase better than the substrate (I) by a factor of 16. Unfortunately, the methods used for alkylation of uracil (2) on the 1-position gave alkylation of 5-fluorouracil on the 3-position, as reported in the accompanying paper (8).

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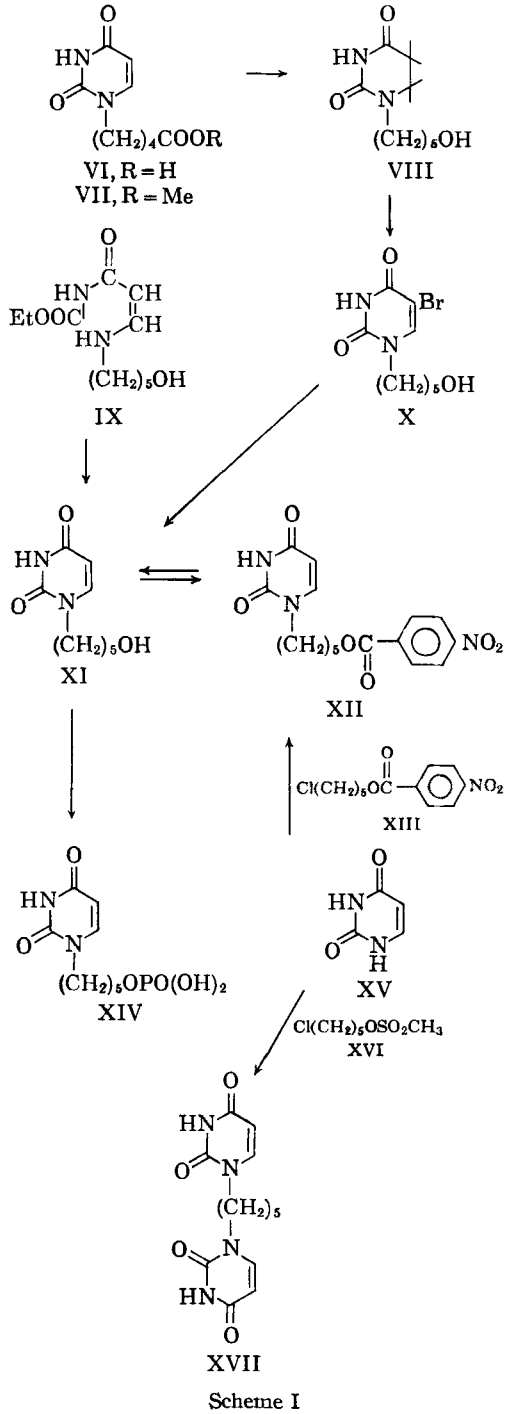
Methods.—In a previous paper of this series (6), a method for alkylation of uracil on the 1-position with halogen compounds of average reactivity, such as 5-bromovaleronitrile, was described; hydrolysis of the resultant uracil-1-valeronitrile afforded uracil-1-valeric acid (VI). Therefore, the first route to be investigated to the key intermediate, 1-(5′-hydroxypentyl)uracil (XI), needed for the current study was conversion of VI to XI.

Direct lithium aluminum hydride reduction of VI or VII could be expected to reduce the 5,6-double bond of the uracil to give VIII (9, 10). However, a similar reduction of a tetrahydroquinazoline-6-carboxylic ester with sodium borohydride-aluminum chloride in diglyme (11) was successful if the conditions were carefully controlled (9). Unfortunately, the uracil derivative (VI) which is less hindered at the 5,6-double bond, was over-reduced to a 5,6-dihydrouracil under these conditions, as shown by the loss of the ultraviolet maximum at 267 mμ. Since dihydrouracils can be converted to uracils (12) by bromination, then elimination of hydrogen bromide with lithium chloride in *N,N*-dimethylformamide, this sequence was investigated on the reduction product (VIII); this sequence resulted in aromatization, but the reaction could not be controlled to stop introduction of an extra bromine. That the product was a 5-bromo-1-alkyluracil (X) was shown by its ultraviolet maximum at 285 mμ. Although it should be theoretically possible to hydrogenolyze X to the desired XI, the sequence was so long and proceeded in such poor over-all yield that the decision was made to investigate other routes to XI which might be shorter.

The general methods of Shaw (13) for synthesis of 1-substituted-uracils *via* ethyl propiolate and β-ethoxy-*N*-carbethoxyacrylamide proceeds reasonably well with aromatic amines, but much poorer with aliphatic amines. Nevertheless, such a sequence with 5-aminopentanol was investigated; neither the intermediate (IX) nor the product (XI) could be crystallized, but the crystalline *p*-nitrobenzoate (XII) of XI could be isolated in only 2% over-all yield from ethyl propiolate.

The methanesulfonate (XVI) of 5-chloropentanol might be expected to react with uracil to give 1-(5′-chloropentyl)uracil since the sulfonate is a



Scheme I

far superior leaving group than chloro; however, since it is necessary to use excess uracil to avoid disubstitution of the uracil (2), XVI led to appreciable quantities of the bis-uracil (XVII). The desired 1-(5′-chloropentyl)uracil could not be isolated. (Scheme I.)

A smooth and short synthesis of 1-(5′-hydroxypentyl)uracil was finally accomplished with use of 5-chloropentyl *p*-nitrobenzoate (XIII). Alkylation of uracil with XIII in dimethylsulfoxide in the presence of sodium iodide and potassium carbonate afforded XII, the requisite 1-substituted uracil, in 65% yield.

The *p*-nitrobenzoyl blocking group of XII was removed with *n*-butylamine in boiling methanol, thus affording the desired crystalline 1-(5′-hydroxypentyl)uracil in 58% yield. That this sequence was a general one for preparation of 1-(hydroxyalkyl)- and 1-(dihydroxalkyl)uracils has been subsequently demonstrated (14).

The uracil-1-pentanol (XI) was converted to its phosphate ester (XIV) with polyphosphoric acid (4, 15, 16); XIV was isolated as the barium salt in 63% yield, then converted to the sodium salt for enzyme assay.

Synthesis.—Melting points were taken on a Mel-Temp apparatus in capillary tubes and those below 230° are corrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer 137B recording spectrophotometer. Ultraviolet spectra were determined with a Perkin-Elmer 202 recording spectrophotometer. Thin - layer chromatograms (TLC) were run with Brinkmann Silica Gel G, and spots were detected under ultraviolet light or iodine vapor or both.

Methyl Uracil-1-ylvalerate (VII).—To a solution of 636 mg. (3 mmoles) of VI in 6 ml. of methanol was added dropwise 1.25 ml. of acetyl chloride over a period of about 5 min. After being refluxed for 3 hr., the solution was spin-evaporated *in vacuo*. Trituration of the residue with 3 ml. of 5% aqueous sodium bicarbonate gave a solid that was collected on a filter and washed with ice water; yield, 416 mg., m.p. 115–117°. From the combined filtrate and washing was isolated an additional 20 mg. (total 63%), m.p. 114–115°. Two recrystallizations from methanol–water gave white crystals, m.p. 115–116°; $\nu_{max.}$ 3210 (NH), 1730 (ester C=O), 1700–1600 (multiple uracil bands), 1230 cm.$^{-1}$ (ester C—O—C).

Anal.—Calcd. for $C_{10}H_{14}N_2O_4$: C, 53.1; H, 6.24; N, 12.4. Found: C, 53.2; H, 6.01; N, 12.6.

1,5-Bis-(uracil-1-yl)pentane (XVII).—To a stirred solution of 15.9 Gm. (0.126 mole) of 5-chloropentanol-1 in 15.8 Gm. of pyridine cooled in an ice bath and protected from moisture was added 17.2 Gm. (0.150 mole) of methanesulfonyl chloride over a period of 2 hr. After being stirred an additional 3 hr. in the ice bath, the mixture was diluted with 25 ml. of ice water, then strongly acidified with 6 *N* hydrochloric acid. The mixture was extracted with chloroform (3 × 25 ml.). The combined extracts were washed successively with water (2 × 20 ml.), saturated aqueous sodium bicarbonate (2 × 25 ml.), and water (2 × 20 ml.). After being dried with calcium chloride, the chloroform solution was spin-evaporated *in vacuo* (bath 40–50°) leaving 21.4 Gm. (85%) of 5-chloropentyl methanesulfonate (XVI) as a colorless oil; $\nu_{max.}^{film}$ 1165, 1335 (—SO_2—), 715 cm.$^{-1}$ (C—Cl).

A mixture of 6.72 Gm. (60 mmoles) of uracil, 130 ml. of dimethylsulfoxide, 4.00 Gm. (20 mmoles) of XVI, and 8.29 Gm. (60 mmoles) of potassium carbonate was magnetically stirred at 80° for 6 hr., then diluted with 100 ml. of water and acidified to pH 6–7 with 5% hydrochloric acid. The aqueous solution was extracted with chloroform (5 × 80 ml.). The combined extracts were spin-evaporated *in vacuo;* the residual dimethylsulfoxide was then removed at less than 1 mm. in a hot water bath leaving 5.05 Gm. of viscous oil. Trituration with chloroform gave 1.17 Gm. (20%) of crude XVII, m.p. 180–185° (turbid); further trituration of this solid with 50 ml. of hot chloroform afforded 0.70 Gm. (12%), m.p. 215–220° (turbid). Several crystallizations from hot 2-methoxyethanol by addition of water gave white crystals, m.p. 238–240°, which contained no halogen; $\nu_{max.}$ 3450 (NH), 1670–1620 cm.$^{-1}$ (broad uracil bands).

Anal.—Calcd. for $C_{13}H_{16}N_4O_4$: C, 53.4; H, 5.52; N, 19.2. Found: C, 53.2; H, 5.38; N, 19.2.

The filtrate from the 1.17 Gm. showed five spots on TLC with 1:5 methanol–benzene. Although the desired 1-(5′-chloropentyl)uracil was probably present, it could not be crystallized.

5-Chloropentyl p-Nitrobenzoate (XIII).—To a stirred solution of 6.13 Gm. (75 mmoles) of 5-chloropentanol-1 in 25 ml. of reagent pyridine cooled in a water bath and protected from moisture was added 13.9 Gm. (75 mmoles) of *p*-nitrobenzoyl chloride in portions over a period of 20 min. After being stirred at ambient temperature for 16 hr., the mixture was poured on 200 Gm. of crushed ice. When the ice had melted, but the temperature was still below 10°, the mixture was filtered and the product washed with cold water. The solid was dissolved in 100 ml. of chloroform, then the solution was filtered to remove some insoluble material. The solution was washed successively with saturated aqueous sodium bicarbonate (3 × 50 ml.) and water (2 × 50 ml.). Dried with magnesium sulfate, the chloroform solution was spin-evaporated *in vacuo*. The residue was dissolved in a minimum of chloroform, then several volumes of 1:1 benzene–petroleum ether (b.p. 60–110°) were added. *p*-Nitrobenzoic anhydride (2.7 Gm.), m.p. 193–194°, was removed by filtration. Spin-evaporation of the filtrate gave 10.5 Gm. (78%) of product which solidified to yellow crystals at −5° and was suitable for further transformation. Recrystallization from chloroform–petroleum ether (b.p. 60–110°) gave light yellow plates, m.p. 31–32°; $\nu_{max.}$ 1730 (ester C=O) 1605 (C=C), 1530, 1350 (NO_2), 1280 cm.$^{-1}$ (ester C—O—C).

Anal.—Calcd. for $C_{12}H_{14}ClNO_4$: C, 53.0; H, 5.19; N, 5.16. Found: C, 52.8; H, 4.96; N, 5.21.

Scriabine (17) has recorded a m.p. of 29° for this compound prepared by a different route.

Uracil-1-ylpentyl p-Nitrobenzoate (XII).—A mixture of 3.36 Gm. (30 mmoles) of uracil, 35 ml. of dimethylsulfoxide, 4.14 (30 mmoles) of anhydrous potassium carbonate, 1.50 Gm. (10 mmoles) of sodium iodide, and 2.48 Gm. (9.15 mmoles) of XIII was stirred at 80° for 3 hr. The mixture was diluted with 20 ml. of water, then acidified to pH 2 with 5% hydrochloric acid and extracted with chloroform (3 × 50 ml.). The dried chloroform solution was spin-evaporated *in vacuo*, and the residual dimethylsulfoxide was removed in high vacuum

in a hot water bath. Trituration of the residue with 15 ml. of ethanol gave 1.80 Gm. (57%) of product, m.p. 136–141°. From the filtrate was isolated an additional 0.26 Gm. (8%), m.p. 138–140°. Recrystallization from 2-methoxyethanol–water gave pale yellow crystals in 55% yield based on XIII, m.p. 142–144°; $\nu_{max.}$ 1715 (ester C=O), 1660 (uracil C=O), 1600 (sh, C=C), 1510, 1340 (NO_2), 1270 cm.$^{-1}$ (ester C—O—C).

Anal.—Calcd. for $C_{16}H_{17}N_3O_6$: C, 55.3; H, 4.93; N, 12.1. Found: C, 55.5; H, 5.07; N, 11.9.

1-(5′-Hydroxypentyl)uracil (XI).—A solution of 0.500 Gm. (1.37 mmoles) of recrystallized XII in 10 ml. of methanol and 2 ml. of *n*-butylamine was refluxed for 16 hr., then spin-evaporated *in vacuo*. The residue was partitioned between 10 ml. of water and 20 ml. of chloroform. The aqueous phase was washed with an additional 20 ml. of chloroform, then the aqueous phase was spin-evaporated to dryness *in vacuo*. Crystallization of the residue from ethyl acetate gave 0.165 Gm. (58%) of white crystals, m.p. 78–80°; $\nu_{max.}$ 3500 (OH), 1690, 1670, 1615 (uracil), 1040 (C—O—H); no ester absorption near 1720 cm.$^{-1}$; $\lambda_{max.}$ (pH 7, 13) 268 mμ.

Anal.[1]—Calcd. for $C_9H_{14}N_2O_3$: C, 54.5; H, 7.12; N, 14.1. Found: C, 54.8; H, 6.95; N, 14.3.

Barium Uracil-1-ylpentyl Phosphate (XIV).—To a solution of 1.3 Gm. of phosphorus pentoxide in 1.75 Gm. of 86% phosphoric acid was added 198 mg. (1 mmole) of XI. After being magnetically stirred and protected from moisture in a bath at 60° for 7 hr., the solution was diluted with 12 ml. of water and heated on a steam bath for 20 min. to hydrolyze any polyphosphate ester. The pH was adjusted to exactly 6.5 with hot saturated barium hydroxide, then filtered through a Celite pad and washed with 100 ml. of hot water in portions. The combined filtrate and washings were spin-evaporated *in vacuo* to about 10 ml. After standing overnight, the mixture was filtered and the product washed with water; yield, 260 mg. (63%), m.p. over 300°. Recrystallization from water gave white crystals with $\lambda_{max.}$ (H_2O): 270 mμ (ε 9800); $\nu_{max.}$ 1680–1660 (uracil C=O); 1120–1060, 980 cm.$^{-1}$ (PO_3^{2-}).

Anal.—Calcd. for $C_9H_{13}BaN_2O_6P$: C, 26.1; H, 3.17; N, 6.77. Found: C, 25.9; H, 3.30; N, 6.77.

For enzyme assay, a hot solution of 41 mg. (0.1 mmole) of barium salt in 10 ml. of water was treated with 15 mg. (0.11 mmole) of anhydrous sodium sulfate in 5 ml. of water. The mixture was filtered through a Celite pad, and the combined filtrate and washings were spin-evaporated *in vacuo*. The residue was dissolved in cold water, then the solution was clarified by filtration through a Celite pad. The concentration of XIV sodium salt was 28.70 m*M* as determined by the absorbance of the solution at 270 mμ using ε 9800.

[1] This compound was first prepared in this laboratory from XII by Dr. T. J. Schwan.

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